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Anti-collagenolytic mechanism of action of doxycycline treatment in rheumatoid arthritis

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Abstract Tetracyclines exert, independently of their antimicrobial activity, anti-collagenolytic effects by inhibiting activities of human interstitial collagenases and by preventing the oxidative activation of latent pro-collagenases. We tested the clinical response to a 3-month doxycycline in concert with collagenase activity in 12 rheumatoid arthritis (RA) patients. Patients received 150 mg/day of doxycycline for 3 months. Clinical assessments at zero, six and 12 weeks comprised classification of the functional class, joint score index, Hb, CRP, ESR, health assessment questionnaire, visual analogue scale (VAS) of pain, pain disability index, comprehensible psychopathological rating scale (CPRS), SDS-PAGE laser densitometric collagenase activity measurements and Western blots. Significant reductions were seen in joint score index ($P<0.01$), pain VAS ($P<0.05$) and some CPRS parameters. Furthermore, collagenase activities measured from saliva by quantitative SDS-PAGE electrophoresis were significantly reduced during the 12-week intervention ($P<0.01$). Western blots demonstrated intact 75–80 kDa enzyme protein (classic neutrophil collagenase), but also a newly discovered mesenchymal, less glycosylated 40–55 kDa MMP-8 subtype of fibroblast/chondrocytic origin. These results indicate that the documented favourable clinical response may in part be due to in vivo inhibition of classic neutrophil and mesenchymal collagenase/MMP-8 activities produced by doxycycline. This anti-collagenolytic doxycycline effects is mediated through inhibition of the enzyme activity and not through degradation of the enzyme, which may have contributed to the reportedly reduced tissue destruction, as has been seen in clinical studies concerning RA as well as reactive arthritis.

Key words Rheumatoid arthritis · Doxycycline · Collagenases · Matrix metalloproteinases · Therapy

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Introduction

Several clinical studies have concluded that tetracycline derivates are effective in treating patients with rheumatoid arthritis [1–5]. Tetracyclines decrease collagenase activity [6, 7], inhibit leucotaxis [8, 9] and phagocytosis [10], have immunomodulatory effects on the complement cascade [11] and on human mononuclear cells [12, 13], and inhibit angiogenesis [14]. Tetracyclines also possess anti-inflammatory properties probably related to their antioxidant activity [15–17].

Tetracyclines can directly inhibit in vitro the activities of human interstitial collagenases and of other matrix metalloproteinases (MMPs) [18] with preference for "neutrophil" collagenase MMP-8 [19]. More recently, "neutrophil" collagenase has been found to be produced also by articular chondrocytes [20, 21] and by TNF-α stimulated synovial fibroblasts/lining cells (Hanemaaijer, personal communication) [22]. Furthermore, tetracyclines can

prevent oxidative activation of latent pro-collagenases [23, 24]. This oxidative activation liberates the fourth coordination site of the zinc at the active center of the proenzyme from the thiol group of the Cys^{73} located in the propeptide domain [25]. We have shown earlier that MMP-8/collagenase-2 levels are reduced during long-term doxycycline treatment of reactive arthritis in serum and especially in saliva using quantitative SDS-PAGE, ELISA and spectrophotometric assay methods [26, 27]. Reactive arthritis has a spontaneous tendency to heal so that most of the patients will become symptomless within 3–6 months after the disease initiation. It was therefore left unclear whether or not the anti-collagenolytic effect was a direct doxycycline effect or perhaps associated with the favourable course of the disease and of the acute phase response.

As both the in vivo effect of doxycycline on collagenase activity and the mechanism of action responsible for the favourable clinical response in RA have remained uncertain, we conducted a study offering 12 patients with well-documented, active RA 3-month-long treatment with 150 mg doxycycline per day. During the course of the study both the clinical status and the collagenase activity were monitored.

Patients and methods

The study was performed at the outpatient clinic of the Department of Rheumatology, Helsinki University Central Hospital. The open-label study protocol was approved by the local ethics committee prior to initiation. The patients gave their informed concent in writing. The 12 enrolled RA patients fulfilled the 1987 ACR criteria (Table 1) [28]. All received 150 mg/day of doxycycline for 3 months. Patients were allowed to continue their disease modifying anti-rheumatic drug (DMARD) therapy, which had been kept stable for the last 3 months prior to the study. Stable per oral corticosteroids were allowed up to 10 mg prednisolone (or equivalent) per day. Inclusion criteria were: functional class I–III [29], signs of active disease as defined by a sedimentation rate of ≥28 mm/h or CRP ≥15 mg/l and a joint tenderness score index showing at least six tender joints (Table 1) [30]. Exclusion criteria were other ongoing antibiotic treatments, pregnancy, malignancy or tetracycline allergy. Blood (Hb, ESR and CRP) and stimulated saliva samples were collected at baseline, at 6 weeks and at 12 weeks. In addition, the following assessments were made: a four-stage functional classification [29]; joint score index (measuring tenderness in proximal interphalangeal, metacarpophalangeal, wrist, elbow, shoulder, acromioclavicular, sternoclavicular, jaw, cervical, hip, knee, talocrural, talocalcanear, metatarsophalangeal and toe joints on each side; scores 0–3) [30] performed by the principal investigator; the Health Assessment Questionnaire (HAQ) [31]; Visual Analogue Scale of Pain (VAS) [32]; Pain disability index (PDI, 7 VAS variables) [33]; and Comprehensible Psychopathological Rating Scale (CPRS, 5 VAS variables) [34].

Collagenolytic activity was measured against soluble native/triple helical collagen type I monomers. Native type I collagen was extracted from human skin and further purified by selective salt precipitation at acid and neutral pH. The purity of type I collagen substrate was examined by cyanogen bromide cleavage peptide analysis. Salivary samples were centrifuged and supernatants assayed for collagenase activity by the quantitative SDS-PAGE laser densitometric method originally described by Turto et al. [35]. Total collagenase activity was measured in the presence of 1 mM aminophenylmercuric acetate (APMA) and the endogenously in vivo activated collagenase activity without APMA. APMA is an optimal organomercurial activator of latent collagenases, because it removes the thiol group of the Cys^{73} from the active site zinc and thus releases the fourth coordination site of it [25]. The salivary samples were incubated with soluble native 1.5 µM type I collagen at 22°C for 48 h. Incubation was stopped by addition of a modified Laemmli's sample buffer containing 40 mM EDTA, followed by immediate heating at 100°C for 5 min. Subsequently, the degradation products were separated by SDS-PAGE on 10% T 2.6% C gel. The gels were stained with Coomassie brilliant blue and destained in 5% acetic acid –10% methanol in water (v/v). The destained gels were quantified by destitometric scanning using the LKB Ultrascan Laser Densitometric model 2202. The values representing αA-chains were multiplied by 4/3 and their proportion of total collagen in the sample was used as a measurement of collagenase activity, which is expressed as per cent type I collagen degraded.

Collagenase enzyme protein was demonstrated using Western blotting. The samples were treated with Laemmli's buffer, pH 6.8, containing 5 mM dithiothreitol (DTT) and heated for 5 min at 100°C. High- and low-range prestained SDS-PAGE standards (Bio-Rad, Richmond, Calif.) were used as molecular weight markers. The saliva samples were separated on 8–10% SDS-PAGE 10% T 2.6% C gels at 200 V for 45 min and electrophoretically transferred to nitrocellulose membrane at 100 V for 45 min (Bio-Rad). Gelatin (3%) in 10 mM Tris-HCl, pH 8.0, 0.05% Triton X-100, 22 mM NaCl (TST) was used to block non-specific binding sites on the nitrocellulose membrane. After 3×15 min with TST the membrane was incubated with anti-MMP-8 antibody (1:1000 dilution in TST) for 10 h. Polyclonal rabbit anti-human MMP-8 was kindly donated by Dr. Jürgen Michaelis (Department of Pathology, Christchurch Medical School, Christchurch, New Zealand) [36]. After 3×15 min washes with TST

Table 1 Demographic characteristics of 12 RA patients receiving doxycycline for 12 weeks (*ESR* erythrocyte sedimentation rate (mm/h), *CRP* C-reactive protein (mg/l), *C* oral corticosteroid, *NSAID* non-steroidal anti-inflammatory drug, *MTX* methotrexate, *HCQ* hydroxychloroquine, *DP* d-penicillamine, *po. gold* auranofin, *im. gold* aurothiomalate, *SSZ* sulfasalazine, *P* podophyllum emodium)

Patient no.	Duration of disease (years)	ESR/CRP (mm/h/mg/l)	Erosions	Medication	Intra-articular cortisone	Adverse effects
1	8	40/34	Yes	C, NSAID	No	Oral fungus
2	7	78/24	Yes	MTX, C, NSAID	No	No
3	18	22/28	Yes	–	No	No
4	6	28/25	Yes	HCQ, C, NSAID	No	Gastric
5	6	30/19	Yes	HCQ, C, NSAID	Yes	No
6	21	58/40	Yes	DP, C, NSAID	No	No
7	10	28/21	Yes	po. gold, C, NSAID	No	Vaginal fungus
8	12	34/79	Yes	NSAID	No	No
9	23	28/32	Yes	im. gold, HCQ, C, NSAID	No	No
10	4	14/15	No	SSZ, HCQ, NSAID	No	No
11	15	76/36	Yes	P, C, NSAID	Yes	No
12	17	76/130	Yes	MTX, C, HCQ, NSAID	Yes	No

the membrane was incubated with alkaline phosphatase-conjugated goat-anti-rabbit IgG (1:1000 diluation in TST; Sigma, St. Louis, Mo.) for 1 h. After washing with TST for 15 min with 10 mM Tris-HCl, pH 8.0, 22 mM NaCl, the immunoblots were visualized by addition of nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) diluted to *N-N*-dimethyl-formamide (Sigma) in 20 mM Tris-HCl, 5 mM $MgCl_2$, 150 mM NaCl, pH 9.5. All incubations were performed at 22°C. The secondary antibody did not react with the bands detected by Western blotting. Positive controls for neutrophil MMP-8 and mesenchymal MMP-8 were produced as described in detail elsewhere [22, 37].

All values represent mean±SEM. For comparison between groups, *t*-test was used for normally distributed variables and Wilcoxon signed rank test for skewed variables.

Results

All patients completed their 3-month treatment so that there were no drop-outs. Significant reductions in joint score index (tender joints) were seen at 6 and 12 weeks (16±2 vs 11±2, $P<0.01$; 16±2 vs 8±1, $P<0.01$). However, Hb, CRP, ESR or changes in functional class were not seen during the 3-month-long intervention period. Pain VAS was reduced at 12 weeks (50±7 vs 34±8, $P<0.05$). Among the seven PDI variables a significant reduction was seen in domestic disability at 12 weeks (53±8 vs 42±7, $P<0.05$) and in vocational behaviour at 12 weeks (23±5 vs 10±4, $P<0.001$). None of the five CPRS variables changed during the study period. HAQ scores did not change during the trial.

Quantitative SDS-PAGE electrophoresis scanning of the patients' saliva samples showed that collagenase activity was significantly reduced at 12 weeks [15±2 vs 10±2 (% type I collagen degraded), $P<0.01$ (Fig. 1), one patient's samples]. Concomitant Western blots (Fig. 2, two patients' samples) showed intact MMP-8 enzyme (75–80 kDa band) and a smaller 40–55 kDa band. The former moved together with a purified neutrophil MMP-8 and the latter with the mesenchyml MMP-8. Fibroblast-type MMP-1 collagenase was not found in RA saliva.

All patients completed the study. Patient 1, however, contracted an oral candidiasis 3 weeks prior to the final examination. In addition, minor side effects were noted such as gastric discomfort including loose stools (patient 4) and vaginal fungal infection (patient 7), which did not lead to interruptions in the medication.

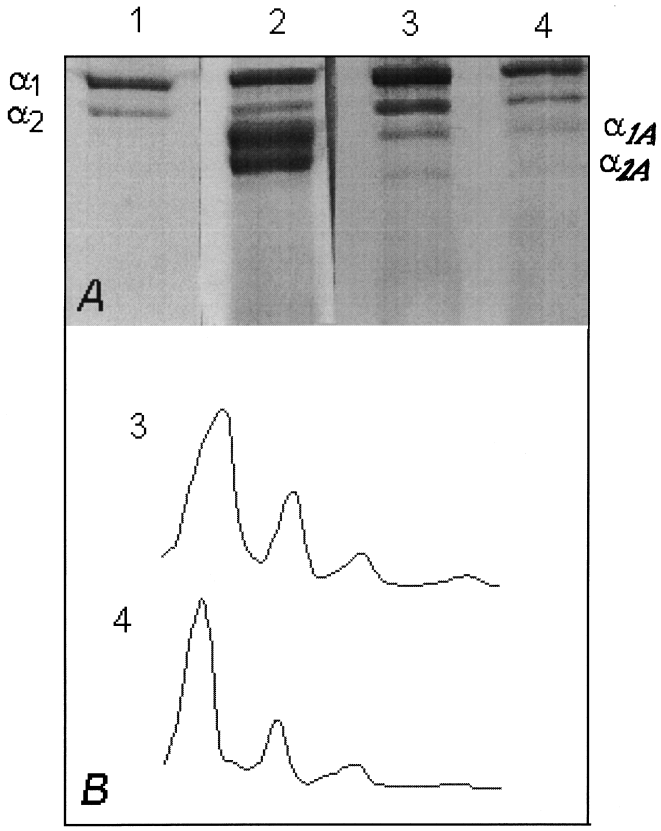

Fig. 1 Quantitative SDS-PAGE electrophoresis scanning of one patient's samples. *Lane 1* shows two type I collagen $\alpha1/\alpha2$ chains. *Lane 2* represents type I collagen incubated with purified and APMA activated MMP-8. Note the formation of degradation products $\alpha1$A and $\alpha2$A. *Lane 3* represents SDS-PAGE scanning of patient's sample before treatment with doxycycline. *Lane 4* shows the same patient's sample after treatment with doxycycline. Note the scanning results in panel B showing the higher activity peaks of degradation products $\alpha1$A and $\alpha2$A (corresponding to lane 3) and reduced in Fig. 4 (corresponding to *lane 4*) because of diminished activity caused by doxycycline treatment

Discussion

Treatment of RA with minocycline (200 mg/day) for 48 weeks in a series of 219 patients was found by Tilley and coworkers to be safe and effective for patients with mild-to-moderate disease [5]. At 48 weeks, more patients in the minocycline group than in the placebo group showed improvement in joint swelling and joint tenderness. Although no time course was indicated, the minocycline group at the end of the trial showed greater improvement in hematocrit and ESR. The present clinical findings are compatible with those recently reported. The lack of response of Hb and acute phase reactants in the present study may be due to a shorter observation period. The other main conclusion drawn by Tilley and coworkers was that the mechanisms of action remain to be determined [5]. We have had another starting point in that we, in cooperation with Professor Lorne M. Golub's group, have been studying the non-antimicrobial, anti-collagenolytic properties of tetracyclines and tetracycline derivatives [6, 7, 19]. Doxycycline inhibits "neutrophil" collagenase MMP-8 in vitro at concentrations (IC_{50} = 26 µM) readily attainable by routine treatment, whereas "fibroblast" collagenase MMP-1 is more resistant (IC_{50} = 280 µM) [19]. In addition, tetracyclines may inhibit the oxidative activation of pro-collagenases [26]. After stopping the tetracycline treatment in patients with reactive arthritis, MMP-8 activity (also in saliva) returns back to higher levels, therefore, explaining the need for long-term tetracycline regimes [38]. Recently, we have documented a beneficial effect of doxycycline

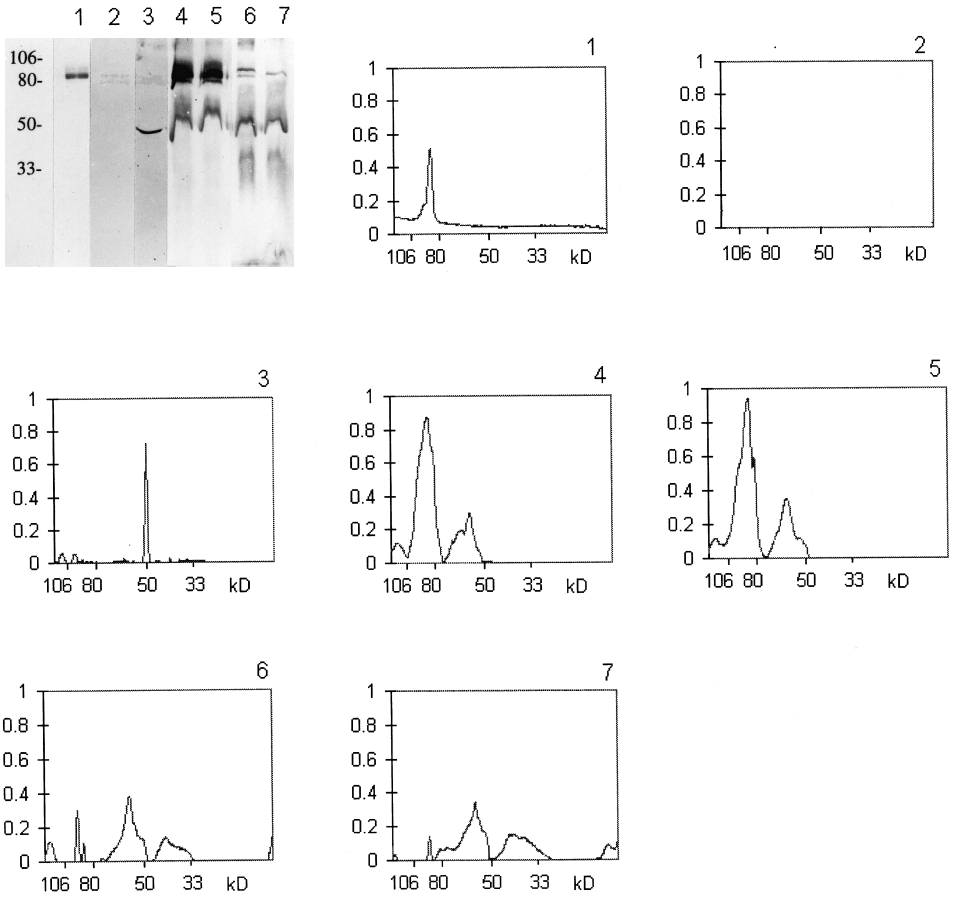

Fig. 2 Western blots of neutrophil extract containing the classic neutrophil collagenase at 75–80 kDa (*lane 1*); fibroblast supernatants without (*lane 2*) and with (*lane 3*) tumour necrosis factor-α stimulation, demonstrating the less glycosylated "mesenchymal" collagenase of MMP-8 type at 40–55 kDa; rheumatoid arthritis (RA, two patients) patient saliva before (*lanes 4 and 6*) and after (*lanes 5 and 7*) and 3-month-long doxycycline 150 mg/day treatment. Notice that (a) RA samples contain both the classic and the more recently discovered mesenchymal form of the "neutrophil" collagenase and (b) there is no fragmentation of the collagenase enzyme protein in spite of significant inhibition of the enzyme activity, as demonstrated using soluble type I collagen monomers as substrate in SDS-PAGE laser densitometric assay (see text for details)

treatment on Chlamydia-triggered reactive arthritis, which may in part be based on the inhibition of collagenase activity [26, 27, 39]. The beneficial effects of doxycycline treatment on the course of reactive arthritis can probably not totally be ascribed to its antimicrobial effects in spite of the clear role of an infection as a trigger for reactive arthritis. On the other hand, the anti-collagenolytic effects seen during doxycycline treatment of reactive arthritis cannot be ascribed to drug effects alone, because reactive arthritis has a tendency to heal spontaneously, so that the clinical symptoms tend to disappear and the acute phase response to cease. We believe the present results demonstrate that doxycycline exerts an inhibitory action on collagenase activity levels, not only in vitro [7] and in experimental animal models [40], but also in vivo in RA patients. Furthermore, this inhibition of collagenase enzymes may in part explain the beneficial effects of doxycycline treatment in arthritis.

Because of the relative resistance of fibroblast collagenase MMP-1 to tetracycline inhibition [19], it was concluded that tetracyclines would be effective mainly in diseases, in which the polymorphonuclear neutrophilic leucocyte (PMN) is the main mediator cell. This is probably the fact in arthritides characterized by a PMN predominance in the synovial fluid and, in particular, in RA which has been considered as an extravascular, intra-articular immune complex disease to PMN-mediated phagocytosis of synovial fluid immune complexes and regurgitation of proteinases during feeding [41, 42]. It was earlier believed that MMP-8 is only synthesized during the myelocyte stage of development of the PMNs and stored in the specific or secondary granules of the mature PMNs. More recently, "neutrophil" collagenase synthesis has been demonstrated also in articular chondrocytes [20, 21] and in TNF-α-stimulated synovial fibroblasts (Hanemaaijer, personal communication) [22]. In the two last mentioned cells, the MMP-8 is less glycosylated and has an apparent molecular weight of 40–55 kDa, which contrasts with the 85–80 kDa molecular weight of the "classic" PMN enzyme. These new findings seem to widen the spectrum of diseases, in which the anti-collagenolytic effects of tetracyclines might be of potential usefulness. Interestingly, the present study demonstrates a significant decrease in the total collagenolytic activity of the stimulated whole saliva. Doxycycline-mediated collagenase inhibition occurs in vitro in the presence of physiological concentrations of Ca^{2+} and Zn^{2+} in a reversible and non-competitive manner [43]. Other inhibitory mechanisms have been proposed. Smith et al. have shown that upon exposure to doxycycline human recombinant MMP-8, is fragmented to inactive low-molecular-weight species [44, 45]. However, our results do not indicate that corresponding fragmentation is associated with

decrease in collagenase activity in vivo. At the same time, Western blotting demonstrates that this total collagenolytic activity is associated with the presence of both the classic neutrophil collagenase and of the "mesenchymal" MMP-8. In fact, this is the first study, in which the mesenchymal MMP-8 isoenzyme has been demonstrated in a body fluid in man.

As this study suggests, the clinical improvement of RA patients during tetracycline treatment is associated with an inhibition of the collagenases. Due to the enzyme-species-specific inhibition of different collagenase isoforms, it seeems that this inhibition is a combined effect of doxycycline on the classic neutrophil and the newly discovered "mesenchymal" collagenases of MMP-8 type. Finally, the anti-microbial and anti-collagenolytic effects of tetracyclines can be dissociated at the molecular level: removal of the dimethylamine group from the carbon number four from the A-ring of the tetracycline (this structurally modified tetracycline is called CMT-1) abolishes its anti-microbial effects, whereas the anti-collagenolytic effects remain. The IC_{50} values of neutrophil and fibroblast collagenases for CMT-1 are 31 and 510 μM, respectively. The promising clinical results and the underlying molecular effects encourage further developments in the field.

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