Ciprofloxacin enhances the stimulation of matrix metalloproteinase 3 expression by interleukin-1β in human tendon-derived cells

A potential mechanism of fluoroquinolone-induced tendinopathy

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Abstract

Objective

To determine whether the fluoroquinolone antibiotic ciprofloxacin, which can cause tendon pain and rupture in a proportion of treated patients, affects the expression of matrix metalloproteinases (MMPs) in human tendon-derived cells in culture.

Methods

Cell cultures were derived from 6 separate tendon explants, and were incubated in 6-well culture plates for 2 periods of 48 hours each, with ciprofloxacin (or DMSO in controls) and interleukin-1β (IL-1β), alone and in combination. Samples of supernatant medium from the second 48-hour incubation were assayed for MMPs 1, 2, and 3 by Western blotting. RNA was extracted from the cells and assayed for MMP messenger RNA (mRNA) by semiquantitative reverse transcription–polymerase chain reaction, with normalization for GAPDH mRNA.

Results

Unstimulated tendon cells expressed low or undetectable levels of MMP-1 and MMP-3, and substantial levels of MMP-2. IL-1β induced a substantial output of both MMP-1 and MMP-3 into

cell supernatants, reflecting increases (typically 100-fold) in MMP mRNA, but had only minor effects on MMP-2 expression. Ciprofloxacin had no detectable effect on MMP output in unstimulated cells. Preincubation with ciprofloxacin potentiated IL-1β–stimulated MMP-3 output, reflecting a similar effect on MMP-3 mRNA expression. Ciprofloxacin also potentiated IL-1β–stimulated MMP-1 mRNA expression, but did not potentiate the output of MMP-1, and had no significant effects on MMP-2 mRNA expression or output.

Conclusion

Ciprofloxacin can selectively enhance MMP expression in tendon-derived cells. Such effects might compromise tendon microstructure and integrity.

The fluoroquinolone antibiotics are a group of broad-spectrum antibiotics that target the bacterial enzyme DNA gyrase, and have been used widely and successfully. The reported side effects associated with the use of these antibiotics include tendon pain and/or rupture in a small proportion of patients, principally

affecting the Achilles tendons (1). Although other adverse reactions, which have been observed in the central nervous system and gastrointestinal tract, may involve antagonism of amino acid neurotransmitter receptors (2), the cellular interactions underlying the effects of fluoroquinolones on the tendons are not clear. Inflammation of the paratenon and degenerative changes in tendon cells have been noted in studies of fluoroquinolone-treated animals (3, 4). Fluoroquinolones have been shown to have a number of effects on various mammalian cell types in culture, including both increased and decreased expression of inflammatory mediators (5, 6), reduced expression of some extracellular matrix proteins (7, 8), reduced mitochondrial activity (8), and noncytotoxic inhibition of canine tendon cell proliferation (7).

The cells of the tendon, predominantly fibroblasts, are responsible for the maintenance of the extracellular matrix. This may involve the modification and turnover of extracellular matrix components, including collagen cross-linking, breakdown, and resynthesis, especially in those tendons that are subject to overuse (9, 10). A large part of this remodeling is likely to be mediated by the group of enzymes known as matrix metalloproteinases (MMPs) (10, 11). The level of MMP

activity in a tissue can be regulated by changes in gene expression, by the cleavage of latent MMP precursors, and by association of the MMP with inhibitory proteins (11). We have previously described differences in the expression of certain MMPs between normal and degenerate human tendons, consistent with a role in tendon pathology (12).

In cultured canine tenocytes, the fluoroquinolone ciprofloxacin decreased matrix synthesis and increased the release of matrix-degrading proteolytic (caseinase) activity (7). In this study, we have examined whether ciprofloxacin can influence the expression of MMPs in human tendon-derived cells in culture.

MATERIALS AND METHODS

Materials.

Ciprofloxacin was a gift from Bayer (Newbury, UK) and a stock (10 mg/ml) was prepared on each day of use by warming in DMSO. Interleukin-1β (IL-1β) was a gift from Glaxo Wellcome (Stevenage, UK) and aliquots (1 μg/ml) were stored at −70°C. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), antibiotics, TRIzol, Western Breeze immunodetection

system, and oligonucleotide primers for reverse transcription–polymerase chain reaction (RT-RCR) were obtained from Invitrogen (Paisley, UK). One-Step RT-PCR reagents and FAM-labeled oligonucleotide probes were obtained from Applied Biosystems (Warrington, UK). The anti–MMP-1 antibody used for Western blotting was a rabbit polyclonal antibody described previously (**13**), and other primary and secondary antibodies were obtained from TCS Biologicals (Buckingham, UK) and Dako (Ely, UK).

Cell isolation and incubation.

Tendon specimens were obtained from tissue discarded during surgery for chronic tendinopathy, following documentation of patient consent and local ethics committee approval. Cells, isolated by outgrowth from 6 separate tendon explants, were maintained and passaged in DMEM containing 10% (volume/volume) FCS, penicillin, streptomycin, and 25 m *M* HEPES, and were used at passages between 3 and 8. Cells were seeded at 10^5/well in 6-well plates, and were incubated for 3 days before the experiment. They were rinsed with 2 ml of serum-free medium containing insulin, transferrin, and selenium, or with medium containing

10% FCS, and were then given 2 ml of the same medium containing ciprofloxacin (50 μg/ml; 0.5% [v/v] DMSO in control wells), with or without IL-1β (1 ng/ml). After 48 hours, the cells were again rinsed and given fresh medium containing ciprofloxacin (DMSO in controls) with or without IL-1β. After a further 48 hours, the supernatant medium was removed and stored at −20°C, and the cells were rinsed with serum-free medium and solubilized in TRIzol (1 ml/well). In some of the experiments, cell counts were performed on parallel wells of cells at the start of the experimental treatment and at the end of each 48-hour incubation.

Western blotting.

Aliquots (20 μl) of serum-free culture supernatants were mixed with sample buffer, boiled for 3 minutes, and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions, using a 10% (weight/volume) polyacrylamide resolving gel with a 4.5% (w/v) stacking gel. The proteins were electroblotted onto polyvinylidene difluoride membranes. These were blocked, incubated with antibodies to MMPs, washed, and developed using standard protocols, and the films were scanned and

analyzed using 1-dimensional software (Eastman Kodak, Rochester, NY).

RNA isolation and analysis by RT-PCR.

RNA was isolated from the TRIzol extracts by phenol-chloroform separation followed by precipitation with isopropanol and ethanol. The RNA was stored at −70°C as aliquots, which were thawed only once, and was assayed for GAPDH, MMP-1, MMP-2, and MMP-3 messenger RNA (mRNA) using one-step RT-PCR reactions in a GeneAmp 5700 (Applied Biosystems). All primers and probes have been described previously (**12**). Standard curves were run in each assay using freshly diluted aliquots of pooled tendon-cell RNA. For each target, this produced a linear plot of the threshold cycle against the log(dilution), whose slope was within 10% of the expected value, indicating a similar, near-maximum efficiency. A single product of the appropriate size was produced, and no signal was produced if the reverse transcriptase step was omitted. All samples from a single experiment were run on the same plate, generally using either 3 or 4 replicate determinations per sample. The values obtained for MMP mRNA

expression were corrected for GAPDH mRNA expression in the same sample.

Presentation of data.

In each experiment, the values for MMP-1 and MMP-3 protein or mRNA expression were normalized to the value for cells treated with IL-1β alone (after a control preincubation), expressed as 100%. The values for MMP-2 were normalized to that for cells undergoing control treatment throughout, expressed as 100%, since MMP-2 (unlike MMP-1 and MMP-3) was readily detectable in the control cells. Results were obtained from 6 experiments, 1 from each cell isolate, and significant differences from the 100% value are expressed as the 95% confidence interval (95% CI) of the treated samples.

RESULTS

Growth of tendon cell cultures.

The majority of the cells in the tendon-derived cultures showed fibroblastic morphology that was maintained for at least 8 passages. The cells were seeded at a density

just below confluence, and control cell numbers at the end of the experiments were within 25% of the initial values; control cell numbers in serum-free medium frequently showed a small decrease, while those in medium containing 10% FCS generally showed a small increase. Of the treatments tested, only incubation of the cells with IL-1β for both 48-hour incubations in FCS-containing medium caused significant changes in cell numbers (mean ± SEM final cell number 173 ± 20% of the initial value, compared with 117 ± 7% in control cell cultures [n = 3]; $P < 0.05$ by t-test). Ciprofloxacin did not cause significant changes in cell numbers and, in additional experiments, had no cytotoxic effects at concentrations up to 100 μg/ml (assayed by release of lactate dehydrogenase; data not shown).

Secretion of MMPs into cell culture supernatants.

Supernatants from unstimulated tendon cells cultured in serum-free medium showed no immunodetectable MMP-1 or MMP-3 on Western blots (Figure 1). Treatment with IL-1β stimulated the output of both of these MMPs, in the latent proenzyme form (Figure 1). This stimulation was at least 50-fold (by comparison

with serial dilutions of MMP standards), and was maintained or increased when IL-1β was also included in the preincubation (Figure 2).

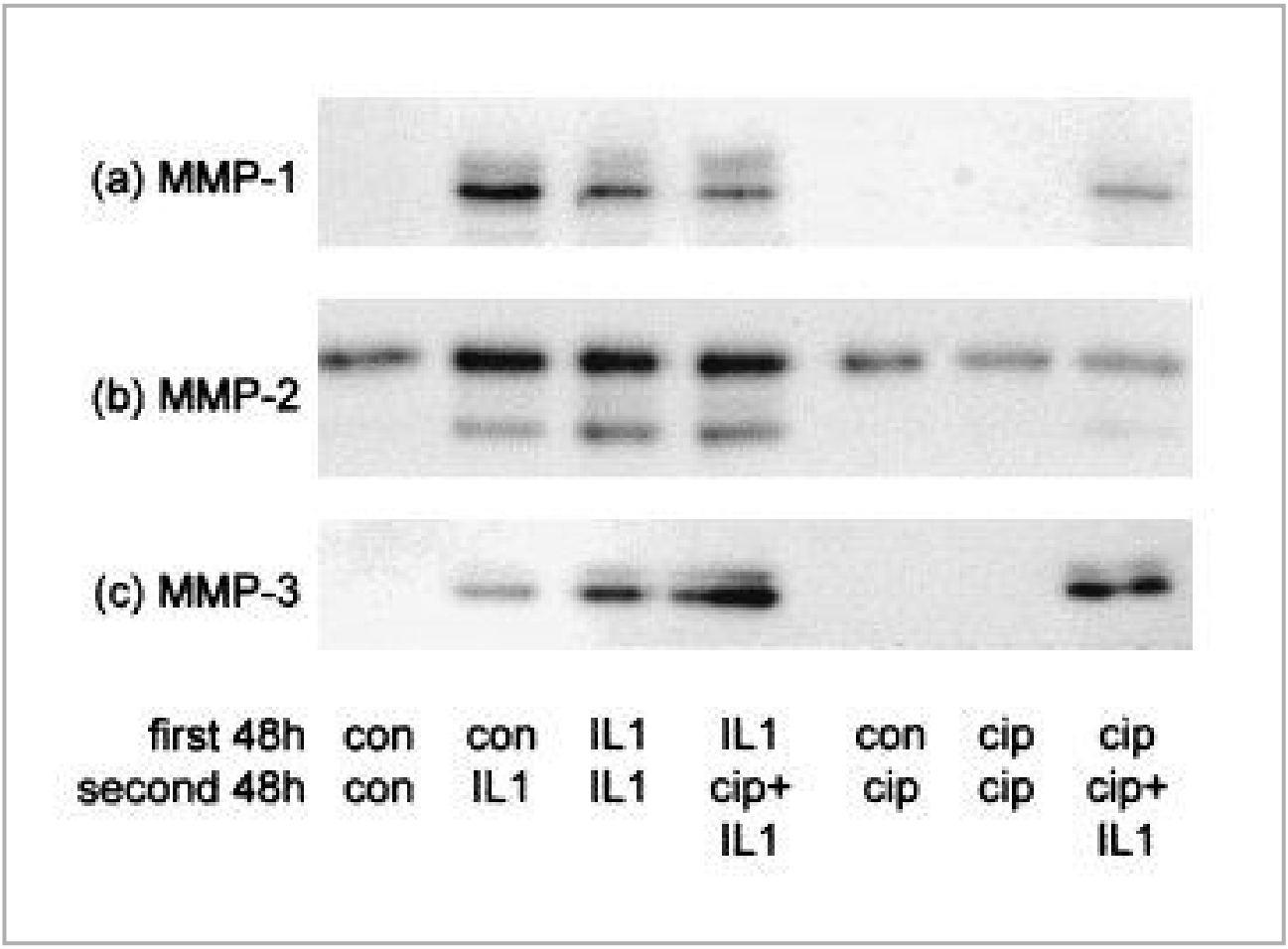

Figure 1.

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Western blot analysis of matrix metalloproteinase (MMP) secretion by human tendon-derived cells. Cells in serum-free medium were treated for 2 periods of 48 hours (48h) with ciprofloxacin (cip) and/or

interleukin-1β (IL1), and supernatants from the second incubation were analyzed by Western blotting. The blots are from a single experiment and show the bands detected using antibodies specific for MMP-1 **(a)**, MMP-2 **(b)**, and MMP-3 **(c)**. By comparison with MMP standards, the principal bands correspond to latent pro–MMP-1 (52 kd), latent and active MMP-2 (72 and 62 kd), and pro–MMP-3 (52 kd). con = vehicle controls.

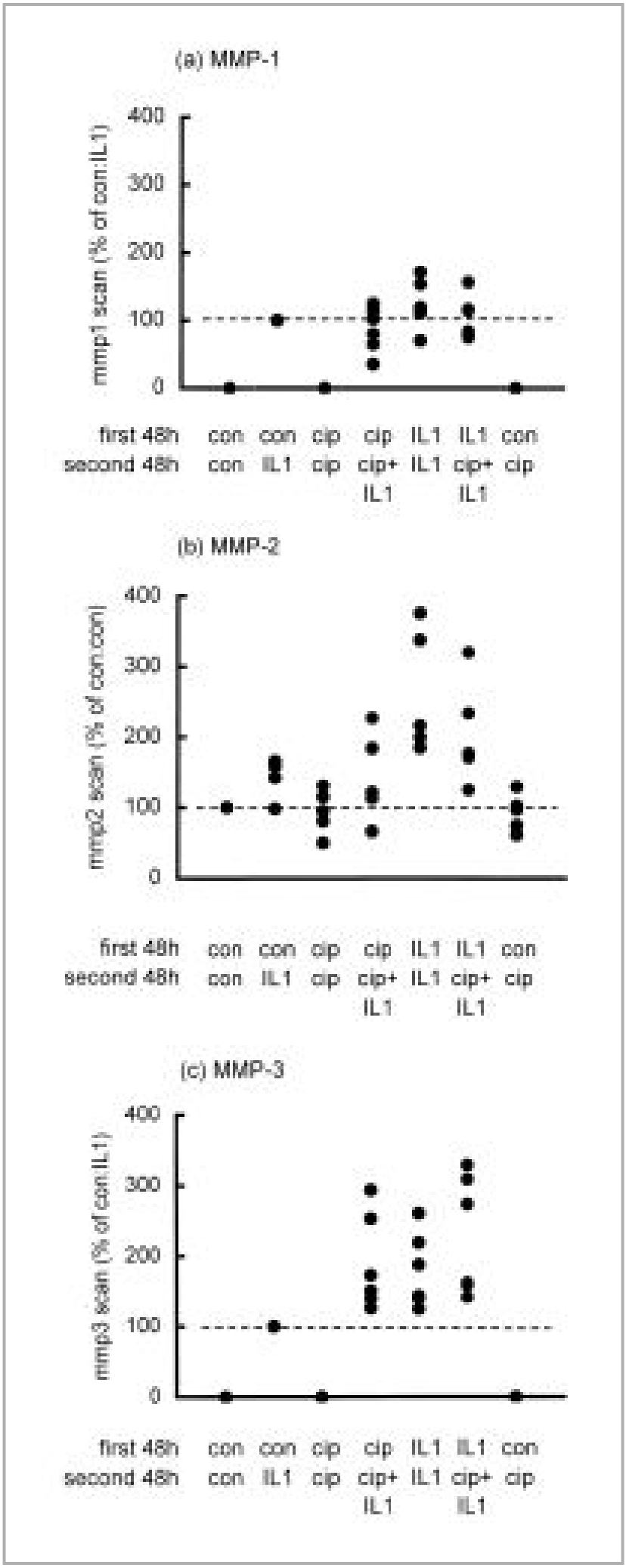
(a) MMP-1
mmp1 scan (% of con:IL1)
400
300
200
100
0
first 48h con con cip cip IL1 IL1 con
second 48h con IL1 cip cip+ IL1 IL1 cip+ IL1 cip
(b) MMP-2
mmp2 scan (% of con:con)
400
300
200
100
0
first 48h con con cip cip IL1 IL1 con
second 48h con IL1 cip cip+ IL1 IL1 cip+ IL1 cip
(c) MMP-3
mmp3 scan (% of con:IL1)
400
300
200
100
0
first 48h con con cip cip IL1 IL1 con
second 48h con IL1 cip cip+ IL1 IL1 cip+ IL1 cip

Figure 2.

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Analysis of matrix metalloproteinase (MMP) protein secretion by human tendon-derived cells. Cells in serum-free medium were treated for 2 periods of 48 hours (48h) with ciprofloxacin (cip) or interleukin-1β (IL1), and supernatants from the second incubation were analyzed by Western blotting. Each point represents the normalized scan value from a single experiment, and the broken line shows the 100% value. For MMP-1 **(a)** and MMP-3 **(c)**, the scan values were normalized to those of cells treated with IL-1β after a control preincubation; for MMP-2 **(b)**, the scan values (combined main bands) were normalized to those of cells given control treatment throughout. The results from 6 separate experiments are shown, with each experiment using cells from a separate tendon isolate. con = vehicle controls.

Incubation of the cells with ciprofloxacin did not stimulate detectable output of either MMP-1 or MMP-3 in control cells, but had differential effects on the stimulation of MMP-1 and MMP-3 output by IL-1β (Figures 1 and 2; compare treatments con/IL1 and cip/cip+IL1). After preincubation with ciprofloxacin, the induction of MMP-1 output by IL-1β in each experiment either increased by <25% or decreased (Figure 2) (mean difference not significant). In marked contrast, the stimulation of MMP-3 output by IL-1β was potentiated by ciprofloxacin in each experiment, increasing up to 3-fold (mean 190%, 95% CI 118–261%). The addition of ciprofloxacin to cells undergoing continuing stimulation after preincubation with IL-1β had no consistent effect on either MMP-1 or MMP-3 output (Figure 2).

In addition to MMP-1 and MMP-3, we examined the expression of MMPs 2, 7, 9, and 13 by the tendon cells. MMP-2 secretion by control cells was readily detected (Figure 1), and showed a small, but significant, stimulation by IL-1β (mean 146%, 95% CI 111–181%, increasing to 263%, 95% CI 154–372% when IL-1β was also included in the preincubation) (Figure 2). Ciprofloxacin had no significant effect on the output of MMP-2 from either control or IL-1β–stimulated cells

(Figure 2). Neither MMP-7 nor MMP-13 was detected under any condition tested, while the secretion of MMP-9 was low and was not affected by either IL-1β or ciprofloxacin (data not shown).

Expression of mRNA for MMPs 1, 2, and 3.

The expression of MMP mRNA in the tendon cell cultures was investigated by semiquantitative RT-PCR. Similar results were obtained in RNA extracted from the serum-free cultures whose supernatants had been used for Western blotting (Figures 3a–c), and from parallel cultures in medium containing 10% FCS (Figures 3d–f). Only low levels of MMP-1 and MMP-3 mRNA were detected in unstimulated cells, and there was a stimulation (typically 100-fold, up to 10,000-fold) by IL-1β, which persisted when IL-1β was also included in the preincubation (Figure 3). Consistent with the data for MMP-2 output, control cells expressed substantial levels of MMP-2 mRNA. This showed variable modulation by IL-1β and/or ciprofloxacin, with no overall significant difference, although stimulation up to 3-fold occurred in some experiments (Figure 3).

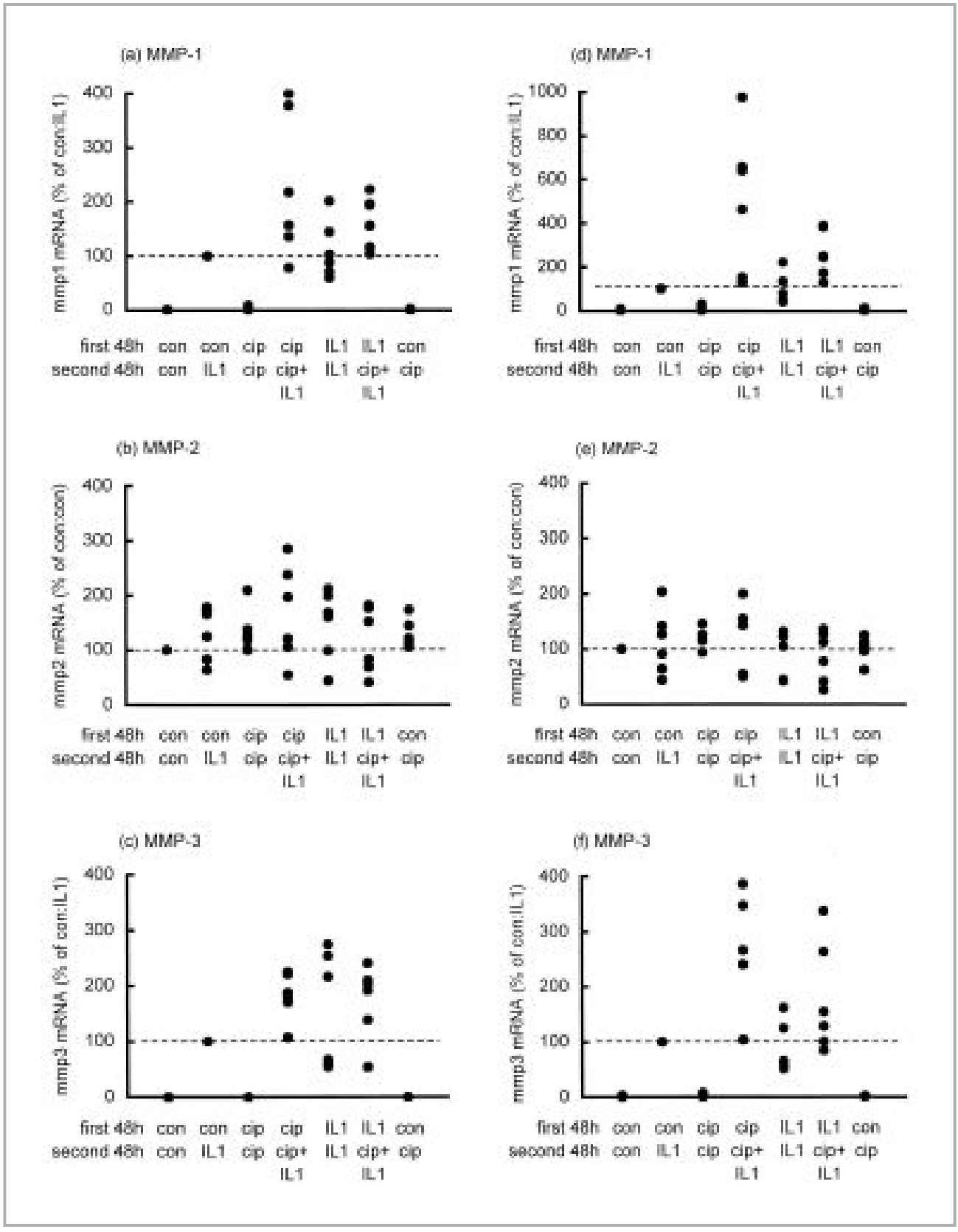

Figure 3.

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Analysis of matrix metalloproteinase (MMP) mRNA expression in human tendon-derived cells in serum-free medium **(a–c)** or medium containing 10% fetal calf serum **(d–f)**. Cells were treated for 2 periods of 48 hours (48h) with ciprofloxacin (cip) or interleukin-1β (IL1). Total RNA was isolated and analyzed relative to GAPDH mRNA. Each point represents the normalized value from a single experiment, and the broken line shows the 100% value. For MMP-1 **(a** and **d)** and MMP-3 **(c** and **f)**, mRNA values were normalized to those of cells treated with IL-1β after a control preincubation; for MMP-2 **(b** and **e)**, mRNA values were normalized to those of cells given control treatment throughout. The results from 6 separate experiments are shown (from which the serum-free supernatants were analyzed for MMP secretion as shown in Figure 2).

Incubation with ciprofloxacin increased both MMP-1 and MMP-3 mRNA levels in unstimulated cells in

medium containing 10% FCS; these effects were significant ($P < 0.05$ by Wilcoxon signed rank test), but were small (mean 4-fold) compared with those induced by IL-1β (Figure 3). Consistent with its potentiation of stimulated MMP-3 output, pretreatment of the cells with ciprofloxacin potentiated the elevation of MMP-3 mRNA induced by IL-1β in both serum-free and serum-containing media (mean 182%, 95% CI 137–276% and mean 265%, 95% CI 160–368%, respectively, of that in the IL-1β–stimulated control cells) (Figure 3). However, in contrast to its lack of effect on MMP-1 output (Figure 2), pretreatment with ciprofloxacin also potentiated the stimulation of MMP-1 mRNA expression by IL-1β, both in serum-containing medium (mean 504%, 95% CI 162–847%) (Figure 3d) and in 5 of the 6 experiments in serum-free medium (mean 228%, 95% CI 88–368%; mean not significantly increased) (Figure 3a). The addition of ciprofloxacin to cells undergoing continuing stimulation after preincubation with IL-1β had no consistent effect on either MMP-1 or MMP-3 mRNA levels (Figure 3).

DISCUSSION

In this study, we have shown that the antibiotic ciprofloxacin, which induces tendon pain in some patients (1) and tendon pathology in rodents (3, 4), can increase MMP expression in human tendon-derived fibroblasts. Specifically, ciprofloxacin potentiated IL-1β–stimulated expression of MMP-3 at both the mRNA and protein level. Ciprofloxacin also enhanced the expression of MMP-1 mRNA in most experiments, frequently more than that of MMP-3 mRNA, but this did not result in increased output of MMP-1 protein, indicating the potential for additional effects on MMP-1 translation or secretion. Since MMP-1 and MMP-3 are often coordinately stimulated by inflammatory mediators (14, 15), as was observed in the present study at the mRNA level, this is a novel differential effect of this compound on IL-1β–stimulated MMP expression.

Tendon pain and degeneration have been associated with an increase in the normal turnover of matrix proteins (9, 10, 12). MMP-3 has a broad substrate specificity; it is able to degrade matrix components including type III collagen and the proteoglycans aggrecan and versican, and is capable of activating a variety of other MMPs and pro–tumor necrosis factor (11). However, its role in tendon physiology and pathology has not been clearly defined. In tendons from

sites exposed to high mechanical demands, such as the Achilles and supraspinatus tendons, the expression and activity of MMP-3 are high compared with that in normal flexor tendons (**10**, **12**). This activity may be necessary for the maintenance of the matrix in response to repeated microinjury; a change in the activity of several MMPs has also been associated with tendon rupture (**10**). Further evidence indicating the importance of MMPs in tendon has been provided by the side-effect profile of broad-spectrum MMP inhibitors such as marimastat, which has been shown to induce shoulder- and hand-tendon lesions (**16**). Whether these effects are mediated by inhibition of specific MMPs is not yet known, although related metalloenzymes may also be implicated. We have not yet investigated the effects of ciprofloxacin on this diverse group of metalloenzymes, but we found no significant effect on the expression of MMP-2, MMP-7, MMP-9, and MMP-13 in this culture system.

A previous study using canine tenocytes showed that ciprofloxacin increased secretion of proteolytic activity, measured as caseinase activity (**7**). There are several differences between the two studies, which may be due, at least in part, to differences in the cell types involved or the culture conditions utilized (although it should be

noted that the dose of ciprofloxacin used in the present study was the same as the highest of the effective range used in the previous study [7], a range which was chosen according to serum values observed in patients undergoing treatment). For example, the proliferation of the canine cells was inhibited by ciprofloxacin, whereas the human cells in the present study showed little proliferation and no effect of ciprofloxacin. Also, active caseinase was secreted by the canine cells, whereas the major form of MMP-3 secreted in our study corresponds (by size on Western blotting) to pro–MMP-3. Finally, the canine cells increased caseinase activity in response to ciprofloxacin alone, but the enhancement of MMP-3 secretion by ciprofloxacin observed herein by Western blotting occurred in cells stimulated with IL-1β. However, as shown by our results, we also found that ciprofloxacin, when added alone, significantly increased the expression of MMP mRNA over basal levels; this increase was similar to or greater than the increase in caseinase activity reported in the previous study (**7**), but we estimate that a corresponding increase in the very low basal level of secreted MMP-3 would not have been detected by Western blotting. We conclude that the two studies, taken together, indicate that ciprofloxacin can

specifically enhance the expression of matrix-degrading protease(s).

Achilles tendon pain is not generally associated with inflammation, at least in the tendon mid-substance (**17**), but 2 lines of evidence suggest that the current model system may be relevant to fluoroquinolone-induced tendon problems. First, fluoroquinolones have been shown to induce localized inflammation of the paratenon surrounding rat Achilles tendon (**3**). Second, fluoroquinolones have been shown to enhance the expression of certain interleukins and other cytokines (**5**, **6**). Our results raise the possibility that a combination of fluoroquinolone and (fluoroquinolone-induced) inflammatory mediators might result in the inappropriate or unbalanced expression of MMPs. Interestingly, although the mechanisms by which fluoroquinolones initiate changes in mammalian cells are not known, some reports have indicated that their effects on interleukin expression may coincide with an increased activity of transcription factors, such as activator protein 1 (**5**), which are also known to influence expression of MMPs (**14**, **15**). Changes in expression of matrix components such as collagen and proteoglycans have also been reported in response to various fluoroquinolones (**7**, **8**). Using the present

system, it will be of interest to determine 1) whether the expression of these genes is coordinated, and 2) whether other members of the fluoroquinolone class have similar effects.

Finally, there was some variation between experiments in the degree of enhancement of MMP-1 and MMP-3 output and mRNA expression by ciprofloxacin. These observations may reflect differences in the cultured cell populations rather than in the patients from whom the cells were isolated. However, we conclude that changes in the expression of MMPs, such as those that we have observed, could contribute to damage of tendon microstructure and the onset of pain in patients susceptible to the effects of fluoroquinolones.

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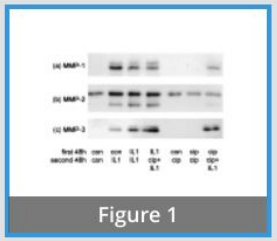

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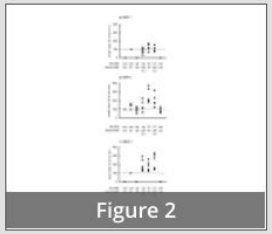

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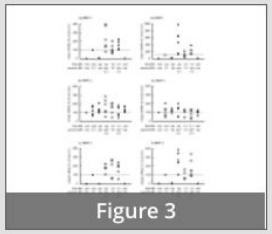

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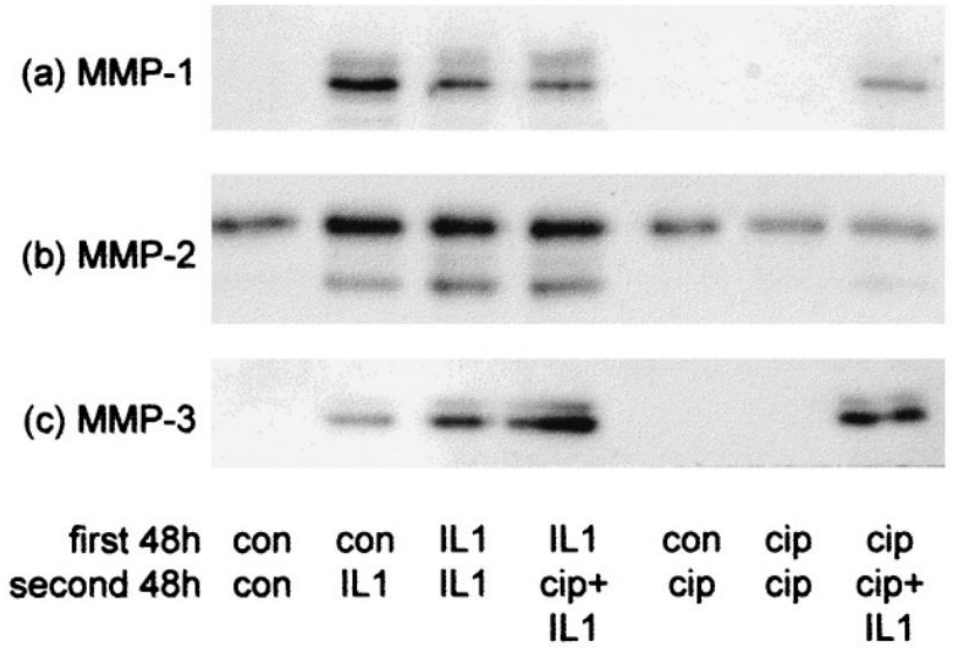

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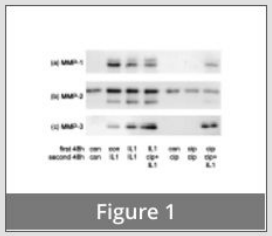

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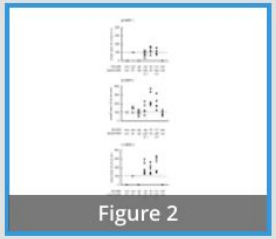

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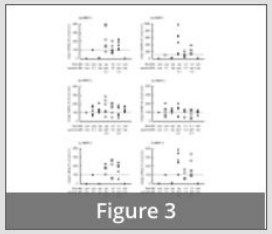

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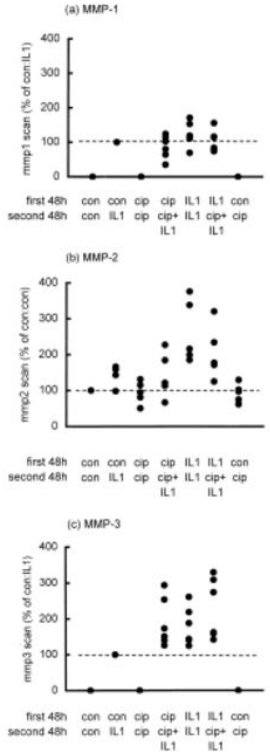

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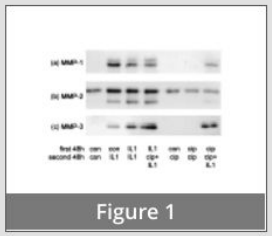

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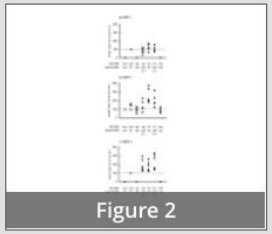

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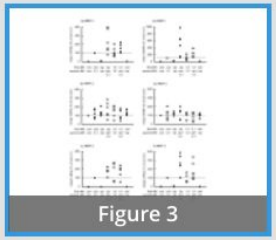

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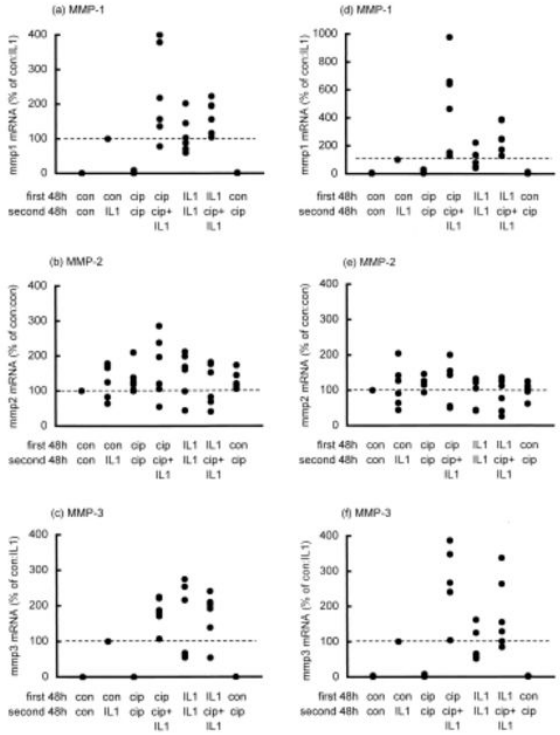

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