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Doxycycline-induced inhibition of prolidase activity in human skin fibroblasts and its involvement in impaired collagen biosynthesis

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Abstract

Several lines of evidence suggest that doxycycline, a semi-synthetic derivative of tetracycline, may be a useful agent in the treatment of osteoarthritis. It inhibits collagen synthesis and collagenase activity in hypertrophic chondrocytes, slowing the process of collagen turnover. However, the mechanism of doxycycline-induced inhibition of these processes has not been established. We considered prolidase, an enzyme involved in collagen metabolism, as a possible target for the doxycycline-induced inhibition of collagen synthesis. Cultured human skin fibroblasts, specialized for collagen synthesis, were used as model cells. Prolidase [E.C. 3.4.13.9] is a manganese-dependent cytosolic exopeptidase that cleaves imidodipeptides containing C-terminal proline, thus providing large amounts of proline for collagen resynthesis. Enzyme activity is regulated through the β_1 integrin receptor. Therefore, we compared the effect of doxycycline on prolidase activity and expression, collagen biosynthesis, gelatinolytic activity and β_1 integrin expression in 24-h treated cultured human skin fibroblasts. We found that doxycycline induced coordinately inhibition of prolidase activity and collagen biosynthesis (IC_{50} at about 150 μg/ml) and gelatinolytic activity in cultured human skin fibroblasts. The inhibitory effect of doxycycline on the processes was not due to the cytotoxicity of this drug, as shown in the cell viability tetrazoline test. However, an inhibitory effect of the drug on DNA synthesis was observed (IC_{50} at about 100 μg/ml). The decrease in prolidase activity in fibroblasts treated with doxycycline was not accompanied by any differences in the amount of prolidase or β_1 integrin recovered from these cells, as shown by Western immunoblot analysis. This suggests that the doxycycline-induced down-regulation of prolidase is a post-translational event. The data presented here raise the possibility that the doxycycline-induced decrease in collagen biosynthesis is mostly due to the inhibition of prolidase activity.

Keywords: Doxycycline; Collagen; Prolidase; Fibroblast

1. Introduction

Doxycycline, a member of the tetracycline family, has been shown to inhibit collagen turnover (Yu et al., 1992) and biosynthesis (Davies et al., 1996). It was also shown to decrease collagenase and gelatinase activities and thus matrix degradation in an avian explant culture system (Cole et al., 1994). This inhibitory activity appears to be independent of its antibacterial properties, since a chemically modified tetracycline retains this inhibitory ability, though it is not an active antimicrobial agent (Golub et al., 1991, 1992). Since doxycycline may be a useful agent in the treatment of osteoarthritis and approval for doxycycline has been sought for clinical trials, it has become important to understand the mechanism by which it affects collagen metabolism.

We considered prolidase as a possible target for the doxycycline-induced inhibition of collagen synthesis. Prolidase [E.C.3.4.13.9] is a cytosolic exopeptidase that cleaves imidodipeptides with a C-terminal proline (Endo et al., 1989; Phang and Scriver, 1989). The enzyme contributes to the recovery of imino acids (proline) from endogenous and exogenous protein sources, mainly collagen. The biological function of the enzyme involves the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen resynthesis (Chamson et al., 1989). The efficiency of proline recycling from imidodipeptides is about 90% (Jackson et al., 1975). Therefore, prolidase activity (despite collagen gene expression) may be a limiting factor in the regulation of collagen biosynthesis and turnover.

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A possible mechanism for the regulation of prolidase activity was suggested by experiments with fibroblast cultures in which the extracellular matrix protein composition had been altered by collagenase treatment. It was found that prolidase activity responded to extracellular matrix metabolism through a signal mediated by the β_1 integrin receptor (Pałka and Phang, 1997).

Therefore, in the present study, we compared the effect of doxycycline on prolidase activity and expression, collagen biosynthesis, gelatinolytic activity and β_1 integrin expression in cultured human skin fibroblasts.

2. Materials and methods

2.1. Materials

Glycyl–proline (Gly–Pro), bacterial collagenase (type VII), trypsin, bovine serum albumin, doxycycline, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), gelatin, Nonidet P-40, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, Protein A-Sepharose, anti-mouse IgG (whole molecule) alkaline phosphatase conjugate antibody and Sigma-Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system were purchased from Sigma (USA), as were most other chemicals used. The Dulbecco's minimal essential medium (DMEM) and fetal bovine serum used for cell culture were obtained from Life Technologies (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals (USA). Monoclonal anti-β_1-integrin antibody was obtained from ICN Biomedicals (USA). Polyclonal anti-human prolidase antibody (Boright et al., 1989) was a gift from Dr. Charles Scriver (McGill University, Montreal, Canada). Monoclonal anti-human matrix metalloproteinases MMP-2 and MMP-9 antibodies were received from R&D System, Germany. Nitrocellulose membranes (0.2 μm) and sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) molecular weight standards were received from Bio-Rad Laboratories (USA). Horseradish peroxidase-labeled anti-rabbit immunoglobulin G antibody was purchased from Promega (USA). 5[^{3}H] proline (28 Ci/mmol) and ECL-Western detection system were purchased from Amersham (UK). [^{3}H]Thymidine (6.7 Ci/mmol) came from NEN (USA). Scintillation fluid, Ultima Gold XR, was obtained from Packard (USA).

2.2. Fibroblast cultures

Normal human skin fibroblasts, obtained by punch biopsy from an 11-year-old male donor, were maintained in growth medium containing 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin at 37 °C under 5% CO_2 in an incubator. The cells were used between the 8th and 12th passages. The fibroblasts were subcultivated by trypsinization. Subconfluent cells from Costar flasks were detached with 0.05% trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA) in the calcium-free phosphate-buffered saline (PBS). For the assays, cells were cultured in six-well plates (Costar). For these experiments, cells were counted in hemocytometers and cultured at 1×10^5 cells per well in 2 ml of growth medium. Cells reached confluency at day 6 and in most cases such cells were used for the experiments. Confluent cells were treated for 24 h with the studied drug in the growth medium.

2.3. Collagen production

Incorporation of radioactive precursor into extracellular matrix proteins was measured after labeling confluent cells in serum-free medium for 24 h with 5[^{3}H]proline (5 μCi/ml, 28 Ci/mmol), as described previously (Oyamada et al., 1990). Incorporation into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase according to the method of Peterkofsky et al. (1982). Results are shown as combined values for cell plus medium fractions.

2.4. Prolidase activity assay

The activity of prolidase was determined according to the method of Myara et al. (1982), which is based on the measurement of proline with Chinard's reagent (Chinard, 1952). Briefly, the monolayer was washed three times with 0.15 M NaCl. Cells were collected by scraping and suspended in 0.15 M NaCl, centrifuged at low speed ($200 \times g$) and the supernatant was discarded. The cell pellet (from two wells of Costar plates) was suspended in 0.3 ml of 0.05 M Tris–HCl, pH 7.8, and sonicated three times for 10 s at 0 °C. Samples were then centrifuged ($18{,}000 \times g$, 30 min) at 4 °C. Supernatant was used for protein determination and then for the prolidase activity assay. Activation of prolidase requires preincubation with manganese, and therefore 0.1 ml of supernatant was incubated with 0.1 ml of 0.05 M Tris–HCl, pH 7.8, containing 2 mM $MnCl_2$ for 24 h at 37 °C. After preincubation, the prolidase reaction was initiated by adding 0.1 ml of the preincubated mixture to 0.1 ml of 94 mM Gly–Pro to a final concentration of 47 mM. After an additional incubation for 1 h at 37 °C, the reaction was terminated with 1 ml of 0.45 M trichloroacetic acid. In parallel tubes, the reaction was terminated at time "zero" (without incubation). The released proline was determined by adding 0.5 ml of the trichloroacetic acid supernatant to 2 ml of a 1:1 mixture of glacial acetic acid/Chinard's reagent (25 g of ninhydrin dissolved at 70 °C in 600 ml of glacial acetic acid and 400 ml of 6 M orthophosphoric acid) and incubated for 10 min at 90 °C. The amount of proline released was determined colorimetrically by reading absorbance at 515 nm and calculated by using a calibration curve for proline standards. Protein

concentration was measured by the method of Lowry et al. (1951). Enzyme activity is reported as nanomoles per minute per milligram of supernatant protein.

2.5. Immunoprecipitation

Confluent cells in six-well plates were rinsed with PBS, scraped out of the wells and centrifuged at 1000 × g for 3 min. Then the cells (from six wells) were solubilized with lysis buffer containing 10 mM Tris–HCl, pH 7.4, 250 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, at 4 °C for 10 min. The insoluble material was removed by centrifugation at 10,000 × *g* for 5 min at 4 °C. Supernatant containing 100 μg of protein was added to 100 μg of Protein A-Sepharose that had been linked to anti-MMP-2 or anti-MMP-9 antibody in the following manner. Protein A-Sepharose was washed three times with lysis buffer and 100 μl of suspension containing about 100 μg of beads was incubated for 1 h at 4 °C with 20 μl of the respective antibodies. Then, the conjugate was incubated for 1 h at 4 °C with shaking. The immunoprecipitate was washed four times with lysis buffer. Proteins were released from the beads by boiling in SDS sample buffer and loaded into a 10% SDS–polyacrylamide gel. The immunoprecipitates were analyzed by Western immunoblot.

2.6. SDS–PAGE

Slab SDS–PAGE was used, according to the method of Laemmli (1970). Samples of cell supernatants (50 μg of protein) were incubated for 5 min at 100 °C in 62.5 mM Tris–HCl, pH 6.8, containing 2.0% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.001% bromophenol blue. Samples were subjected to electrophoresis on a 0.1% SDS–polyacrylamide slab gel (composed of 4% stacking gel and a 7.5% separating gel) at 50 V per gel for 1.5 h at room temperature in a pH 8.3 running buffer containing 25 mM Tris, 192 mM glycine and 0.1% SDS. The following unstained high-molecular weight standards (from Bio-Rad) were used: galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa).

2.7. Western blot analysis

After SDS–polyacrylamide gel electrophoresis, the gels were allowed to equilibrate for 5 min in 25 mM Tris, 0.2 M glycine in 20% (v/v) methanol. The protein was transferred to a 0.2-μm pore-sized nitrocellulose membrane at 100 mA for 1 h by using a LKB 2117 Multiphor II electrophoresis unit according to the method described in the manual accompanying the unit. A nitrocellulose membrane containing molecular weight standards was stained for 1 min with 0.2% Panceau S, and the positions of standards were marked with S&S NC marker (Schleicher and Schuell, Germany) and destained in TBS-T solution (20 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl and 0.05% Tween 20). The nitrocellulose membrane was blocked with 5% dried milk in TBS-T for 1 h in room temperature, with slow shaking, and then incubated with polyclonal antibody against human prolidase at a concentration of 1:3000 or with anti-integrin β_1 monoclonal antibody at a concentration of 1:1000 in 5% dried milk in TBS-T for 1 h. After the incubation, the nitrocellulose membrane was washed with TBS-T (1 × 15 min and 2 × 10 min) with slow shaking. In order to analyze prolidase, second antibody, horseradish peroxidase-labeled antibody against rabbit Fc immunoglobulin G, was added at a concentration 1:5000 in TBS-T and incubated for 30 min with slow shaking. Then the nitrocellulose membrane was extensively washed with TBS-T (5 × 10 min) and submitted to ECL Western blotting detection system (Amersham) for 1 min. The nitrocellulose was covered with Saran Wrap and exposed to film for about 1 min. In order to analyze β_1 integrin subunit, second antibody (alkaline phosphatase conjugated) against mouse IgG (whole molecule) was added at a concentration of 1:7500 in TBS-T and incubated for 30 min with slow shaking. Then the nitrocellulose membrane was washed with TBS-T (5 × 5 min) and incubated with Sigma-Fast BCIP/NBT reagent.

2.8. SDS–substrate gels for analysis of proteinases

Fibroblast supernatants were subjected to substrate gel electrophoresis in 10% polyacrylamide gels impregnated with 1 mg/ml gelatin (Unemori and Werb, 1986). Samples of cell supernatants (20 μg of protein) were mixed with Laemmli sample buffer, containing 62.5 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol (lacking β-mercaptoethanol and modified to contain a final concentration of 2.5% SDS, and electrophoresed under non-reducing conditions at 50 V per gel for 1.5 h at room temperature in a pH 8.3 running buffer containing 25 mM Tris, 192 mM glycine and 0.1% SDS. In parallel gels, the following unstained high-molecular weight standards were used: galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa) and ovalbumin (45 kDa). After electrophoresis, the gel was incubated in 2% Triton X-100 for 30 min at 37 °C to remove SDS and then incubated for 18 h at 37 °C in substrate buffer (50 mM Tris–HCl buffer, pH 8, containing 5 mM $CaCl_2$). After staining with Coomassie blue R250, gelatin-degrading enzymes were identified as clear zones against a blue background.

2.9. Mitogenic assay

To examine the effect of doxycycline on fibroblast proliferation, the cells were seeded in 24-well tissue culture dishes at 1×10^5 cells/well with 1 ml of growth

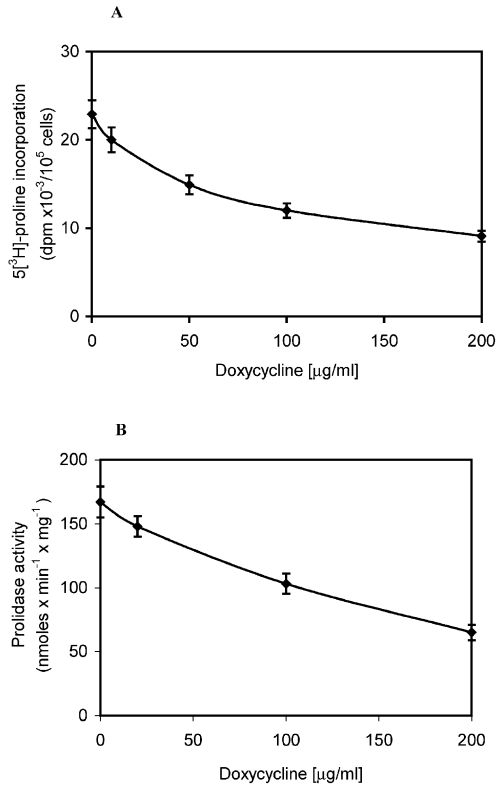

Fig. 1. Collagen biosynthesis (A) and prolidase activity (B) in confluent human skin fibroblasts cultured for 24 h with different concentrations of doxycycline. Collagen biosynthesis is reported as dpm of 5[^{3}H]proline incorporated into proteins susceptible to the action of bacterial collagenase per milligram of cell protein. The enzyme activity is reported as nanomoles of proline released during 1 min per milligram of supernatant protein.

medium. After 48 h, subconfluent cells ($1.8 \pm 0.1 \times 10^5$ cells/well) were incubated in DMEM containing 10% of fetal bovine serum and various concentrations of doxycycline (10–200 μg/ml) and 0.5 μCi of [^{3}H] thymidine for 24 h at 37 °C. After that time, the cells were rinsed three times with PBS, solubilized with 1 ml of 0.1 M sodium hydroxide containing 1% SDS, and scintillation fluid "Ultima Gold XR" (9 ml) was added and the incorporation of radioactivity into DNA was measured in a scintillation counter.

2.10. Cell viability assay

The assay was performed according to the method of Carmichael et al. (1987) using tiazolyl blue (MTT). Confluent cells, cultured in six-well plates, were washed three times with PBS and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37 °C in 5% $C0_2$ in an incubator. The medium was removed and 1 ml of 0.1 M HCl in absolute isopropanol was added to the attached cells. The absorbance of converted dye in living cells was measured at a wavelength of 570 nm. The viability of fibroblasts cultured in the presence of doxycycline was calculated as a percentage of control cells.

2.11. Statistical analysis

In all experiments, the mean values for six independent experiments ± standard deviations (S.D.) were calculated, unless otherwise indicated.

3. Results

Confluent human skin fibroblasts were used to test the effect of doxycycline on collagen biosynthesis and prolidase activity. The rationale for the use of confluent cells in the experiments was that prolidase activity (Myara et al., 1985) and collagen biosynthesis (Makela et al., 1990) are dependent on cell density and increase when the cell density increases. Collagen biosynthesis and prolidase activity were measured in fibroblasts treated for 24 h with different concentrations of doxycycline. The drug decreased, in a dose-dependent manner, collagen biosynthesis (Fig. 1A) and prolidase activity (Fig. 1B) in confluent human skin fibroblasts. The concentration of the drug required for 50% inhibition (IC_{50}) of both processes was about 150 μg/ml. In both experiments, IC_{50} values were calculated on the basis of the doxycycline concentration in the medium of cultured cells.

The decrease in prolidase activity due to the treatment of confluent cells with doxycycline may result from the suppression of enzyme expression or enzyme inactivation during post-translational modification. It has been postulated that prolidase expression is mediated through the β_1 integrin receptor (Pałka and Phang, 1997). Therefore, the Western immunoblot analysis for prolidase (Fig. 2, lanes 1 and 2) and β_1 integrin (Fig. 2, lanes 3 and 4) was

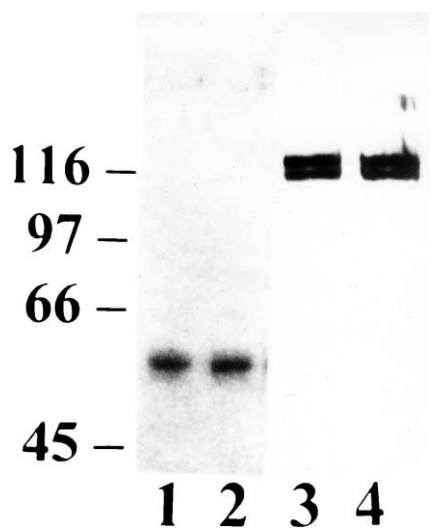

Fig. 2. Western immunoblot analysis of prolidase from control fibroblasts (lane 1), fibroblasts treated for 24 h with 150 μg/ml of doxycycline (lane 2), β_1 integrin from control fibroblasts (lane 3) and fibroblasts treated for 24 h with 150 μg/ml of doxycycline (lane 4). The same amounts of supernatant protein (20 μg) were run in each lane.

Table 1
Viability of confluent human skin fibroblasts treated for 24 h with different concentrations of doxycycline

Doxycycline (μg/ml)	Cell viability (% of control)
0	100
25	99 ± 1
100	98 ± 2
200	97 ± 2
500	87 ± 3

Mean values ± S.D. from six independent experiments are presented.

performed in control (Fig. 2, lanes 1 and 3) and doxycycline (150 μg/ml)-treated cells (Fig. 2, lanes 2 and 4). The decrease in prolidase activity due to the treatment of cells with doxycycline shown in Fig. 1B was not accompanied by a difference in the amount of the enzyme (Fig. 2, lane 2) or receptor (Fig. 2, lane 4) proteins recovered from these cells, compared to control. This suggests that the inhibitory effect of doxycycline on prolidase activity is a post-translational event.

Because the effects of doxycycline on prolidase activity and collagen biosynthesis may be a result of drug cytotoxicity, cell viability in the presence of different concentrations of doxycycline was assayed. Cell viability was measured by the method of Carmichael et al. (1987), using tetrazolinum salt. Because doxycycline might interact with tetrazolinum salt, the control test (non-specific reaction) was performed with both reagents incubated for 24 h in a cell-free system. Values were corrected for the non-specific reaction. The viability of cells incubated for 24 h with the indicated concentrations of doxycycline is presented in Table 1. As can be seen, doxycycline at concentrations up to 200 μg/ml (concentrations at which prolidase activity and collagen biosynthesis are strongly inhibited) was not cytotoxic for confluent human skin fibroblasts. An increase in the drug concentration to 500 μg/ml resulted in a 25% reduction in the viability of these cells. This suggests that the inhibitory effect of doxycycline (at concentrations up to 200 μg/ml) on prolidase activity and collagen biosynthesis is not due to the cytotoxicity of this drug. DNA synthesis was found to be inhibited by doxycycline with an IC_{50} of about 100 μg/ml (Fig. 3) in confluent fibroblasts during 24 h of incubation.

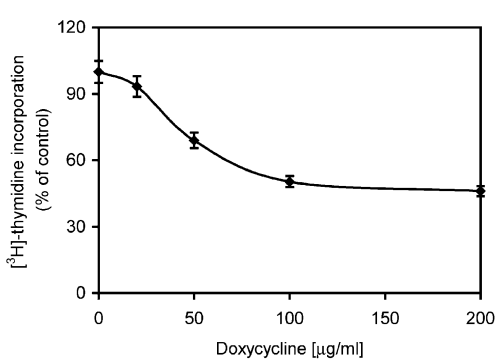

Fig. 3. DNA biosynthesis (measured as [^{3}H]thymidine incorporation into DNA) in confluent human skin fibroblasts cultured for 24 h with different concentrations of doxycycline.

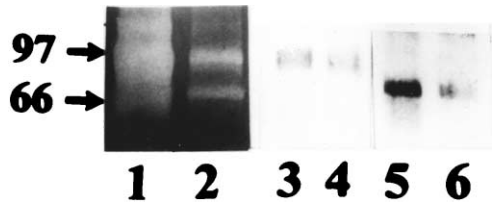

Fig. 4. Zymography (lanes 1 and 2) and Western immunoblot for immunoprecipitated MMP-9 (lanes 3 and 4) and MMP-2 (lanes 5 and 6) from conditioned medium of control fibroblasts (lanes 1, 3 and 5) and cells treated with 150 μg/ml of doxycycline for 24 h (lanes 2, 4 and 6).

The dose-dependent effects of doxycycline on collagen biosynthesis and prolidase activity suggest that it may affect the degradation of extracellular matrix collagen. As can be seen from Fig. 4, lane 1, conditioned medium from control fibroblasts contained very active gelatinases with a molecular weight between 100 and 60 kDa, as detected by zymography. However, in the cells cultured with 150 μg/ml doxycycline for 24 h, the activity of these gelatinases significantly decreased (Fig. 4, lane 2). In this case, two main gelatinases were observed: a 92-kDa protein (latent form of active 82 kDa gelatinase, MMP-9) and a 66-kDa protein (probably active form of latent 72 kDa gelatinase, MMP-2). Both of them are well-defined tissue collagenases (Brown et al., 1993). Supporting evidence for the presence of these gelatinases in conditioned media of control and doxycycline-treated cells came from the study of specific gelatinase expression by Western immunoblot analysis. As can be seen from Fig. 4, lane 3, conditioned medium of control cells contained the 92 kDa immunoprecipitable gelatinase; however, it was weakly expressed. In the cells treated with 150 μg/ml doxycycline, this gelatinase was not detected (Fig. 4, lane 4). A similar experiment was done for 72 kDa gelatinase. In this case, a large amount of 72 kDa gelatinase was recovered from the medium of control cells (Fig. 4, lane 5) and only trace amounts were recovered from the medium of doxycycline-treated (150 μg/ml) cells (Fig. 4, lane 6). This suggests that doxycycline may inhibit both the activity and expression of MMP-9 and MMP-2.

4. Discussion

The data presented here show that doxycycline inhibits collagen and DNA biosynthesis, gelatinolytic activity and prolidase activity in cultured human skin fibroblasts. The IC_{50} of doxycycline for inhibition of collagen and DNA biosynthesis and prolidase activity in fibroblast cultures was estimated to be about 150 μg/ml. At this concentration, gelatinolytic activity and the expression of MMP-2 and MMP-9 were also significantly reduced. The effects of

doxycycline were not due to the cytotoxicity of the drug. Cell viability, measured in the tetrazoline test after a 24-h incubation with the above concentration of doxycycline, was not different from that of control fibroblasts cultured without doxycycline.

The doxycycline-induced inhibition of gelatinolytic activity suggests that it may be a useful agent for the pharmacotherapy of diseases accompanied by excessive degradation of extracellular matrix proteins. Similar results were obtained with human osteoarthritic cartilage, where doxycycline was found to inhibit collagenase and gelatinase activities (Smith et al., 1998; Nordstrom et al., 1998). This activity of doxycycline seems to be promising for the treatment of rheumatoid arthritis because it reduces cartilage destruction. However, the mechanism by which doxycycline suppresses collagen biosynthesis has not been established.

The question arises as to which of the studied processes is a primary target for the doxycycline-induced effects. The doxycycline-induced decrease in collagen biosynthesis found in our studies is not likely to be due to suppression of gelatinolytic activity. In fact, a decrease in tissue collagen degradation is usually accompanied by an increase in tissue collagen content, probably as a result of the sustained capacity of cells to produce collagen (Alexander and Werb, 1991). Therefore, we considered whether the inhibition of collagen biosynthesis by doxycycline might be due to the inhibition of prolidase activity.

The biological function of prolidase involves the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen synthesis (Chamson et al., 1989). Once native extracellular collagen is cleaved into two fragments by tissue collagenases (Sunada and Nagai, 1983), the fragments themselves are susceptible to further degradation by a wide variety of extracellular and intracellular (mainly lysosomal) proteases (Sunada and Nagai, 1983). The final step of collagen degradation is mediated by cytosolic prolidase that cleaves X-pro dipeptides, which are not degraded in lysosomes (Yaron and Naider, 1993). Released proline serves as a substrate for collagen biosynthesis. It is evident that the absence of prolidase (as in prolidase deficiency) will severely impede the efficient recycling of collagen proline. The clinical symptoms related to collagen deficit can be attributed to this effect (Goodman et al., 1968). Enhanced liver prolidase activity was found during the fibrotic process (Myara et al., 1987). This suggests that prolidase, by providing proline for collagen biosynthesis, may regulate the turnover of collagen and may be a rate-limiting factor in the regulation of collagen production. Recently, a link has been found between collagen production and prolidase activity in cultured human skin fibroblasts treated with anti-inflammatory drugs (Miltyk et al., 1996), anthracyclines (Muszyńska et al., 2001), during experimental aging of these cells (Pałka et al., 1996), fibroblast chemotaxis (Pałka et al., 1997), and cell surface integrin receptor ligation (Pałka and Phang, 1997). Thus, prolidase may be a primary target for doxycycline-induced inhibition of collagen biosynthesis.

The decrease in enzyme activity due to the treatment of fibroblasts with doxycycline was, however, not accompanied by a difference in the amount of enzyme protein recovered from these cells, as shown by Western immunoblot analysis. This suggests that the inhibitory effect of doxycycline on prolidase activity is a post-translational event. It can be suggested that the phenomenon may be due to the ability of doxycycline to form a complex with Mn(II), a metal required for prolidase activity. This activity of tetracyclines is a well-known phenomenon (Gulbis et al., 1975; Williamson and Everett, 1975). It may also explain the doxycycline-induced inhibition of gelatinases, metalloproteases that require metal for catalytic activity. Similarly, the decrease in prolidase activity in doxycycline-treated cells cannot be attributed to the decrease in cell surface β_1 integrin receptors, which are known to transmit the signal for prolidase activity and expression (Pałka and Phang, 1997), because no differences in the amount of the receptor protein between control and doxycycline-treated cells were observed, as detected by Western immunoblot analysis. Another point that should be addressed is whether the doxycycline-induced inhibition of prolidase activity and collagen biosynthesis is a result of a suppression of DNA synthesis. As we found, doxycycline induced inhibition of DNA synthesis at concentrations that were shown to inhibit both prolidase activity and collagen biosynthesis. However, the decrease in prolidase activity cannot be explained by DNA suppression because the expression of prolidase in fibroblasts treated with doxycycline was the same as in control cells. The anti-mitotic effect of doxycycline in vivo may result in a decrease in the number of tissue fibroblasts, thus contributing to a decrease in the overall biosynthesis of collagen by these cells. Our results do not provide an experimental basis for the measurement of such a possibility, since in order to provide optimal conditions for the assay of collagen biosynthesis (Makela et al., 1990) and prolidase activity (Myara et al., 1985) we used confluent cells. Nevertheless, it seems possible that in vivo, both processes (decrease in cell number and decrease in prolidase activity), may be responsible for the suppression of collagen synthesis in cells exposed to doxycycline.

The data presented here raise the possibility that the doxycycline-induced inhibition of collagen synthesis is mostly due to the inhibition of prolidase activity, possibly at the post-translational level.

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