Journal of Cellular Biochemistry 57:619–629 (1995)

Interleukin-1β Induction of Tissue Inhibitor of Metalloproteinase (TIMP-1) Is Functionally Antagonized by Prostaglandin E_2 in Human Synovial Fibroblasts

John A. DiBattista, Jean-Pierre Pelletier, Muhammad Zafarullah, Kazushi Iwata, and Johanne Martel-Pelletier

University of Montreal, and the Louis-Charles Simard Research Centre, Notre-Dame Hospital, Montreal, Canada H2L 4K8 (J.A.D., J.-P.P., M.Z., J.M.-P.); Research Laboratories I, Fuji Chemical Industries, Ltd., Chokeiji, Takaoka, Toyama 933, Japan (K.I.)

Abstract Elevated levels of tissue inhibitor of metalloproteases-1 (TIMP-1) have been demonstrated in inflamed synovial membranes, and it is believed that the inhibitor may play a critical role in the regulation of connective tissue degradation. The present study was undertaken to define the cellular mechanism of action of the inflammatory mediators, interleukin-1β (IL-1β) and prostaglandin E_2 (PGE_2), in the control of TIMP-1 synthesis and expression in human synovial fibroblasts. Recombinant human IL-1β induced a time- and dose-dependent saturable response in terms of TIMP-1 mRNA expression (effective concentration for 50% maximal response, EC_{50} = 31.5 ± 3.3 pg/ml) and protein synthesis (EC_{50} = 30 ± 3.3 pg/ml). The protein kinase C (PKC) inhibitors, H-7, staurosporine, and calphostin C, reversed the rhIL-1β induction of TIMP-1 mRNA. PGE_2 also inhibited rhIL-1β-stimulated TIMP-1 mRNA expression and protein secretion in a dose-dependent fashion. The concentration of PGE_2 necessary to block 50% of rhIL-1β-stimulated TIMP-1 secretion, IC_{50}, was 1.93 ng/ml (4.89 nM). Forskolin, and other stable derivatives of cAMP, mimicked, to a large extent, the effects of PGE_2. The phorbol ester, PMA, up-regulated considerably the mRNA expression of TIMP-1 but had no effect on protein production. Calphostin C substantially reduced PMA-activated TIMP-1 expression. Staurosporine, calphostin C, H-7, and substances that elevate cellular levels of cAMP, like PGE_2, also reduced basal expression and synthesis of TIMP-1. Taken together, the data suggest that PKA and C may mediate opposing effects in terms of TIMP-1 expression and secretion in human synovial fibroblasts.

Key words: interleukin-1, prostaglandin E_2, TIMP-1, human synovial fibroblasts

Extracellular matrix destruction of connective tissues is a hallmark of arthritic diseases and it is believed that, among other factors, matrix metalloproteases (MMPs) play an important role in mediating the progressive degradation of connective tissue matrix components such as collagens and proteoglycans [Harris, 1990; Dean, 1991; Martel-Pelletier et al., 1984; Pelletier et al., 1983]. There is evidence that net tissular proteolytic activity is elevated and that natural protease inhibitors are unable to contain the destruction [Dean et al., 1989]. One very important inhibitor is the tissue inhibitor of metalloproteases-1 (TIMP-1), which is capable of interacting with active collagenase and stromelysin irreversibly and with considerable specificity and affinity [Cawston, 1986; Stricklin and Welgus, 1983; Welgus and Stricklin, 1983; Welgus et al., 1985; Herron et al., 1986]. This ability has focussed much attention on the possible role of TIMP-1 in the regulation of matrix stability and turnover. Indeed TIMP-1 has been detected in many tissues and body fluids [Yamashita et al., 1992; Kodama et al., 1990], with considerable quantities having been discovered in the synovial fluids and cartilage of arthritic patients [Martel-Pelletier et al., 1994; Lohmander et al., 1993; Hayakawa et al., 1991]. In human osteoarthritic (OA) cartilage, for example, the normal basal quantitative relationship between TIMP-1 and MMPs is disturbed in favour of an increased presence of active enzymes [Dean et al., 1989]. Thus, defining the mechanism(s) governing TIMP-1 expression in

Received August 10, 1994; accepted August 31, 1994.

Address reprint requests to Dr. John A. DiBattista, Unité des Maladies Rhumatismales, Hôpital Notre-Dame, 1560 Est, Rue Sherbrooke Est, Montreal, Quebec, Canada H2L 4K8.

a temporal and tissue-specific manner may provide insight into the pathophysiology of several forms of arthritides.

TIMP-1 is expressed in a variety of cell types and can be transcriptionally regulated in cultured cells (in vitro) by serum, various growth factors, phorbol esters, cytokines, retinoids, and glucocorticoids [Clark et al., 1987; Murphy et al., 1985; Campbell et al., 1991; Takahashi et al., 1993; Wright et al., 1991; Kordula et al., 1992]. Other studies have shown that TIMP-1 mRNA expression is augmented considerably in OA synovial membranes [Spence McCachren, 1991; Zafarullah et al., 1993] in tandem with MMPs. Interestingly, tissue interleukin-1β (IL-1β) mRNA levels are also found elevated in OA synovia compared to normal synovial membranes [Zafarullah et al., 1993], suggestive of a potential relationship between the presence of IL-1β and TIMP-1/MMP production in vivo.

IL-1 is a pleiotropic cytokine and a potent mediator of the inflammatory response in connective tissues with the capacity to induce the synthesis and secretion of collagenase (MMP-1), stromelysin-1 (MMP-3), gelatinase A (MMP-2), and prostaglandin E_2 (PGE_2) by synoviocytes and chondrocytes [Wood et al., 1983; Gowen et al., 1984; McGuire-Goldring et al., 1984]. It also stimulates the production of human chondrocyte/synoviocyte plasminogen activator, which mediates the formation of plasmin, a known activator of latent MMPs [Werb et al., 1977]. Thus, much of the joint destruction, inflammation, and destabilization observed in osteo- and rheumatoid arthritis could be the result of IL-1 action.

The signal transduction pathways associated with the effects of IL-1 on a number of target genes are a matter of conjecture. Generally, secretagogues that bind to membrane receptors stimulate, among other pathways, the activity of intracellular protein kinases in order to transduce the signal from the cellular membrane to the nucleus [Meyer and Habener, 1993; Karin, 1992; Nishizuka, 1986]. IL-1 has been shown to induce the translocation of protein kinase C (read activation) from the cytoplasm to the cellular membrane in certain cell lines with the resultant induction of the effectory cellular oncogenes, *c-fos* and *c-jun* [Bunning et al., 1986; Conca et al., 1989; Schontal et al., 1988]. We and others have shown that the cytokine induces MMP-1, MMP-3, plasminogen activator, and plasminogen activator inhibitor-1 (PAI-1) expression through a protein kinase C (PKC)-mediated mechanism in normal and rheumatoid human synovial fibroblasts [Case et al., 1990; DiBattista et al., 1994a,b]. There are reports however, which suggest that PKC does not mediate IL-1 induction of MMP and eicosanoid synthesis in bovine chondrocytes [Hulkower et al., 1991; Conquer et al., 1992]. Furthermore, IL-1 has been purported to activate adenylate cyclase with the resultant increase in cellular cAMP levels in certain blood-borne derived cell lines [Munoz et al., 1990]. At the nuclear level, IL-1 can alter the genetic program of a cell by activating certain transcription factors, such as nuclear factor-κB (NF-κB) [Stylianou et al., 1992].

Prompted by our previous studies [Zafarullah et al., 1993], which showed elevated coexpression of IL-1β and TIMP-1 (MMP-1 and MMP-3 as well) in OA synovial membranes compared to normal controls, we studied the effect of rhIL-1β on TIMP-1 expression and synthesis in normal human synovial fibroblasts and investigated the possible intermediary role of PKC. In this regard, we also explored the potential role of PGE_2, a potent stimulator of cAMP-activated protein kinase (PKA) and a product of IL-1β action on connective tissue cells. Our results suggest that PKC and PKA associated signal transduction pathways may have opposing effects on TIMP-1 mRNA expression and protein secretion.

MATERIALS AND METHODS

PGE_2, dibutyryl cAMP, forskolin, diethylpyrocarbonate (DEPC), polyvinylpyrrolidone (PVP), Ficoll, salmon testes DNA, 2′,3′-dideoxyadenosine (DDA), 3-isobutyl-1-methylxanthine (IBMX), ethylenediamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), sodium acetate, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Staurosporine, H-7, calphostin C, cycloheximide, Sp-cAMP, and phorbol 12-myristate-13-acetate (PMA) were the products of Calbiochem (San Diego, CA). Recombinant human interleukin-1β (rhIL-1β, SA: 1 U/10 pg protein) was obtained from Genzyme Corporation (Boston, MA). Dulbecco's modified Eagles' medium (DMEM), Hepes, heat-inactivated fetal calf serum (FCS), stock antibiotic–antimycotic mixture (10,000 U/ml of penicillin base, 10,000 μg/ml of streptomycin base, and 50 μg/ml of amphotericin-B), agarose, and phenol were products of Gibco BRL (Gaithersburg, MD). TRIS (tris(hydroxymethyl)aminomethane), NaCl,

$MgCl_2$, $CaCl_2$, 8-hydroxyquinoline, formamide, formaldehyde, and ethanol are products of Fisher Scientific (Montreal, Quebec).

Cell Culture

Synovial fibroblasts (synoviocytes) were acquired by necropsy from the knee joints of adult cadavers within 12 h of death. Cells were isolated by sequential enzymatic digestion with filter-sterilized solutions of trypsin (1 mg/ml; 1 h) dissolved in DMEM supplemented with an antibiotic mixture followed by 6 h in the presence of collagenase (1 mg/ml) in DMEM supplemented with 10% heat-inactivated FCS and the antibiotic mixture as previously described [DiBattista et al., 1994a,b]. After digestion, the cells were collected by brief low-speed centrifugation, washed, and cultured in DMEM supplemented with 10% heat-inactivated FCS and antibiotics. Cells were released with a solution of trypsin-EDTA (0.05%–0.53 mM) and passaged 3–6 times. Unless otherwise indicated, cells were innoculated into 6-well cluster plates (1-52795A, Nunclon, Denmark) at high density, and all experiments were conducted at confluence when the cells reached a stationary phase (1.2×10^6 cells/well). In general, before experimentation, cells were exposed to low serum (0.5%) conditions for 24–48 h.

Interleukin-1β Stimulation of TIMP-1: Time Course and Dose Response

In order to ascertain optimal temporal conditions for TIMP-1 expression and synthesis, cell cultures were incubated with 50 pg/ml of rhIL-1β for 0–72 h. Medium was collected for analysis of TIMP-1 protein by enzyme immunoassay (EIA) and total cellular RNA was recovered from the cell monolayers and analyzed by Northern hybridization using labeled cRNA probes (see below). Dose–response experiments were conducted and the effective concentration necessary for 50% activation (EC_{50}) of total TIMP-1 synthesis in synovial fibroblasts was determined in experiments where cultures were exposed to 0–500 pg/ml rhIL-1β for 18–24 h at 37°C.

Effect of PMA on TIMP-1 Expression

To determine whether the effects of rhIL-1β on TIMP-1 expression could be mimicked qualitatively and quantitatively by a known potent and specific activator of PKC, the following experiments were performed. Cultures, having been preincubated as described above, were exposed to PMA (100 nM) in the presence or absence of the PKC inhibitor calphostin C (250 nM) for 24 h at 37°C in a 5% CO_2–95% air mixture. The effects of rhIL-1β (50 pg/ml) on TIMP-1 expression in cells pretreated for 24–48 h with 100 nM PMA (to down-regulate PKC) was also studied. TIMP-1 steady-state mRNA expression levels were estimated as described below.

Effects of PGE_2, cAMP Mimetics, and Protein Kinase Inhibitors on Cytokine-Activated TIMP-1 Expression and Synthesis

To ascertain the role of intracellular protein kinases in the up-regulation of TIMP-1 expression by rhIL-1β and the possibility that PGE_2, a major product of IL-1 action on connective tissue cells, might mediate/influence cytokine action, we performed the following experiments. Cells were incubated in the presence or absence of 50 pg/ml of rhIL-1β with or without increasing concentrations of PGE_2 (0.1 ng to 1 μg/ml) for 24 h at 37°C in a 5% CO_2–95% air mixture. PGE_2 was added from 1,000× concentrated ethanolic stock solutions. Where indicated, additions to the culture medium of protein kinase inhibitors, H-7, calphostin C, staurosporine, H-8, and dibutyryl cAMP, 2′,3′ dideoxyadenosine, Sp-cAMP, or forskolin, were made from concentrated stock solutions. Compounds that are water soluble were prepared as concentrated stock solutions in DMEM, whereas those that are lipid soluble were dissolved in dimethylsulfoxide (DMSO). DMSO was present at no more than 0.1% (v/v) in the culture medium. Protein kinase inhibitors were added to the culture medium 1 h prior to the addition of rhIL-1β. The inhibitory activity of calphostin C on PKC was initiated by a 1-h exposure to fluorescent light at 37°C as previously described [Bruns et al., 1991]. The concentrations of protein kinase inhibitors and cAMP mimetics used in these experiments were chosen based on prior experimentation and our previous reports [DiBattista et al., 1994a,b]. They have been optimized in terms of biological effect and maintenance of cellular protein synthesis and viability.

Companion experiments were also conducted to determine the effects of PGE_2 and cAMP mimetics/inhibitors, and protein kinase inhibitors on baseline (control) levels of TIMP-1 in the

absence of rhIL-1β stimulation. TIMP-1 protein and mRNA were measured as described below.

Quantitation of TIMP-1 by EIA

The concentration of TIMP-1 in conditioned medium of human synoviocytes was determined by the recently established one-step sandwich EIA system [Kodama et al., 1990]. The assay measures both free and complexed TIMP-1 protein although, in human synoviocyte culture medium, we detect exclusively zymogenic forms of MMPs [DiBattista et al., 1994a], suggesting that assayable TIMP-1 is in its free form. The sensitivity 0.24 ng/ml (1.5 pg/microtiter well) and linearity was obtained at 1.2–49 ng/ml (7.5–300 pg/microtiter well).

Northern Blot Analysis of TIMP-1 mRNA

Total cellular RNA was isolated as previously described [Aiba et al., 1981] with minor modifications. Briefly, cells were lysed in a preheated buffer (60°C) containing 20 mM sodium acetate, pH 5.0, 1% SDS, and 1 mM EDTA. The lysate was then extracted 3 times with preheated phenol (equilibrated in 20 mM sodium acetate, pH 5.0) and RNA in the resultant aqueous phase was precipitated with 2 vol of absolute ethanol overnight at −20°C. Following solubilization of the RNA pellet in DEPC-treated sterile H_2O, RNA was quantitated spectrophotometrically at 260 nm, and the OD_{260}/OD_{280} was 1.7–2.0, with no detectable genomic DNA contamination, as judged by agarose gel electrophoresis.

Generally, 5 μg of total RNA was resolved on 1.2% agarose–formaldehyde gels as previously described [DiBattista et al., 1994a]. Following transfer to nylon membranes (Zetaprobe, Bio-Rad, Richmond, CA) in Tris-acetate buffer, pH 7.8 overnight at 4°C, the RNA was crosslinked to the membranes by exposure for 10 min to ultraviolet (UV) light. Prehybridization was performed for 18 h at 68°C in SET buffer (60 mM Tris, pH 7.4, 450 mM NaCl, 3 mM EDTA) containing 10× Denhardt's solution, 250 μg/ml yeast RNA, 50 μg/ml denatured salmon testes DNA, 10 μg/ml polyadenylic acid, 0.1% SDS, and 0.1% sodium pyrophosphate. Hybridization was carried out in the same buffer containing ^{32}P-labeled cRNA probes (10^8–10^9 cpm/μg RNA; 6×10^6 dpm/ml buffer) for TIMP-1 or GAPDH for 24–36 h at 68°C. The labelling nucleotide was [α-^{32}P]-CTP (3,000 Ci/mmol, Amersham Canada Ltd., Oakville, Ontario).

The human TIMP-1 cDNA probe (0.8 kb inserted into *Eco*RI sites of Bluescript) was kindly provided by Dr. R.H.L. Pang (Creative Biomolecules, Hopkinton, MA). An antisense cRNA was generated by linearizing the plasmid with *Xba*I and polymerizing with T3 RNA polymerase. A 780-bp *Pst*I/*Xba*I fragment from GAPDH cDNA (1.2 kb; American Type Culture Collection, Rockville, MD) was subcloned into pGEM-3Z vector (Promega Biotech, Madison, WI), from which was synthesized a cRNA probe after linearization with PstI. This latter probe served as a control of RNA loading, as GAPDH is constitutively expressed.

Stringent serial post-hybridization washes were conducted at 68°C, with a final wash of 0.1 × SET, 0.1% SDS and 0.1% sodium pyrophosphate. Following brief rinsing at RT in 3 × SET, the membranes were subjected to autoradiography using Kodak XAR5 films (Eastman Kodak LTD, Rochester, NY) and Cronex intensifying screens (DuPont Canada, Mississauga, Ontario) at −80°C. All blots were subjected to laser scanning densitometry (GS-300 Hoefer Scientific Instruments, San Francisco, CA) for semiquantitative measurements and the results expressed as the relative amount of TIMP-1 mRNA normalized to the level of GAPDH mRNA.

DNA, Protein, and Data Analysis

Values were expressed as mean ± SD of the mean, and n refers to the number of different cell lines (i.e., patients). Cellular DNA content was determined by the method of Burton [1956], using salmon sperm DNA as a standard. Cytosolic protein was estimated by the BioRad protein assay reagent using a mixture of γ-globulin and BSA (80/20, respectively) as a standard. Statistical significance was assessed using the Student's *t*-test. Significant differences were confirmed only when the probability was less than or equal to 5%. Where appropriate, analysis of variance (ANOVA) was performed.

RESULTS

Effect of rhIL-1β on TIMP-1 Synthesis and Expression

Preliminary experiments revealed that rhIL-1β-stimulated TIMP-1 production increased in a linear fashion ($r^2 = 0.987$) as a function of time up to 72 h (Fig. 1A). As such, we found it appropriate to perform subsequent experiments, dealing with TIMP-1 protein synthesis and secre-

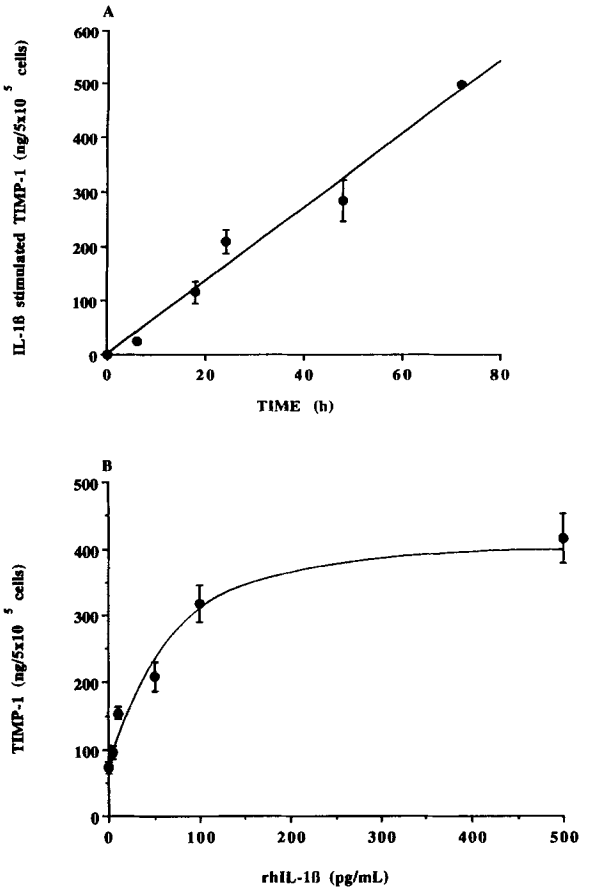

Fig. 1. Time course (**A**) and dose–response effects (**B**) of rhIL-1β stimulation of TIMP-1 secretion. Cultured human synoviocytes were incubated with (A) rhIL-1β (50 pg/ml) at 0–72 h at 37°C or with (**B**) increasing concentrations of rhIL-1β (0–500 pg/ml) for 24 h at 37°C. Conditioned medium was then recovered, and total TIMP-1 levels were quantitated by a specific EIA assay, as described under Materials and Methods. TIMP-1 synthesis (ng/5 × 10^5 cells) in the absence of rhIL-1β (control, see intercept y-axis)) was 78.9 ± 6.3 for (**B**). Values represent the mean ±SD (n = 3–4 cell lines). One-way ANOVA established a statistical difference for curve (**B**) at a level of $P < 0.001$.

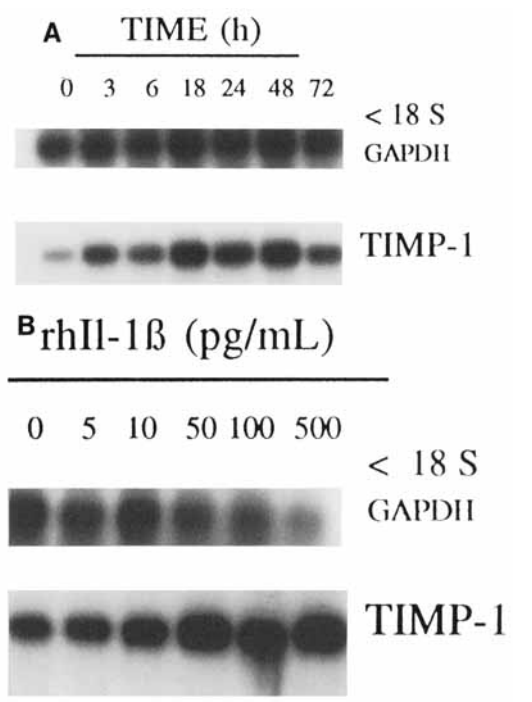

Fig. 2. Time course (**A**) and dose–response effects (**B**) of rhIL-1β stimulation of TIMP-1 mRNA expression. Cultured human synoviocytes were incubated with (**A**) rhIL-1β (50 pg/mL) for 0–72 h at 37°C or with (**B**) increasing concentrations of rhIL-1β (0–500 pg/ml) for 24 h at 37°C. Cell monolayers were then extracted for total RNA; 5 μg was electrophoresed through a formaldehyde–agarose gel and transferred to nylon membranes. The blots were pre-hybridized for 18 h and hybridized for 24 h at 68°C with a ^{32}P-labeled-cRNA TIMP-1 probe. Membranes were reprobed with GAPDH ^{32}P-labeled-cRNA to obviate differences in RNA loading. A representative of three experiments is shown. Densitometric analysis revealed the following average TIMP-1/GADPH ratios. A: 0 h, 0.40; 3 h, 0.44; 6 h, 0.64; 18 h, 0.70; 24 h, 0.66; 48 h, 0.62; 72 h, 0.45. B: Control, 0.85; 5 pg/ml, 1.32; 10 pg/ml, 1.43; 50 pg/ml, 1.62; 100 pg/ml, 1.76; 500 pg/ml, 1.74.

tion, for 24 h. Dose-response studies revealed that IL-1 stimulation of TIMP-1 production was dose dependent and apparently saturable with an EC_{50} of about 30 ± 3.3 pg/ml (i.e., 3 U/ml; second-degree polynomial function, $r^2 = 0.964$, Fig. 1B). IL-1β-induced TIMP-1 mRNA expression reached a zenith after about 18 h, remained stable up to 48 h, and then appeared to decline (Fig. 2A). TIMP-1 mRNA levels increased in an apparently dose-dependent and saturable fashion in the presence of increasing concentrations of rhIL-1β, with the maximum observed at 31.5 ± 5.2 pg/ml of cytokine (Fig. 2B). These latter values were calculated from a second order polynomial function ($r^2 = 0.982$) of the optical density of hybridization bands of TIMP-1 versus the concentration of rhIL-1β.

Effect of PGE_2 on TIMP-1 Synthesis and Expression

PGE_2, a by-product of IL-1β stimulation of arachidonic acid metabolism in connective tissue cells, inhibited rhIL-1β-stimulated TIMP-1 secretion in a dose-dependent fashion, with an IC_{50} of about 1.93 ng/ml (4.89 nM; linear regression, $r^2 = 0.93$, Fig. 3A). As can be seen in Figure 3A, PGE_2, at higher concentrations, depressed TIMP-1 levels below controls (0 on the ordinate; 81.6 ± 9.1 ng/5 × 10^5 cells) confirming other results showing that PGE_2 inhibits basal, resting levels of TIMP-1 secretion (data

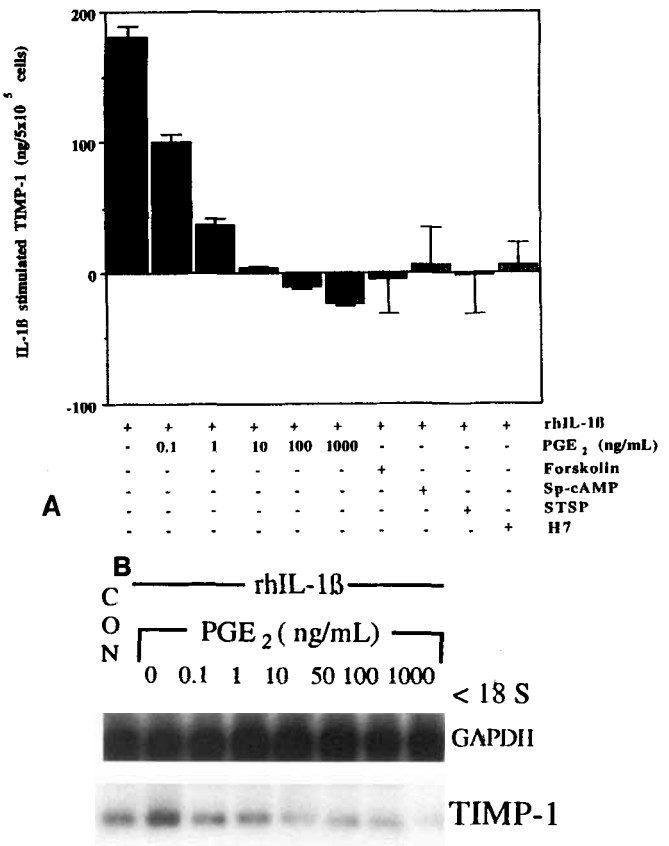

Fig. 3. Dose dependent suppression by PGE_2 of TIMP-1 secretion (**A**) and mRNA expression (**B**). A: Cultured human synoviocytes were incubated with rhIL-1β (50 pg/ml) for 24 h at 37°C with or without increasing concentrations of PGE_2 (0–1,000 ng/ml) or with forskolin (60 μM), H-7 (20 μM), Sp-cAMP (200 μM), staurosporine (STSP, 50 nM), Spent medium was collected and analyzed (n = 5) for TIMP-1 protein by EIA, as described under Materials and Methods. Control values, i.e., 0 on the graph = 81.6 ± 9.1 ng/5 × 10^5 cells. B: Cell monolayers were then extracted for total RNA; 5 μg was electrophoresed through a formaldehyde–agarose gel and transferred to nylon membranes. The blots were prehybridized for 18 h and hybridized for 24 h at 68°C with a ^{32}P-labeled-cRNA TIMP-1 probe. Membranes were re-probed with GAPDH ^{32}P-labeled-cRNA to obviate differences in RNA loading. A representative of two experiments is shown. Densitometric analysis revealed the following average TIMP-1/GADPH ratios: control, 0.62; rhIL-1β, 1.42; rhIL-1β + 0.1, 1, 10, 50, 100, and 1,000 ng/ml PGE_2, 0.73, 0.71, 0.59 0.57, 0.58, and 0.47, respectively.

not shown). PGE_2 also down-regulated TIMP-1 mRNA expression in a dose-dependent fashion (Fig. 3B) reminiscent of that seen for TIMP-1 protein.

Effect of cAMP Mimetics, Protein Kinase Inhibitors, and PMA on TIMP-1 Secretion and Expression

cAMP mimetic compounds like forskolin (60 μM), and Sp-cAMP (200 μM), a metabolically stable derivative of cAMP, significantly ($P < 0.001$) reversed the cytokine-induced increase in TIMP-1 secretion giving 78.2 ± 17.3 ng/5 × 10^5 cells and 85.6 ± 20.5, respectively, as did the protein kinase inhibitors H7 (87.0 ± 18.5) and staurosporine (79.0 ± 29.5) (Fig. 3A).

Forskolin (60 μM) and dibutyryl cAMP (500 μM) also reversed cytokine-induced TIMP-1 mRNA expression, while the adenylate cyclase inhibitor, 2′,3′ dideoxyadenosine was without effect in this regard (Fig. 4A). The protein kinase inhibitors H7 (20 μM), staurosporine (50 nM), and calphostin C (250 nM) also abrogated the effects of rhIL-1β in terms of TIMP-1 mRNA expression, while H8, a PKA inhibitor, had a lesser effect.

The phorbol ester, PMA (100 nM), strongly upregulated the expression of TIMP-1 mRNA, an effect that was substantially reversed by preincubating cells with the PKC inhibitor, calphostin C (Fig. 5). PMA did not, however, increase TIMP-1 protein secretion (data not shown). Protein kinase inhibitors not only block the effects

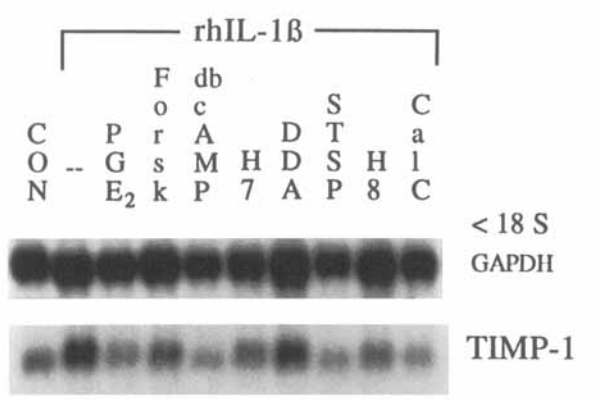

Fig. 4. Effects of PGE_2, forskolin, dibutyryl cAMP (db-cAMP), 2′,3′-dideoxyadenosine (DDA), H-7, and staurosporine (STSP), H8, and calphostin C on rhIL-1β-activated TIMP-1 mRNA expression. Cultured human synoviocytes were incubated in the presence or absence (control) of rhIL-1β (50 pg/ml) plus either 10 ng/ml of PGE_2, forskolin (60 μM), H-7 (20 μM), cAMP (500 μM), DDA (140 μM), STSP (50 nM), H-8 (2 μM), or calphostin C (Cal C, 250 nM) for 24 h at 37°C. The cell monolayer was extracted for total RNA; 5 μg was electrophoresed through a formaldehyde–agarose gel and transferred to nylon membranes and processed for Northern analysis, as described under Materials and Methods. A representative of two experiments is shown. Densitometric analysis revealed the following average TIMP-1/GADPH ratios: control, 0.20; rhIL-1β, 0.61; rhIL-1β + PGE_2, forskolin, db-cAMP, H-7, DDA, STSP, H-8, Cal C, gave 0.18, 0.21, 0.15, 0.25, 0.63, 0.15, 0.49, and 0.15, respectively.

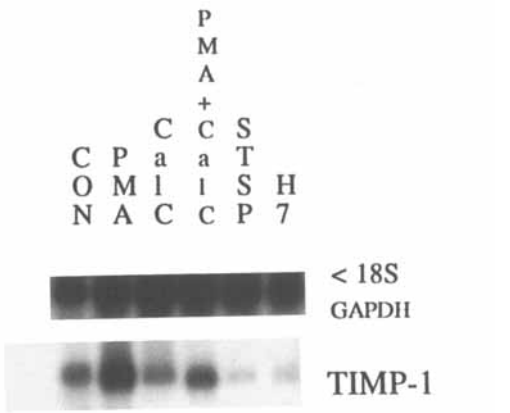

Fig. 5. Effect of PMA and protein kinase C inhibitors on TIMP-1 mRNA expression. Cultured human synoviocytes were incubated with PMA (100 nM) for 24 h at 37°C with or without the specific protein kinase C inhibitor calphostin C (250 nM). Additionally, cells were exposed to calphostin C (Cal C), staurosporine (STSP, 50 nM), and H7 (20 μM) alone. The cell monolayer was extracted for total RNA; 5 μg was electrophoresed through a formaldehyde–agarose gel and transferred to nylon membranes and processed for Northern analysis, as described under Materials and Methods. A representative of three experiments is shown. Densitometric analysis revealed the following average TIMP-1/GADPH ratios: control, 0.40; PMA, 1.05; Cal C, 0.32; PMA + Cal C, 0.46; STSP, 0.25; H7, 0.29.

of rhIL-1β but also can suppress basal resting levels of TIMP-1 mRNA expression (e.g., calphostin C, staurosporine and H7; Fig. 5). This was not due to inhibitor-induced cell death, since, at the concentrations employed in these experiments, we recovered >95% of cells initially plated; also, GAPDH mRNA levels were consistent. Results of the effects of rhIL-1β on TIMP-1 expression in the presence of PMA-down-regulated PKC (preincubation of PMA for 24–48 h) were ambiguous due to persistently elevated expression levels of TIMP-1 (data not shown).

DISCUSSION

The present study was prompted by, among other basic interests, the previous observation that inflamed human OA synovial membranes coexpressed elevated levels of TIMP-1 and IL-1 mRNA compared to normal controls [Zafarullah et al., 1993]. Despite the plethora of cytokines (e.g., tumor necrosis factor-α [TNFα] and interleukins-6 and -1 [IL-6, IL-1]) and growth factors (e.g., IGF-1, TGF-β, bFGF) present in diseased synovial membranes, we tested in vitro whether the coexpression was merely fortuitous or was significant in terms of the pathophysiology of the disease. In general, coexpression of other cytokines/growth factors and TIMP-1 in diseased synovial membranes has not been observed. We demonstrate here that indeed IL-1β can significantly upregulate the expression and secretion of TIMP-1 by a mechanism probably involving PKC.

IL-1 is pluripotent cytokine and an important mediator of inflammation by virtue of its ability to activate immune cell trafficking and responses, promote the expression and synthesis of matrix destructive metalloproteases (e.g., collagenase, stromelysin, and gelatinase), and stimulate the production of the immune-system modulator and inflammatory mediator, PGE_2 [Dayer et al., 1986]. No consensus has emerged as to how the IL-1 signal is transduced in target cells, and it is likely that this will vary, depending on the cell type and cell response. We and others have recently shown that IL-1β-induced upregulation of collagenase and stromelysin expression in human synovial fibroblasts is mediated, at least partially, by activation of PKC [Case et al., 1990; DiBattista et al., 1994a]. Acting potentially in a negative feedback loop, PGE_2, reverses the effects of IL-1 by potently inhibiting metalloprotease expression, a process that was shown to be mediated by the activation of PKA, via cAMP [DiBattista et al., 1994a].

Our data presented here strongly favour a similar mechanism of IL-1β action on TIMP-1 expression and synthesis in connective tissue cells. Substances which either inhibit PKC (ex. H7, staurosporine, and calphostin C) or elevate cellular levels of cAMP and activate PKA, significantly abrogate IL-1β induced effects. These findings are essentially identical to what we observed for MMP expression. Admittedly, H7 and staurosporine do not display an absolute specificity for PKC [Gadbois et al., 1992; Kobayashi et al., 1989]; however, calphostin C binds specifically to the regulatory domain of the kinase [Kobayashi et al., 1989] and has effects that are quantitatively and qualitatively similar to those observed using H7 and staurosporine. The concentrations of the latter two inhibitors that we used were about 3–5 times the IC_{50} observed using isolated extracts of PKC [Gadbois et al., 1992], ensuring that we saturate most, if not all, binding sites on the kinase. Furthermore, under similar conditions, H7 has been shown to block the cytosolic to membrane transition of PKC following activation and to inhibit its activity in mouse neuroblastoma 2a C-1300 cells [Felipo et al., 1990]. In support of our findings, Edwards et al. [1992], using transient transfection experiments and gel retardation assays, identified consensus binding sites for AP-1 (inducible by IL-1) in the promoter region of the murine TIMP gene identical to those found in the promoter region of collagenase and stromelysin genes. Furthermore, in proximity to AP-1 motifs, binding sites for PEA-3 (polyomavirus enhancer) transcription factor are found in TIMP-1, stromelysin-1, and collagenase promoter regions. Both AP-1 and PEA-3 factors are likely to be involved in the transcriptional regulation of these genes by PMA and IL-1. Thus data from various types of experiments seem to suggest that TIMP-1, collagenase, and stromelysin-1 (also 92-kd gelatinase B) may be coordinately regulated through the actions of a shared set of *cis* elements and *trans*-acting factors, although there may be promoter elements in each of these genes that mitigate gene-specific mechanisms of regulation.

Interestingly, H7, staurosporine, and calphostin C reduced the basal levels of TIMP-1 mRNA expression and protein secretion suggesting that "constitutive" TIMP-1 production may be also under the positive influence of PKC activity within the resting cell. These results were observed in the absence of any inhibitor effects on cell protein synthesis or cell viability. In this context it is noteworthy that, in cell-free transcription systems, AP-1 binding sites were also found necessary to the maintenance of "constitutive" TIMP-1 expression [Ponton et al., 1992]. In this regard, relatively high levels of PGE_2 (50–100 ng/ml) produced the same effect although resting levels of TIMP-1 production were apparently not as sensitive to the eicosanoid as those stimulated by IL-1β.

To further dissect signal transduction pathway governing TIMP-1 expression initiated by the binding of IL-1β to its cell surface receptor, we used a specific and potent activator of PKC, namely PMA. As mentioned above, phorbol ester response elements (AP-1) have been identified in the promoter region of the TIMP-1 gene, which likely binds one of the family of AP-1 proteins (dimers of C-FOS/C-JUN). Indeed, as has been reported in studies using many different cells lines [Murphy et al., 1985; Campbell et al., 1991], we observed a rather large increase in the expression levels of TIMP-1 that was completely reversed by pre-incubating cells with calphostin C for 60 min. These results appeared to reflect very closely those observed when using IL-1β, strenghthening the notion that the latter cytokine acts to upregulate TIMP-1 expression by activating PKC. Parenthetically, IL-1 also rapidly (30–60 min after binding) upregulates c-*fos* and c-*jun* expression in human connective tissue cells [Conca et al., 1989]. Furthermore, PMA and IL-1 apparently induce similar nuclear DNA binding proteins as judged by gel retardation assays using ^{32}P-labeled fragments of the stromelysin promoter [Sirum-Connolly and Brinckerhoff, 1991].

While dramatically stimulating TIMP-1 expression, PMA does not augment the level of TIMP-1 protein secretion, in contrast to what we observed with IL-1β. This same phenomenon was reported in HepG2 cells [Kordula et al., 1992]; indeed, we and others have reported that plasminogen activator inhibitor-1 (PAI-1) and interleukin-1 receptor antagonist (IL-1ra) mRNA were stimulated severalfold in the presence of the phorbol ester, but there was no protein production [DiBattista et al., 1994b; Krzesicki et al., 1993]. These results have not been fully rationalized but are unrelated to PMA-induced prolongation of mRNA half-life (accumulation of cytoplasmic mRNA), since the phorbol ester increases the transcriptional rates of the latter two genes.

The present study compared alongside our previous work on cytokine and prostaglandin control of MMP expression and synthesis suggests that both matrix proteases and their natural inhibitor are coordinately regulated by several important secretagogues (e.g., IL-1). It is known that similar cell types in human synovial membranes express collagenase, stromelysin, and TIMP-1, as judged by in situ hybridization and immunohistochemistry [Gravallese et al., 1992; Pelletier et al., 1992]. This parallelism may not be limited to the latter enzyme/inhibitor system as we and others observed a similar form of regulation for plasminogen activator (PA), a serine protease and activator of plasmin, and plasminogen activator inhibitor-1 (PAI-1) in connective tissue cells [DiBattista et al., 1994b; Mochan et al., 1986; Kooistra et al., 1991]. Careful analysis of data on production rates revealed that the molar ratio of TIMP-1:MMP-1 (this study and that reported in DiBattista et al. [1994a] are identical experiments) in resting synoviocytes is at least greater than 10, whereas the ratio drops to 1.56 ± 0.95 (mean ±SD; n = 8 different human cell lines) in cells activated by saturating concentrations of rhIL-1β. Despite the impressive increase in MMP production in the presence of the cytokine, TIMP-1 levels rise as well seemingly to ensure a kind of stoichiometric balance between enzyme and inhibitor at critical times. The in situ hybridization studies conducted by Spence McCachren [1991] also show an altered balance in the expression of MMPs and TIMP-1 during inflammation (OA and RA synovia), one in which TIMP-1 is still prominent. It may be that MMPs and their inhibitors evolved in tandem sharing common regulatory mechanisms but then diverging to develop specialized gene-specific modes of regulation to deal with various physiological or environmental challenges.

In summary, we believe that the present data improve our understanding of connective tissue homeostasis and provide new insights into the functional profile of IL-1. Also, this work, together with previously published data, indicate that IL-1 is capable of inducing and possibly coordinating the expression of TIMP-1 and MMPs in human synovial fibroblasts through a series of common cytoplasmic signal transducing pathways and nuclear cis elements and *trans*-acting factors. PKA and PKC may mediate opposing effects in terms of TIMP-1 expression and secretion in connective tissue cells.

ACKNOWLEDGMENTS

We thank Santa Fiori for help in the preparation of the manuscript and Nathalie Morin M.Sc., and Francois Cyril Jolicoeur, B.Sc., for expert technical assistance. This research was supported by grants (J.D.B.) from the Canadian Arthritis Society and the Medical Research Council of Canada. This work is dedicated to Prof. Thomas Sandor, F.R.S.C., on his 70th birthday; a mentor and a friend.

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