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Doxycycline Inhibits Collagen Synthesis by Bovine Chondrocytes Cultured in Alginate

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Doxycycline is known for its ability to inhibit matrix metalloproteinases (MMPs), a family of enzymes that play a role in cartilage breakdown in arthritides. Its prophylactic effect in reducing joint degradation in osteoarthritis is mainly attributed to this property. In this study, we show that doxycycline exhibits a profound inhibition of collagen synthesis by bovine articular chondrocytes cultured in alginate. At 25 μM doxycycline, collagen synthesis was decreased by 50 %; no effect on cell proliferation (DNA levels) or general protein synthesis (^{35}S-Met and ^{35}S-Cys incorporation) was observed. Messenger RNA levels of type II collagen were also reduced, indicating an effect of doxycycline at the transcriptional level. The concentration of doxycycline needed to downregulate collagen synthesis was > 10-fold lower than that needed to inhibit most of the MMPs. Inasmuch as differentiated chondrocytes in the early stages of osteoarthritis exhibit increased collagen synthesis, the beneficial effect of doxycycline *in vivo* may involve prevention of changes in chondrocyte phenotype. © 1997 Academic Press

Tetracyclines possess anti-microbial properties as well as inhibitory effects towards matrix metalloproteinases (MMPs; collagenases, stromelysins and gelatinases) [1-4]. Inhibition of MMPs by for instance doxycycline, minocycline and lymecycline occurs independently of their antimicrobial activity. MMPs play an important role in the physiological and pathophysiological breakdown of extra-cellular matrix (ECM) [5-7]. The beneficial effect of doxycycline on ECM degradation in animal models and humans in periodontal and arthritic diseases [8-12] has been attributed to the inhibition of MMPs. At least two mechanisms seem to be involved: inhibition of active MMPs by chelation of calcium and zinc ions [2,13], and impairment of activation of neutrophil procollagenase (MMP-8) [14]. In spite of the large number of studies on matrix degradation, no reports exist on the effects of the drug on matrix synthesis by chondrocytes. In the present study, we investigated the direct effect of doxycycline on matrix synthesis by bovine chondrocytes and found a profound effect on collagen synthesis.

MATERIAL AND METHODS

Cell Isolation and Culture

Chondrocytes from the metacarpophalangeal joint of calves (12-14 months old) were isolated by collagenase digestion following established procedures [15-18]. Cell entrapment was performed according to Guo *et al.* [16]. Briefly, cells were suspended in 1.2 % (w/v) alginate (Keltone LVCR, Kelco, Chicago, USA) in 0.9 % (w/v) NaCl at a density of 4×10^6 cells/ml, which was passed dropwise through a 22 gauge needle into 102 mM $CaCl_2$ [15-18]. After 10 minutes of polymerization, beads were washed in 0.9 % (w/v) NaCl (3 times) and cultured in DMEM-glutamax (Gibco-BRL) supplemented with 100 U/ml of penicillin/streptomycin, 10% (v/v) FCS (Gibco-BRL) and 50 μg/ml ascorbic acid. Ten beads were cultured in 0.5 ml medium in a humid atmosphere of 5 % CO_2 in air at 37°C and medium was renewed twice a week. Doxycycline (Sigma) was dissolved in culture medium in the appropriate concentration. Treatment with doxycycline was started after 3 days of culture. At several time points, beads were harvested and stored frozen (−20°C) until analysis. Cultures and experiments were at least performed in triplicate.

Assays

DNA content. Chondrocyte containing alginate beads were digested for 2 hours at 65°C in 250 μl papain buffer (126 μg/ml papain in 50 mM potassium phosphate buffer, pH 6.5, 2 mM L-cysteine and 2 mM EDTA [19]). To 2 ml of Hoechst dye 33258 (0.1 μg/ml in 10 mM Tris, pH 7.4, 1 mM EDTA and 0.1 M NaCl) 50 μl of digest was added and fluorescence was measured in a Kontron SFM-25 fluorometer, using calf thymus DNA as a standard.

Viability test. After 7 days of culture beads were lyzed in 50 mM sodium citrate in PBS (1 hour 37°C), and the cell pellet was trypsinized to remove extracellular matrix. Cell viability was performed using the trypan blue assay.

Collagen content. Harvested beads were washed twice, once in 0.9 % (w/v) NaCl containing 10 mM $CaCl_2$ and once in saline, and were hydrolyzed in 500 μl 6 M HCl per bead at 108°C for 24 hrs. An aliquot of the hydrolysate was subjected to amino acid analysis (HPLC) to determine the amounts of the collagen-specific amino acid hydroxyproline [20]; collagen levels were determined based on 300 residues per triple helix [24].

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Protein synthesis. Chondrocytes encapsulated in alginate beads were cultured for 7 days in DMEM-glutamax (Gibco-BRL) supplemented with 100 U/ml of penicillin/streptomycin, 10% (v/v) FCS (Gibco-BRL) and 50 μg/ml ascorbic acid, and were treated with doxycycline (0-75 μM) from day 3 to 7. Thereafter, the beads were cultured in the same medium with 1 μCi Tran^{35}S label (containing ^{35}S-Cys and ^{35}S-Met, ICN Biomedicals, Netherlands) for 24 h to label newly synthesized proteins. The beads were washed three times in saline containing 1.5 mM Cys and 1.5 mM Met to remove non-incorporated radiolabel. Beads were digested in papain buffer (see above) and the resulting digest was analyzed for DNA (see above) and counted in a liquid scintillation counter (Packard Tri-Carb 1900CA). General protein synthesis was expressed per μg DNA, with data from control culture (no doxycycline) set to 100%.

Collagen mRNA Analysis

Total RNA was isolated according to Chomczynski and Sacchi [21] from $2 \cdot 10^6$ cells cultured in alginate for 7 days in complete medium (see above) containing 0, 10 or 25 μM Doxycycline. Alginate beads were lyzed in 50 mM EDTA in 50 mM sodium citrate buffer (pH 7.5) for 30 minutes at 37°C prior to RNA isolation. Ten μg of total was fractionated by electrophoresis on a 1 % (w/v) denaturing agarose gel containing 0.75% formaldehyde and transferred to a nylon membrane (Hybond N, Amersham, UK) using a Vacugene system (Pharmacia, Sweden). A 3 kB EcoR1 fragment of the human type II collagen cDNA (HC22, a kind gift of Dr. Ramirez [22]), and a 1.2 kB PstI fragment of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were used as probes. The cDNA fragments were labeled with ^{32}P-dCTP using the random primer method (Multiprime, Amersham, UK). Membranes were hybridized with 1 ng ^{32}P-labeled cDNA fragments (approximately 10^9 cpm/μg DNA) per ml in 0.5 M sodium phosphate buffer (pH 7.2) containing 7 % (w/v) SDS and 10 mM EDTA at 60°C for 18 h and subsequently washed two times for 30 minutes with 1 % (w/v) SDS in 150 mM NaCl/15 mM sodium citrate at 60°C. The membranes were exposed to Fujix intensifying screens for 16 h and relative intensities of the bands were quantified by a Fujix Bas 1000 Phosphor-imager using Tina software. The amount of type II collagen mRNA was expressed as a percentage of control culture (no Doxycycline), relative to GAPDH mRNA.

RESULTS

Effect of Doxycycline on Chondrocyte DNA Content and Matrix Synthesis

To investigate the effect of doxycycline on collagen synthesis, the antibiotic was added to the medium from culture day 3 onwards (10-75 μM). After 15 days of culture, the beads were harvested and analyzed. Only at the highest dose used (75 μM) cell proliferation, measured as DNA content of the bead, was slightly inhibited (fig.1A). At all doxycycline concentrations cell viability was $>$ 96 %, indicating that doxycycline is not toxic under the conditions used.

Remarkably, the amount of collagen synthesized was significantly lowered in the presence of doxycycline (fig.1B). For all concentrations of doxycycline, collagen/DNA curves were sigmoidal and reached a plateau after 15 days of culture. The rate of the collagen synthesis (slope at the steepest part of the sigmoidal curve) as well as the final collagen levels (plateau) were lowered in a dose- dependent manner, with an IC_{50} of approximately 20 μM (fig.2A).

In contrast to collagen synthesis, the rate of general protein synthesis (determined at day 7) was not significantly affected for up to 25 μM doxycycline; at 75 μM the rate of general protein synthesis was slightly reduced by 20 % (fig.1C).

The lower levels of collagen in the presence of doxycycline can result from either a decreased synthesis, an increased degradation or a combination thereof. Inasmuch as levels of hydroxyproline secreted in the medium were not elevated (results not shown), increased intracellular lysosomal degradation is unlikely.

Altogether, this suggests that the mode of action of doxycycline will be on the level of collagen synthesis and therefore mRNA of type II collagen, the major collagen (>95 %) synthesized by chondrocytes, was quantified.

Type II Collagen mRNA

In the presence of doxycycline, a concentration-dependent inhibition of type II collagen mRNA levels was found. At 25 μM doxycycline, type II collagen mRNA was reduced by 30 %, suggesting inhibition of collagen synthesis at the transcriptional level (Fig.2B).

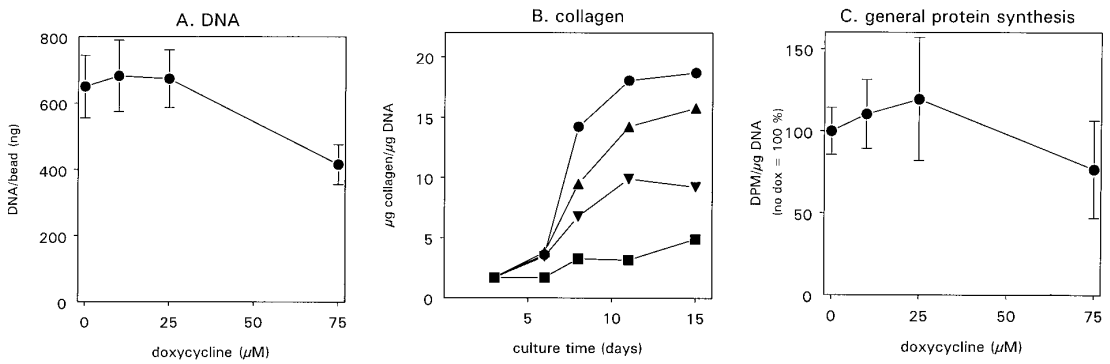

FIG. 1. Effect of doxycycline on cell proliferation (DNA, A), collagen synthesis (B) and protein synthesis rate (C) by articular chondrocytes cultured in alginate. Doxycycline was added at day 3 and maintained in the medium throughout the culture period of 15 days; see Material and Methods for details. Values for A and C were obtained after a culture period of 15 and 7 days, resp. For panel B: ●, control; ▲, 10 μM doxycycline; ▼, 25 μM; ■, 75 μM.

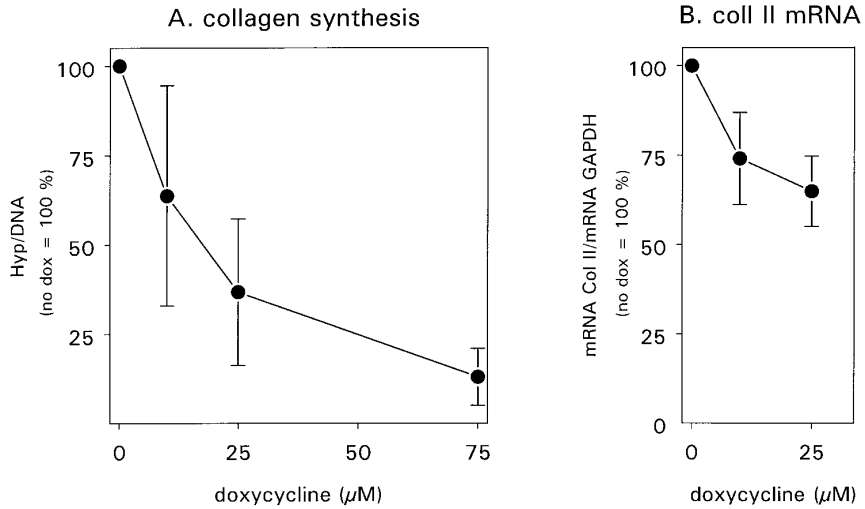

FIG. 2. Effect of doxycycline on collagen levels in the bead after 15 days of culture (A) and on type II collagen mRNA after 7 days of culture (B). Chondrocytes in alginate were cultured in the presence of doxycycline (0, 10 or 25 μM) from day 3 until the cells were harvested (see Material and Methods). The ratio of type II collagen mRNA/GAPDH mRNA from cultures without doxycycline was set to 100 %.

Reversibility of Collagen Synthesis Inhibition

To investigate whether the inhibitory effect of doxycycline on collagen synthesis is irreversible, chondrocytes were cultured in the presence of doxycycline (25 μM) from day 3 until day 11 or 15. Thereafter, the chondrocytes were cultured under control conditions to evaluate whether the chondrocytes regain their capacity to synthesize collagen. At this concentration (25 μM), doxycycline had no effect on cell proliferation (Fig.1A). After excluding the drug from the culture medium, the amount of collagen in the bead increased sigmoidally, comparable to that in control cultures (fig.3). The delay of synthesis was related to the presence of doxycycline: even after 12 days in the presence of doxycycline (from day 3-15) collagen synthesis was regained, indicating the reversibility of the effect of doxycycline on collagen synthesis.

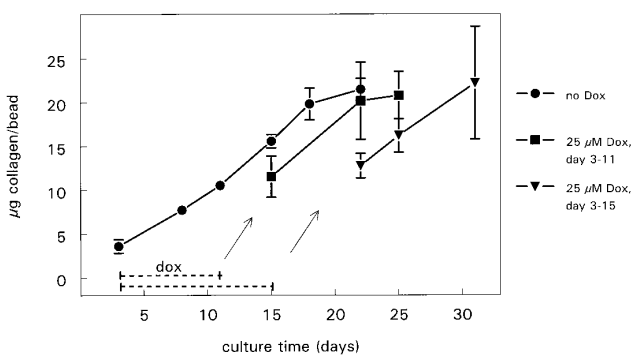

FIG. 3. Reversibility of collagen synthesis inhibition by doxycycline. Chondrocytes in alginate were cultured in the absence of doxycycline (control, ●), or with 25 μM doxycycline from day 3 until day 11 (■) or from day 3 until day 15 (▼).

CONCLUSION AND DISCUSSION

The present study shows that doxycycline selectively and reversibly inhibits collagen synthesis by articular chondrocytes (mRNA and collagen protein measurements). Under the experimental conditions doxycycline had little or no effect on cell proliferation or chondrocyte viability, consistent with findings of others [23]. Up to 25 μM, general protein synthesis (rate of incorporation of radiolabeled Cys and Met) was not affected by doxycycline. The low levels of Met and Cys in type II collagen (5 and 0 residues per 1000 amino acids, respectively [24]), the major collagen synthesized by chondrocytes cultured in alginate [15,25], explains why the strong inhibition of collagen synthesis is not reflected in the determination of the rate of general protein synthesis.

It has been suggested that doxycycline presents a new therapy for reducing joint degradation in arthritis by inhibition of MMPs [8,10-12,26]. However, doxycycline only exerted a protective effect in OA when given prophylactically [11,12], and doxycycline concentrations attained *in vivo* ($\leqslant$10 μM [12]) are not high enough to effectively inhibit MMPs (IC_{50} 80-400 μM; with the exception that MMP-8 may be substantially inhibited ($IC_{50} \approx 20$ μM; [5,27,28]). These observations suggest that MMP inhibition may not be the only or major mechanism of action of doxycycline. Interestingly, the inhibition of collagen synthesis in our study was achieved at doxycycline concentrations attained *in*

vivo, thus lower than those required to inhibit MMPs: at 10 μM substantial reduction of type II collagen mRNA and total collagen levels was observed. In early phases of OA, an increase in type II collagen mRNA as well as newly synthesized type II collagen is an important feature [29-33] that was shown to be correlated to the change in chondrocyte phenotype [32]. Our findings that doxycycline effectively suppresses collagen synthesis by chondrocytes suggests that it may delay the change in chondrocyte phenotype typically seen in OA. This is supported by the suppression of type X collagen specifically deposited by hypertrophic chondrocytes [23], a phenotype encountered in OA [32,34].

Altogether, the combined *in vitro* and *in vivo* findings suggest that the protective effect of doxycycline in OA may involve mechanisms other than MMP-inhibition alone, such as the prevention of phenotypic changes in the chondrocyte.

REFERENCES

1. Greenwald, R. A., Golub, L. M., Lavietes, B., Ramamurthy, N. S., Gruber, B., Laskin, R. S., and McNamara, T. F. (1987) *J. Rheumatol.* **14,** 28–32.
2. Golub, L. M., Ramamurthy, N. S., and McNamara, T. F. (1991) *Crit. Rev. Oral Biol. Med.* **2,** 297–322.
3. Greenwald, R. A., Simonson, B. G., Moak, S. A., Rush, S. W., Ramamurthy, N. S., Laskin, R. S., and Golub, L. M. (1988) *J. Orthop. Res.* **6,** 695–703.
4. Fife, R. S., and Sledge, G. W. (1995) *J. Lab. Clin. Med.* **125,** 407–411.
5. Golub, L. M., Sorsa, T., Lee, H. M., Ciancio, S., Sorbi, D., Ramamurthy, N. S., Gruber, B., Salo, T., and Konttinen, Y. T. (1995) *J. Clin. Periodontol.* **22,** 100–109.
6. Cawston, T. E. (1996) *Pharmacol. Ther.* **70,** 163–182.
7. Martel-Pelletier, J. McCollum, R. Fujimoto, N., Obata, K., Cloutier, J. M., and Pelletier, J. P. (1994) *Lab. Invest.* **70,** 807–815.
8. Brandt, K. D. (1995) *J. Rheumatol.* (suppl 43) **22,** 149–151.
9. Lauhio, A., Salo, T., Ding, Y., Konttinen, Y. T., Nordstrom, D., Tschesche, H., Lahdevirta, J., Golub, L. M., and Sorsa, T. (1994) *Clin. Exp. Immunol.* **98,** 21–28.
10. Greenwald, R. A., Moak, S. A., Ramamurthy, N. S., and Golub, L. M. (1992) *J. Rheumatol.* **19,** 927–938.
11. Greenwald, R. A., Chowdhury, M. H., Moak, S. A., Wang, H., Crossley, E., and Caterson, B. (1994) *Trans. Orthop. Res. Soc.* **19,** 473.
12. Yu, L. P., Smith, G. N., Brandt, K. D., Myers, S. L., O'Connor, B. L., and Brandt, D. A. (1992) *Arthritis Rheum.* **35,** 1150–1159.
13. Smith, G. N., Brandt, K. D., and Hasty, K. A. (1994) *Ann. N.Y. Acad. Sci.* **732,** 436–438.
14. Ramamurthy, N. S., Vernillo, A. T., Greenwald, R. A., Lee, H. M., Sorsa, T., Golub, L. M., and Rifkin, B. R. (1993) *J. Bone Miner. Res.* **8,** 1247–1253.
15. Beekman, B., Verzijl, N., Bank, R. A., Von der Mark, K., and Te Koppele, J. M. (1997) [Unpublished data]
16. Guo, J., Jourdian, G. W., and McCallum, D. K. (1989) *Conn. Tiss. Res.* **19,** 277–297.
17. Häuselmann, H. J., Aydelotte, M. B., Schumacher, B. L., Kuettner, K. E., Gitelis, S. H., and Thonar, E. J.-M. A. (1992) *Matrix* **12,** 116–129.
18. Häuselmann, H. J., Fernandes, R. J., Mok, S. S., Schmid, T. M., Block, J. A., Aydelotte, M. B., Kuettner, K. E., and Thonar, E. J.-M. A. (1994) *J. Cell Sci.* **107,** 17–27.
19. Kim, Y. J., Sah, R. L., Doong, J. Y., and Grodzinsky, A. J. (1988) *Anal. Biochem.* **174,** 168–176.
20. Bank, R. A., Jansen, E. J., Beekman, B., and Te Koppele, J. M. (1996) *Anal. Biochem.* **260,** 167–176.
21. Chomczynski, P., and Sacchi, N. (1987) *Anal Biochem* **162,** 156–159.
22. Su, M. W., Lee, B., Ramirez, F., Machado, M., and Horton, W. (1989) *Nucl. Acid Res.* **17,** 9473.
23. Cole, A. A., Chubinskaya, Luchene, L. J., Chlebek, K., Orth, M. W., Greenwald, R. A., Kuettner, K. E., and Schmid, T. M. (1994) *Arthritis Rheum.* **37,** 1727–1734.
24. Nimni, M. E. (1983) *Sem. Arthr. Rheum.* **13,** 1–86.
25. Petit, B., Masuda, K., D'Souza, A. L., Otten, L., Pietryla, D., Hartmann, D. J., Morris, N. P., Uebelhart, D., Schmid, T. M., and Thonar, E. J-M. A. (1996) *Exp. Cell Res.* **225,** 151–161.
26. Greenwald, R. A. (1994) *Ann. N.Y. Acad. Sci.* **732,** 181–198.
27. Paemen, L., Martens, E., Norga, K., Masure, S., Roets, E., Hoogmartens, J., and Opdenakker, G. (1996) *Biochem. Pharmacol.* **52,** 105–111.
28. Sorsa, T., Ding, Y., Salo, T., Lauhio, A., Teronen, O., Ingman, T., Ohtani, H., Andoh, N., Takeha, S., and Konttinen, Y. T. (1994) *Ann. N.Y. Acad. Sci.* **732,** 112–131.
29. Eyre, D. R., McDevitt, C. A., Billingham, M. E. J., and Muir, H. (1980) *Biochemical J.* **188,** 823–837.
30. Floman, Y., Eyre, D. R., and Glimcher, M. J. (1980) *Clin. Orthop.* **147,** 278–286.
31. Aigner, T., Stöß, H., Weseloh, G., Zeiler, G., and von der Mark, K. (1992) *Virchows Archiv B Cell Pathol.* **62,** 337–435.
32. Aigner, T., Glückert, K., and von der Mark, K. (1997) *Osteoarthritis Cart.* **5,** 183–189.
33. Aigner, T., Bertling, W., Stöß, Weseloh, G., von der Mark, K. (1993) *J. Clin. Invest.* **91,** 829–839.
34. Girkontaite, I., Frischholz, S., Lammi, P., Wagner, K., Swoboda, B, Aigner, T., and von der Mark, K. (1996) *Matrix Biol.* **15,** 231–238.