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Transcriptional Downregulation of Stromelysin by Tetracycline

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Abstract We investigated the role of tetracycline in the transcriptional regulation of matrix metalloproteinases. Using interleukin-1β (IL-1) induced stromelysin as a model system, we describe the repression of the endogenous stromelysin RNA accumulation, as well as the transcriptional inhibition of various stromelysin promoter/chloramphenicol-acetyltransferase constructs in transient transfection assays. The inhibition occurred in a dose-dependent fashion, with an IC_{50} of about 1 μM. Our results suggest that the transcriptional inhibition by tetracycline is not due to a block of activity of the activating protein complex 1 (AP-1) but is mediated by sequences upstream of the AP-1 binding site. © 1996 Wiley-Liss, Inc.

Key words: matrix metalloproteinases, antibiotics, interleukin, transcriptional regulation

The modulation of extracellular matrix plays a pivotal role in physiologic processes such as wound healing [Gross et al., 1983], inflammation [Woessner], differentiation and development [Baldwin and Kayalar, 1986; DeLotto and Spierer, 1986] and in the pathology of tumor invasion [Liotta et al., 1988; McDonnell and Matrisian, 1990], rheumatoid arthritis [Brinckerhoff, 1991], and periodontal disease [Ciancio, 1994]. Degradation of extracellular matrix is a complex event involving several different matrix-metalloproteinases (MMPs), including stromelysin (MMPIII). In contrast to a normal tissue in which stromelysin is expressed only to a very low extent, it is overexpressed in the pathologic situation and thought to be pivotal, if not causal for the development of the disease [Conca, 1993; Mauviel, 1993; Martel-Pelletier et al., 1994]. Therefore, a successful treatment of such diseases as destructive joint disease and peridontal disease could be achieved through the inhibition of the enzyme expression and/or activity. Several factors are known to block stromelysin and collagenase transcription, the most prominent of which are glucocorticoid hormones [Jonat et al., 1990; Frisch and Ruley, 1987; DiBattista et al., 1991]. Although glucocorticoids have long since been used very successfully, they are restricted mainly to the treatment of the most serious forms of degenerative diseases due to their severe side effects on the human body. Other factors capable of blocking MMP synthesis, such nonsteroidal anti-inflammatory drugs (NSAIDs) [Rashad et al., 1989; Mauviel et al., 1994; Pelletier et al., 1992], retinoic acid [Nicholson et al., 1990], or transforming growth factor-β (TGF-β) [Kerr et al., 1990], either lack efficacy or are of unknown clinical value. The search for novel agents with MMP-inhibiting potential therefore remains a focal point in research. Tetracylines have been used as antimicrobial agents in the treatment of various diseases, including periodontal disorders [Krane, 1994; Kinane, 1992]. The mechanism of their action, however, is still not completely understood. Only recently have tetracyclines been recognized as having nonantimicrobial properties: tetracyclines block mammalian collagenase and other MMP activity [Golub et al., 1987; 1991; Rifkin et al., 1993; Vernillo et al., 1994]. It has been suggested that this MMP-inhibitory effect occurs at a post-translational level and that it is based on tetracycline's ability to chelate bivalent metal ions necessary for MMP activity [Golub et al., 1987]. However, it also has been shown that tetracyclines possess a protein ki-

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nase C (PKC) inhibitory function [Webster et al., 1994]. This protein kinase reportedly plays a pivotal role in the transcriptional activation of many MMPs, namely stromelysin and collagenase [Takahashi et al., 1993; Case et al., 1990]. PKC is part of the intracellular signal pathway leading to the activating protein factor-1 complex (AP-1), which is supposed to be the transcription-stimulating factor binding to the MMP promoter [Angel et al., 1987]. In this study we investigated the involvement of tetracycline in the transcriptional regulation of MMPs. Using interleukin-1β (IL-1)-induced stromelysin as a model system, we describe the transcriptional inhibition of MMPs by tetracycline. This inhibition occurred in a dose-dependent fashion, with an IC_{50} of about 1 μM. Our results also suggest that tetracycline blocks stromelysin transcription in a manner independent of AP-1 and is mediated through sequences upstream of the AP-1 binding site.

RESULTS

Negative Regulation of Stromelysin RNA Accumulation

The negative effect of tetracycline on stromelysin expression is reflected in the level of stromelysin RNA accumulation. Northern blot analysis of RNA prepared from human GM01604A skin fibroblasts, probed with the full-length human cDNA, revealed no noticeable accumulation of stromelysin transcripts in nontreated cells (Fig. 1, lane 1). A strong signal resulted in cells treated for 24 h with IL-1 (Fig. 1, lane 2). Tetracycline, administered 15 min prior to IL-1 and present throughout duration, abolished the cytokine-induced RNA accumulation almost completely (Fig. 1, lane 4). Treatment with tetracycline alone had no visible effect due to the lack of basal activity (Fig. 1, lane 3). Rehybridization of the stripped filter with a probe for human glutaraldehyde 3-phosphate-dehydrogenase (GADPH) confirmed that equal amounts of total RNA had been loaded in each lane (Fig. 1, lanes 1–4).

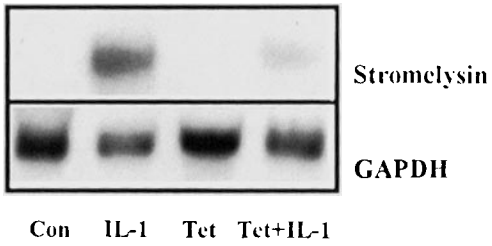

Fig. 1. Tetracycline repression of stromelysin RNA accumulation. Twenty μg of total RNA from human GM01604A skin fibroblasts was analyzed by Northern blotting. The cells had been serum deprived for 24 h previous to another 24 h of no treatment, treatment with IL-1β (30 U/ml) alone or IL-1 plus tetracycline (10 μM). The blot was probed with radioactive human stromelysin c-DNA or, as control for equal amounts of RNA per lane, human GAPDH.

Inhibition of Stromelysin Transcription

To determine whether tetracycline affects transcriptional initiation and to delimit the target of negative regulation, a series of chimaeric stromelysin–chloramphenicolacetyltransferase (CAT) constructs were analyzed in transient transfection experiments. The regulatory sequences influencing stromelysin expression are located in the 5′ flanking region of the gene [Buttice and Kurkinen, 1993], and this region also confers the inhibition of expression by tetracycline. Transfection of a promoter construct carrying human stromelysin sequences from positions −751 to +63 connected to the bacterial CAT coding region revealed that the IL-1 induction of CAT synthesis (Fig. 2, lane 3) correlates with that of the endogenous stromelysin gene shown in the Northern analysis. Although the basal activity in untreated cells was rather high (Fig. 2, lane 2), probably due to a large number of expressed genes in the transient assay, 24 h of IL-1 treatment still led to a roughly six-fold elevation of CAT accumulation. In the presence of tetracycline, IL-1 induction of CAT synthesis was suppressed in a dose-dependent manner (Fig. 2, lanes 4–6). These results suggest regulation at the transcriptional level, a finding that is confirmed by a more precise characterization of the target of negative tetracycline regulation within the stromelysin promoter (Table I).

Sequences Upstream of the AP-1 Binding Site Are Required for Tetracycline Regulation of Stromelysin

The stromelysin promoter contains two transcription factor binding sites described as key sequence elements required for the transcriptional activation of the gene by various factors, including IL-1: an AP-1 binding site at position −73 [Angel et al., 1987; Buttice and Kurkinen, 1993] and a tandem repeat element recognized by members of the Ets-family of transcription factors centered around position −208 [Wasylyk et al., 1991]. Removal of sequences upstream of the latter element led to a slight reduction in IL-1 inducibility, from 6.6-fold for the longest

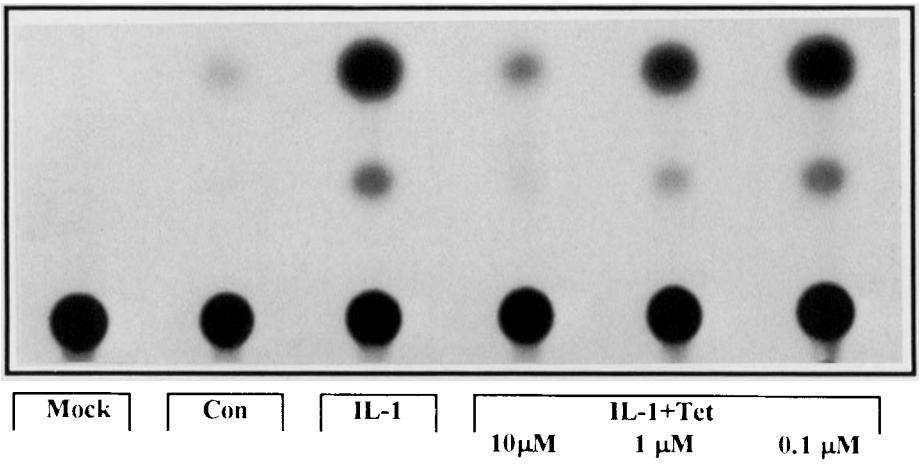

Fig. 2. Tetracycline repression of stromelysin expression. The Cat assay was performed using thin-layer chromatography and visualized by autoradiography. The panel shows the expression of the stromelysin construct −751/+63CAT. Transfection conditions are described in Table I. The concentration of IL-1 was 30 U/ml; tetracycline treatments are indicated. The amount of cell extract per lane was normalized for alkaline phosphatase activity; Cat conversion was determined by densitometry.

TABLE I. Deletion Analysis of the Stromelysin Promoter*

Stromelysin and control constructs	Basal activity[a]		Relative activity	IL-1-induced activity[a]		Relative activity
	−Tet	+Tet		−Tet	+Tet	
−751/+63	1.32 ± 0.21	1.27 ± 0.31	0.96 ± 0.09	8.71 ± 1.98	4.71 ± 0.96	0.54 ± 0.11
−478/+63	0.94 ± 0.31	1.02 ± 0.17	1.08 ± 0.12	5.35 ± 1.79	2.62 ± 0.91	0.49 ± 0.08
−208/+63	2.09 ± 0.41	1.53 ± 0.28	0.73 ± 0.19	8.77 ± 1.14	3.68 ± 0.34	0.41 ± 0.04
−130/+63	2.14 ± 0.38	2.05 ± 0.21	0.96 ± 0.13	7.70 ± 0.57	4.92 ± 0.92	0.63 ± 0.12
−75/+63	0.84 ± 0.20	0.93 ± 0.18	1.11 ± 0.21	1.09 ± 0.17	1.03 ± 0.18	0.94 ± 0.10
Δ−191/−75	1.43 ± 0.30	1.37 ± 0.17	0.95 ± 0.11	2.14 ± 0.11	2.07 ± 0.22	0.97 ± 0.06
CMV-CAT	14.3 ± 1.6	15.2 ± 2	1.06 ± 0.17	18.1 ± 1.3	15.4 ± 1.7	0.85 ± 0.06

*Five μg of various human stromelysin promoter sequences cloned into a CAT expression vector (materials and methods) was transfected into human GM01604A skin fibroblasts using the calciumphosphate method [Graham and van der Eb, 1973]. Also, 2 μg of a construct expressing SV40-promoter driven human placental alkaline phosphatase [Henthorn et al., 1988] was co-transfected to normalize for equal transfection efficiency. At 12 h after transfection, the cells were serum starved for 24 h, followed by another 24 h in serum-free medium with the indicated treatment (IL-1β: 30 U/ml; Tet: 1 μM). After harvest and protein extract preparation, phosphatase and CAT activity were determined (see Materials and Methods). The values are the mean of at least three determinations; standard deviations are indicated. Relative Activity is tetracycline treated over nontreated activity. 5′ and 3′ endpoints of stromelysin sequences are indicated by numbers, with the exception of the internal deletion in Δ−191/−75. CMV-CAT was used as an IL-1-independent control construct.
[a] % of CAT conversion.

analyzed fragment, −751/+63 (Table I, row 1) to 5.3-fold for the construct −318/+63 (data not shown). Deletion of the Ets-binding elements either in half (−208/+63, Table I, row 3, 4.2-fold) or complete (−130/+63, Table I, row 4, 3.6-fold) also led only to a slight gradual loss of IL-1 inducibility. No significant IL-1 activation could be detected when sequences farther downstream of position −130 had been deleted (−75/+63, Table I, row 5, 1.3-fold; note that this construct still contains the intact AP-1 binding site) or sequences between Ets- and AP-1 binding sites had been removed (Δ-191/−75, Table I, row 6, 1.5-fold). Tetracycline showed a repression of promoter activation on all IL-1-responsive constructs (Table I, rows 1–4, right panel). It had no effect on either construct not induced by IL-1 (Table I, rows 5–6). Furthermore, it had no effect on the basal activity of the constructs analyzed (Table I, rows 1–6, left panel). These findings prove that the tetracycline repression of stromelysin does occur at the transcriptional level, since the IL-1- and tetracycline-independent expression of the control construct CMV-CAT excluded a post-transcriptional regulation of CAT by either factor (Table I, row 7). The sequence element(s) mediating the stromelysin repression by tetracycline are

located in the stromelysin promoter between positions −130 and −75.

Specificity of Tetracycline Repression

Even at the highest concentration used in our experiments (10 μM), tetracycline did not cause a general repression of gene expression. The negative regulation is rather specific, as shown by the lack of repression of GADPH (Fig. 1). Also, in the transfection experiments, constructs carrying promoters other than stromelysin were not inhibited by tetracycline. Specifically, neither a construct driven by the cytomegalovirus-LTR (CMV-CAT, Table I, row 7) nor a SV40-promoter driven construct expressing human alkaline phosphatase [Henthorn et al., 1988] exhibited regulation by tetracycline. The latter construct was used as a cotransfected control to normalize the results of CAT expression. Since the above promoters also lack induction by IL-1, it is not clear, however, whether tetracycline interacts with the function of a selected transcriptional element or interferes with IL-1 activation of genes in general.

Downregulation of Stromelysin by Tetracycline Is Dose Dependent

The application of tetracycline in three different concentrations of 0.1, 1, and 10 μM led to a dose-dependent repression of IL-1-induced expression of both the endogenous stromelysin gene and transfected stromelysin-CAT constructs (Fig. 3). To estimate half-maximal inhibition (IC_{50}), the relative stromelysin activity of the endogenous gene, based on RNA accumulation and normalized against GAPDH activity, was plotted against the respective tetracycline concentration, with the activity in the absence of tetracycline set at 100%. Similarly, the relative CAT activity of the transfected constructs −751/+63, −130/+63, and CMV-CAT in cells treated with IL-1 and the indicated Tet concentrations (corrected to equal expression of cotransfected SV40-driven alkaline phosphatase) was plotted against the respective Tet concentrations. The resulting graph (Fig. 3) indicates that the IL-1-induced activity of both the endogenous gene and the transfected stromelysin promoter constructs is repressed halfmaximally with a tetracycline concentration of approximately 1 μM. However, a complete reduction of stromelysin or CAT expression to basal activity or below could not be reached, even with a tetracycline concentration of 30 μM (data not shown).

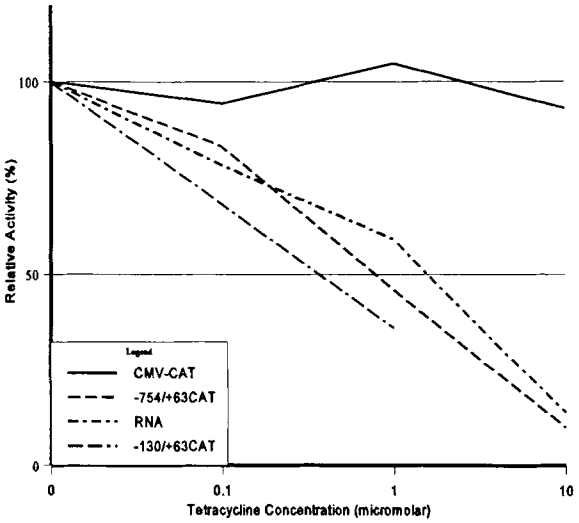

Fig. 3. Tetracycline repression of IL-1-induced stromelysin expression. The relative expression of the indicated CAT constructs (percentage of CAT conversion) and the relative amount of accumulated stromelysin RNA (densitometrically determined from Northern analysis) in cells treated with IL-1β (30 U/ml) and the indicated concentrations of tetracycline were plotted against tetracycline concentration. In each case, the activity of the gene treated with IL-1 alone was arbitrarily set at 100%.

DISCUSSION

Tetracyclines have been used due to their anti-microbial activity in the treatment of various diseases caused by gram-negative bacteria since their introduction in the late 1940s. More recently, a new feature of this class of agents has been discovered: the inhibition of matrix degrading metalloproteinases. The inhibition of collagenase has been shown in germfree and conventional animals [Golub et al., 1983], in uninfected tissues and in sterile in vitro systems [Golub et al., 1983, 1987; Greenwald et al., 1987], demonstrating an inhibitory mechanism independent of the antimicrobial property of tetracyclines. Moreover, chemically modified analogues of tetracyclines (CMTs) that lack antimicrobial activity still retained their ability to inhibit collagenase [Golub et al., 1987]. The mechanism of this collagenase inhibition was thought to occur on a post-translational level: tetracyclines may chelate bivalent cations essential for the activation of MMPs [Golub et al., 1991]. Only recently the block of RNA accumulation of Gelatinase A (MMPII) by doxycycline and CMTs has been reported [Uitto et al., 1994], indicating an inhibi-

tory regulation of MMPs at the transcriptional level. The results of our study confirm this novel tetracycline activity. We demonstrate that tetracycline downregulates the transcription of another MMP gene, stromelysin. In contrast to the inhibition by retinoic acid and glucocorticoid hormones, transcriptional inhibition by tetracycline seems not to be due to a block of AP-1 activity, since no effect on the basal activity of the gene could be detected. Rather, the inhibition depended on the presence of promoter sequences upstream of the AP-1 binding site. Deletion of these sequences, located between the binding sites for AP-1 and Ets, not only elimited tetracycline inhibition but also led to a lack of IL-1 inducibility of the remaining promoter, confirming reports suggesting a role for AP-1 only in the maintanance of basal activity [Buttice et al., 1991; Sirum-Connolly and Brinckerhoff, 1991; Auble and Brinckerhoff, 1991]. Since tetracycline inhibition in our experiments occurred only on promoter constructs that were IL-1 inducible, the precise mechanism of repression remains to be investigated; at this point, it is not clear whether tetracycline activates transcription factor(s) that inhibit IL-1-induced factor(s) or bind to the promoter DNA itself or whether tetracycline blocks the IL-1 signalling pathway. The answer to this question may give rise to a chemically modified tetracycline with a highly specific MMP inhibitory function, potentially eliciting fewer side effects in disease treatment than currently available agents.

MATERIALS AND METHODS

Plasmid Constructions

−751/+63 CAT. The construct was obtained by PCR amplification of parts of the genomic human stromelysin clone (obtained from Dr. C.E. Brinckerhoff). The commercially available M13 sequencing primer served as 5′ end oligonucleotide, binding 5′ to the insert outside the multiple cloning site of the pUC19-based genomic clone. The oligonucleotide 5′ GGATCC-TTCCACTGGCTTTACT-3′ served as 3′ end primer. With its 6-nucleotide overhang, it created a unique *Bam*HI restriction site. The initial PCR product (2.3 kb), containing 1 kb of stromelysin promoter DNA on the 3′ end, was digested with *Kpn*I to isolate 0.8 kb of stromelysin promoter sequences. After filling the ends with T4-DNA polymerase and digestion with *Bam*HI, the fragment was ligated into *Hind*III/*Bam*HI-digested pUC19. To create the CAT construct, the promoter-containing cassette was isolated by *Hind*III/*Bam*HI digestion and subcloned into *Hind*III/*Bam*HI-digested pGem7-754CAT [Kerr et al., 1990].

−478/+63CAT. For this construct, the initial 2.3-kb PCR product was digested with *Xba*I and *Bam*HI and subcloned into *Xba*I/*Bam*HI-digested pUC19. The CAT construct was obtained as described above.

−208/+63CAT. The *Xmn*I/*Bam*HI fragment from −478/+63pUC was subcloned into *Hind*III/*Bam*HI-digested pUC19. Cat construction as above.

−130/+63CAT, −75/+63CAT. Both deletion mutants were obtained by Nuclease Bal31 digestion of *Hind*III-linearized −208/+63CAT. The resulting 5′ ends were blunted with T4 DNA polymerase, and the fragment resulting from *Bam*HI digestion was subcloned into *Hind*III/*Bam*HI-digested pUC19. Construction of the CAT vectors as above.

Cell Culture

Human skin fibroblasts GM01604A (NIGMS Human Genetic Mutant Cell Depository, Camden, NJ) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Transfections and Analysis of Gene Expression

At 24 h after seeding 6–8 × 10^6 cells in 90-mm petri dishes, the DNA constructs were transiently transfected using the calcium phosphate method [Graham and van der Eb, 1973]. After transfection, the cells were serum deprived for 24 h, followed by the indicated treatment for 24 h under serum-free conditions. The cells were then harvested, and protein extracts were prepared for CAT- and alkaline phosphatase assays. CAT activity was determined with 75% of maximal available extract (approx. 150 μg) either by standard enzymatic assay [Gorman et al., 1982], followed by densitometry of the autoradiogram or by ELISA Kit (5′-3′ Inc., Boulder, CO). Alkaline phosphatase activity was determined with a heat inactivated aliquot of the cell extract (20%) according to the original protocol [Henthorn et al., 1988].

RNA Analysis

Treatment of cells in serum-free medium occurred as described above. Total RNA from 5–6 ×

10^6 cells in 140-mm petri dishes was isolated based on the guanidinium chloride method, using RNAzol B (Cinna/Biotex Laboratories, Inc., Houston, TX). Northern analysis with 20 μg of RNA was performed as previously described [Thomas et al., 1980]. The stromelysin cDNA probe was obtained by *Eco*RI digestion of a cDNA clone [Brinckerhoff et al., 1992], GAPDH was commercially available (ATCC). Both probes were radioactively labeled, using a random priming kit (Boehringer). Autoradiographs were exposed for 1–5 days.

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