Cyclooxygenase inhibitors augment the production of pro-matrix metalloproteinase 9 (progelatinase B) in rabbit articular chondrocytes

Akira Ito[a,*], Takashi Nose[a], Shuya Takahashi[b], Yo Mori[a]

[a]*Department of Biochemistry, Tokyo College of Pharmacy, 1432 Horinouchi, Hachioji, Tokyo 192-03, Japan*
[b]*Department of Pharmacology, Research Center, Taisho Pharmaceutical Co., Yoshino-Cho, Ohmiya, Saitama 330, Japan*

Received 11 January 1995

Abstract Matrix metalloproteinase 9 (MMP-9/gelatinase B) has recently been proposed to participate in the destruction of articular cartilage. Here, we report that interleukin 1 (IL-1) enhances the production of the precursor of MMP-9 in rabbit articular chondrocytes in primary culture, and this IL-1-mediated production of proMMP-9 is greatly augmented by cyclooxygenase inhibitors such as diclofenac and indomethacin, whereas the constitutive production of proMMP-2 (progelatinase A) is not modulated by IL-1 and/or cyclooxygenase inhibitors. Exogenous prostaglandin (PG) E1 and PGE2 suppress the proMMP-9 production in a dose-dependent manner. Similar results are also obtained with cultured rabbit synoviocytes. These results provide the first evidence that PGE down-regulates the production of proMMP-9 in chondrocytes and synoviocytes. Thus, cyclooxygenase inhibitors probably exert undesirable catabolic actions on the maintenance of articular cartilage under inflammatory conditions.

Key words: Matrix metalloproteinase 9; Gelatinase B; Cyclooxygenase inhibitor; Prostaglandin E; Indomethacin; Diclofenac; Rabbit chondrocyte

1. Introduction

The destruction of connective tissue matrix components under pathological conditions such as in rheumatoid arthritis (RA) and osteoarthritis (OA) causes the impairment of joint functions, and matrix metalloproteinases (MMPs) are considered to play a critical role in these processes [1,2]. In particular, MMP-1 (interstitial collagenase, EC 3.4.24.7), which specifically degrades native types I, II and III collagen [3], and MMP-3 (stromelysin 1, EC 3.4.24.17), which digests proteoglycans and collagen types IX and X [4–8], are probably key enzymes involved in the pathological destruction of cartilage. In addition to these two enzymes it has been suggested that MMP-9 (gelatinase B, EC 3.4.24.35) plays a role in the degradation of cartilage since it is expressed in osteoarthritic cartilage [9] and is able to degrade proteoglycans [10].

In articular chondrocytes and synoviocytes, IL-1 is known to induce and/or augment the production of proMMPs-1 and -3 as well as prostaglandin (PG) E. These MMPs eventually participate in the destruction of connective tissue matrices [11,12]. Recently, it was also reported that PGE suppresses the production of proMMP-1 in human fibroblasts [13,14] and rabbit synoviocytes [13]. Like proMMPs-1 and -3, the production of proMMP-9 is elevated in rabbit articular chondrocytes when treated with IL-1 [15,16]. However, little is known about the regulatory effect of PGE on the production of proMMP-9 in chondrocytes.

We have therefore examined the effects of cyclooxygenase inhibitors and PGE on the production of proMMP-9 in rabbit articular chondrocytes, and report here that IL-1 enhances the production of proMMP-9, and PGE1 and PGE2 down-regulate the production of proMMP-9. On the other hand, cyclooxygenase inhibitors such as diclofenac and indomethacin further augmented the IL-1-mediated production of proMMP-9, indicating that these inhibitors may exert unfavorable effects for the maintenance of the cartilage under inflammatory conditions.

2. Materials and methods

2.1. Materials

The following reagents were obtained commercially: Dulbecco's modified Eagle's medium (DMEM) from Gibco, Grand Island, NY, USA; bacterial collagenase (CLS1) from Worthington Biochemicals Corp., Freehold, NJ, USA; fetal-bovine serum (FBS) from Whittaker Bioproducts Inc., Walkersville, MD, USA; diclofenac, indomethacin, lactalbumin hydrolysate (LAH), Nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, alkaline phosphatase-conjugated donkey anti-(sheep IgG)IgG, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) from Sigma Chemical Co., St. Louis, MO, USA; PGE1 and PGE2 from Funakoshi, Tokyo, Japan; trypsin from Difco Laboratories, Detroit, MI, USA. Recombinant human IL-1α (rhIL-1; 2×10^7 units/mg) was kindly supplied by Dainippon Pharmaceutical Co., Suita, Osaka, Japan. Sheep anti-(human proMMP-9)antiserum and purified proMMP-9 of HT1080 fibrosarcoma cells were kindly provided by Dr. H. Nagase of the University of Kansas Medical Center, Kansas City, KS, USA. Other reagents used were the same as in a previous paper [17].

2.2. Cell culture of rabbit chondrocytes and synoviocytes

Rabbit articular chondrocytes were prepared by the method of Green [18] with slight modifications. Articular cartilage removed from the knee joints of male Japanese white rabbits weighing 0.3–0.5 kg was initially digested with 0.125% (w/v) trypsin/DMEM, 200 units/ml penicillin and 200 μg/ml streptomycin at 37°C for 1 h and then treated with 0.1% (w/v) bacterial collagenase, 10% (v/v) FBS/DMEM for 2 h. The dispersed chondrocytes were recovered by centrifugation at $700 \times g$ and 4°C for 5 min and then washed once with PBS(−). Chondrocytes were suspended in 10% (v/v) FBS/DMEM/antibiotics, filtered through a 150-mesh nylon screen and resuspended at a cell density of 5×10^5 cells/ml. An aliquot (1 ml) of the cell suspension was placed in each well of 24 multiwell plates and cultured to confluence under 5% CO_2/95% air at 37°C. Cells at the primary culture were used for all experiments.

Rabbit synoviocytes were also prepared and subcultured in a culture of 10% (v/v) FBS/DMEM/antibiotics as described by Vater et al. [19]. For subsequent experiments, confluent cells were trypsinized, sus-

*Corresponding author. Fax: (81) (426) 76-5734.

Abbreviations: MMP, matrix metalloproteinase; DMEM, Dulbecco's modified Eagle's medium; LAH, lactalbumin hydrolysate; FBS, fetal bovine serum; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; rhIL-1, recombinant human interleukin 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PG, prostaglandin.

SSDI 0014-5793(95)00085-2

pended in 10% (v/v) FBS/DMEM/antibiotics and then plated in 24 multiwell plates. Cells up to the 4th passage were used for the experiments.

2.3. Treatment of rabbit cells and preparation of culture media

To estimate the production proMMP-9 and proMMP-2, the culture medium was changed to DMEM/0.2% (w/v) LAH after confluence, and cyclooxygenase inhibitors and other reagents were added to the medium as an ethanol solution. The final ethanol concentration was 0.1% (v/v) in all cultures, and the same amount of vehicle was added to the control cultures. The harvest culture media were stored at −20°C until use.

2.4. Gelatin zymography

Gelatinase activity in the culture media was analyzed by gelatin zymography as described previously [17]. An aliquot (12 μl) of harvested culture media was subjected to SDS-PAGE [20] with an 8.5% (w/v) acrylamide slab gel containing 0.6 mg/ml gelatin under non-reducing conditions. Then the SDS in the gel was removed by rinsing with 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% (w/v) NaN_3, 2.5% (v/v) Triton X-100 (pH 7.5). The gel was then incubated in 20 ml of the same buffer without Triton X-100 for 2 h at 37°C. After incubation, the gel was stained with 0.1% (w/v) Coomassie brilliant blue in 50% (v/v) methanol/20% (v/v) acetic acid, and destained with 1% (v/v) formic acid/30% (v/v) methanol.

2.5. Western blotting for proMMP-9

Each sample (1.5 ml) of harvested and mixed culture media from triplicate wells was mixed with a 1/5 vol. of 20% (w/v) trichloroacetic acid. The resultant precipitates were dissolved in reducing SDS-PAGE sample buffer [20], and then subjected to SDS-PAGE with an 8.5% (w/v) acrylamide slab gel under reducing conditions. Afterwards the electrophoresis proteins in the gel were electro-transferred onto a nitrocellulose membrane. The membrane was reacted with sheep anti-(human proMMP-9)antiserum which was then complexed with alkaline phosphatase-conjugated donkey anti-(sheep IgG)IgG. Immunoreactive proMMP-9 was visualized indirectly with 5-bromo-4-chloro-3-indolyl phosphate and Nitro blue tetrazolium as described previously [21].

2.6. Detrmination of protein and radioimmunoassay for PGE2

Cellular protein was dertermined by the method of Lowry et al. [22] using bovine serum albumin as a standard. PGE2 in culture media was measured by a commercial radioimmunoassay kit (Daiichikagaku-Yakuhin Co., Tokyo, Japan) according to the manufacturer's instructions.

3. Results

3.1. Gelatinolytic activities in rabbit articular chondrocytes

When the culture media of rabbit articular chondrocytes were subjected to gelatin-zymography, gelatinolytic activity of 68 kDa corresponding to proMMP-2 (gelatinase A, EC 3.4.24.24) was constitutively expressed (Fig. 1A, lane 1). The expression of proMMP-2 was not modulated even when cells were treated with rhIL-1 and/or TPA (Fig. 1A, lanes 2–4). In addition to proMMP-2, chondrocytes produced a trace amount of 88 kDa-gelatinolytic activity (Fig. 1A, lane 1), and this activity was augmented by treating cells with rhIL-1 (Fig. 1A, lane 2). TPA synergistically enhanced the IL-1-mediated production of gelatinolytic activity (Fig. 1A, lane 4), although alone it was not effective, as reported previously [16]. Western blotting analysis of the 88 kDa-gelatinolytic activity with an antibody of human proMMP-9 has identified it as rabbit proMMP-9 (Fig. 1B).

3.2. Effects of cyclooxygenase inhibitors, diclofenac and indomethacin on the production of proMMP-9 in rabbit chondrocytes and synoviocytes

When rabbit chondrocytes were treated with rhIL-1 or both TPA and rhIL-1 in the presence of a cyclooxygenase inhibitor, diclofenac, the production of proMMP-9 was greatly enhanced in a dose-dependent manner of the inhibitor (Fig. 2A). Diclofenac, however, did not modulate the production of proMMP-9 in intact or the TPA-treated cells (Fig. 2A). Indomethacin, another cyclooxygenase inhibitor, similarly enhanced the production of proMMP-9 (Fig. 2B). On the other hand, these cyclooxygenase inhibitors did not modulate the production of proMMP-2, indicating that the stimulatory effects of these inhibitors are mainly on proMMP-9 between the two gelatinases.

High levels of MMPs including MMP-1/interstitial collagenase and MMP-3/stromelysin 1 are found in synovial tissues with RA and OA [11,12], but little is known about the produc-

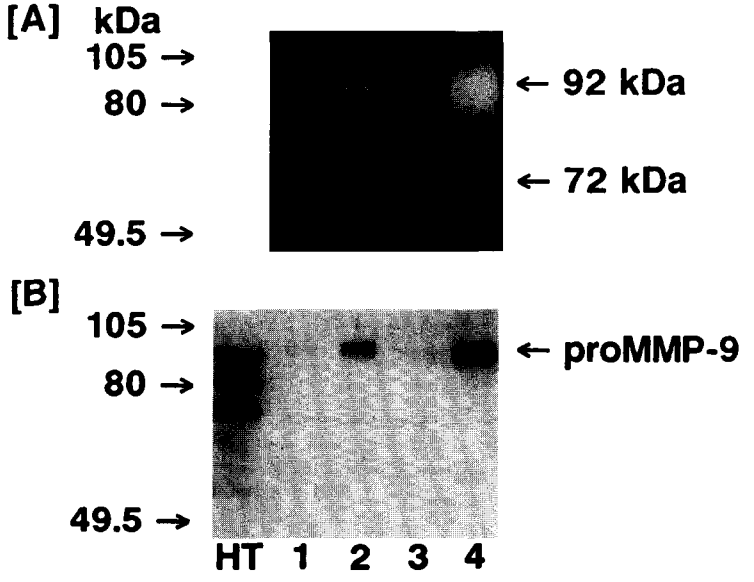

Fig. 1. Identification of 92 kDa-gelatinolytic activity from rabbit articular chondrocytes. Confluent rabbit chondrocytes in 24-multiwell plates were treated with rhIL-1 (1 ng/ml) and/or TPA (10 nM) in 1.0 ml of 0.2% (w/v) LAH/DMEM for 48 h, and then samples of harvested and mixed culture media from triplicate wells were subjected to gelatin zymography (A) and Western blotting (B), as described in the text. Three independent experiments were reproducible, and typical data are shown. Lane 1, non-treated control cells; lane 2, rhIL-1; lane 3, TPA and lane 4, rhIL-1 plus TPA. HT indicates the purified proMMP-9 (1 μg) from HT1080 fibrosarcoma cells.

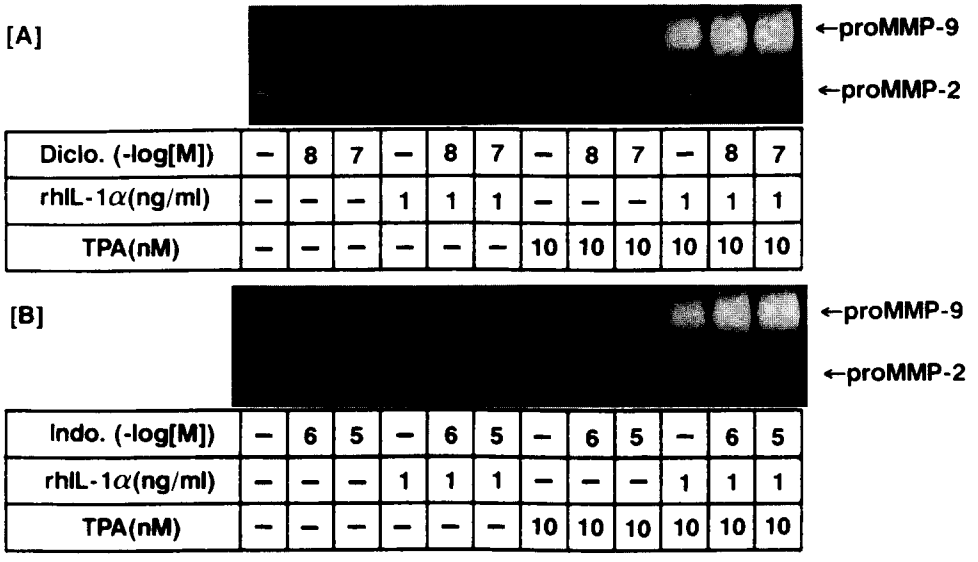

Fig. 2. Effects of cyclooxygenase inhibitors, diclofenac and indomethacin on the production of proMMP-9 in rabbit articular chondrocytes. Confluent rabbit articular chondrocytes in 24-multiwell plates were treated with rhIL-1, TPA and/or a cyclooxygenase inhibitor in 1.0 ml of 0.2% (w/v) LAH/DMEM for 48 h, and then an aliquot (12 μl) of the harvested and mixed culture media from triplicate wells was subjected to gelatin zymography, as described in the text. Three independent experiments were reproducible, and typical data are represented. (A) Diclofenac; (B) indomethacin.

tion of MMP-9 in rabbit synoviocytes. Therefore, similar experiments were conducted with the cultured rabbit synoviocytes. As in the case of chondrocytes, rhIL-1 enhanced the production of proMMP-9, but not proMMP-2 in synoviocytes (Fig. 3A and B, lane 5). Both diclofenac and indomethacin further augmented the IL-1-induced production of proMMP-9 in synoviocytes (Fig. 3A and B, lanes 6–8), whereas the inhibitors did not modulate the production of proMMP-2. These results indicate that the effect of cyclooxygenase inhibitors is not only on articular chondrocytes but also on synoviocytes.

3.3. *Suppression of production of proMMP-9 by PGE1 and PGE2 in rabbit articular chondrocytes*

To investigate the mechanism of the action of cyclooxygenase inhibitors on the production of proMMP-9, we have examined the effects of PGE1 and PGE2, since these inhibitors effectively diminish the production of PGEs in chondrocytes and fibroblasts [23], and rabbit articular chondrocytes are reported to produce predominantly PGE2 [24]. Indeed, when confluent rabbit chondrocytes were treated with 1 ng/ml of rhIL-1, the accumulation of extracellular PGE2 was about 30-

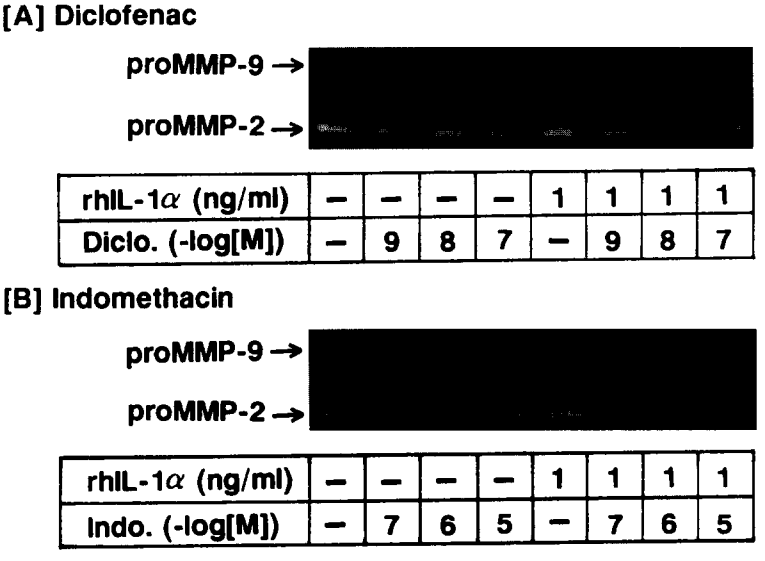

Fig. 3. Augmentation of IL-1-induced production proMMP-9 by diclofenac and indomethacin in rabbit synoviocytes. Confluent rabbit synoviocytes at the fourth passage in 24-multiwell plates were treated with rhIL-1, diclofenac and/or indomethacin for 48 h in 1.0 ml of 0.2% (w/v) LAH/DMEM, and then an aliquot (12 μl) of harvested and mixed culture media from triplicate wells was subjected to gelatin zymography as described in the text. Three independent experiments were reproducible, and typical data are shown. (A) diclofenac; lane 1, non-treated control cells; lanes 2–4, diclofenac (1×10^{-9}, 1×10^{-8} and 1×10^{-7} M, respectively), lane 5, rhIL-1 (1 ng/ml); lanes 6–8, rhIL-1 (1 ng/ml) plus diclofenac (the same as lanes 2–4). (B) indomethacin; lane 1, non-treated control cells, lanes 2–4, indomethacin (1×10^{-7}, 1×10^{-6} and 1×10^{-5} M, respectively), lane 5, rhIL-1 (1 ng/ml) and lanes 6–8, rhIL-1 (1 ng/ml) plus indomethacin (the same as lanes 2–4).

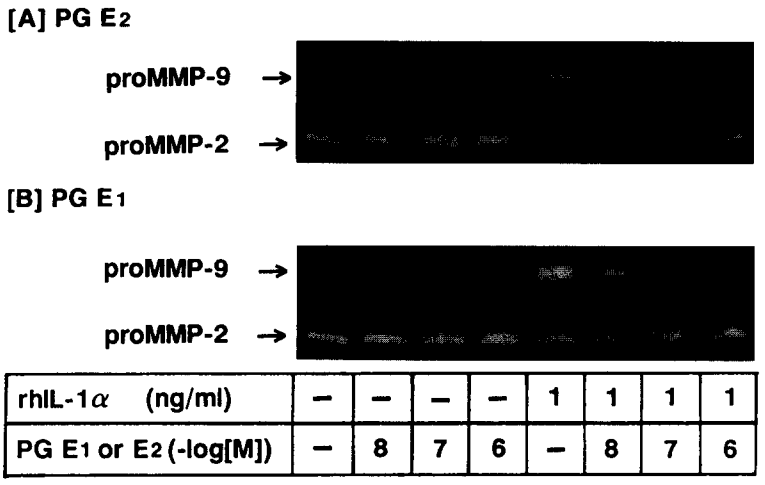

Fig. 4. Effects of PGE1 and PGE2 on the constitutive and IL-1-mediated production of proMMP-9 in rabbit articular chondrocytes. Confluent rabbit chondrocytes were treated with rhIL-1 (1 ng/ml) and/or various concentrations of PGE for 48 h, and other experimental conditions were the same as in Fig. 2. Lane 1, non-treated control cells; lanes 2–4, PGE (1×10^{-8}, 1×10^{-7} and 1×10^{-6} M, respectively); lane 5, rhIL-1 (1 ng/ml) and lanes 6–8, rhIL-1 (1 ng/ml) plus PGE (1 × 10–8, 1 × 10–7 and 1 × 10–6 M, respectively). (A) PGE2; (B) PGE1.

times higher than that by non-treated control cells, and indomethacin reduced the IL-1-mediated accumulation of PGE2 to the level of the non-treated control cells (Table 1). When the chondrocytes were treated with PGE1 or PGE2 both the constitutive and the IL-1-enhanced production of proMMP-9 was suppressed in a dose-dependent manner (Fig. 4A and B, lanes 2–4 and 6–8). The production of proMMP-2 was not affected by PGE1 or PGE2. These results suggest that both PGE1 and PGE2 down-regulate the production of proMMP-9. Thus the enhancement of proMMP-9 production caused by cyclooxygenase inhibitors in chondrocytes and synoviocytes is due to the decreased level of cellular PGEs.

4. Discussion

The roles of PGEs at inflammatory sites are undefined: e.g. PGE is considered to be a mediator of acute-phase inflammation since it increases vascular permeability [25]. In contrast, PGEs also exert anti-inflammatory effects in chronic inflammations [25] and also suppress adjuvant arthritis in rat [26]. Furthermore, PGE2 effectively inhibits the synthesis of inflammatory cytokine of IL-1 in macrophages [27]. In view of these observations, PGEs are very likely to suppress chronic inflammation. This hypothesis might be further supported by the observations concerning indomethacin: i.e. indomethacin suppresses the biosynthesis of proteoglycans in articular cartilage [28–30] and rat chondrocytes [24,31], and accelerates the proliferation of human synoviocytes [32]. It is also reported that cyclooxygenase inhibitors never interfere with the progress of arthritis [33].

Concerning the production of proMMPs, especially proMMP-9, we have observed that both PGE1 and PGE2 suppress proMMP-9 production in cultured rabbit chondrocytes, and the IL-1-mediated production was effectively augmented by cyclooxygenase inhibitors. A similar enhanced production of proMMP-9 by the inhibitors was also detected in human rheumatoid synoviocytes (A. Mukaiyama, A. Ito and Y. Mori, unpublished work). The mechanism of action of both PGE1 and PGE2 on the production of proMMPs, especially proMMP-9, is unclear. From this point of view, we observed that cAMP and intracellular cAMP-modulating reagents, such as forskolin (adenylate cyclase activator) and 3-isobutyl-1-methylxanthine (phosphodiesterase inhibitor) effectively mimicked the suppressive effect of PGEs on the production of proMMPs including proMMP-9 in rabbit chondrocytes (T. Nose, A. Ito, T. Kubo and Y. Mori, unpublished work). In addition, PGE1 enhanced the accumulation of intracellular cAMP: i.e. the level of PGE1-treated cells was about 6-times higher than that of untreated control cells (17.4±0.1 vs. 103.2±14.8 fmol/μg cellular protein/10 min). It is noteworthy, however, that PGE2 did not modulate the intracellular cAMP level in chondrocytes (data not shown), but the cells produced mainly PGE2. These observations therefore suggest that the suppressive effect of PGE1, but not PGE2, on the production of proMMPs may be a result of the increase in intracellular cAMP, whereas the action mechanism of PGE2 on proMMP-9 production is obscure and remains to be clarified in future studies.

In conclusion, we have demonstrated for the first time that in rabbit chondrocytes the production of proMMP-9 is down-

Table 1
Effect of indomethacin on the IL-1-mediated production of PGE2 in rabbit articular chondrocytes

Treatment	PGE2 production pg/μg cellular protein/24 h)
Control	0.52 ± 0.06
rhIL-1	17.15 ± 2.02 ($P < 0.001$[a])
rhIL-1 plus indomethacin	0.52 ± 0.02 ($P < 0.001$[b])

Confluent rabbit articular chondrocytes in 100 mm-diameter dishes were treated with 15 ml of 0.2% (w/v) LAH/DMEM containing rhIL-1 (1 ng/ml) and/or indomethacin (10 μM) for 24 h. PGE2 in harvested culture media was determined by a radioimmunoassay kit, as described in the text. Data are means ± S.D. for three wells. Two independent experiments were reproducible, and typical data are represented. [a] and [b] significantly different from control and rhIL-1-treated cells, respectively.

regulated by PGE1 and PGE2, and that the cyclooxygenase inhibitors indomethacin and diclofenac further augment the IL-1-mediated production of proMMP-9. Therefore, our present findings and previous reports [24,28–33] strongly suggest that the cyclooxygenase inhibitors are very likely to exert undesirable actions on the maintenance of articular cartilage, and that both PGE1 and PGE2 play an anti-inflammatory role in the destruction of articular cartilage.

Acknowledgments: We are grateful to Dr. H. Nagase, University of Kansas Medical Center, Kansas City, KS, USA for generously providing us with sheep anti-(human proMMP-9)antiserum and purified proMMP-9 of HT1080 fibrosarcoma cells, and for his critical reading of the manuscript. We also thank Mr. A. Tsuruta for his technical assistance.

References

[1] Brinckerhoff, C.E. (1991) Arthritis Rheum. 32, 241–246.
[2] Woessner Jr., J.F. and Gunja-Smith, Z. (1991) J. Rheumtol. 18(Suppl.), 99–101.
[3] Welgus, H.G., Jeffrey, J.J. and Eisen, A.Z. (1981) J. Biol. Chem. 256, 9511–9515.
[4] Galloway, W.A.., Murphy, G., Sandy, J.C., Gavrilovic, J., Cawston, T.E. and Reynolds, J.F. (1983) Biochem. J. 209, 741–752.
[5] Chin, J.R., Murphy, G., and Werb, Z. (1985) J. Biol. Chem. 260, 12367–12376.
[6] Okada, Y., Nagase, H. and Harris Jr., E.D. (1986) J. Biol. Chem. 261, 14245–14255.
[7] Okada, Y., Konomi, H., Yada, T., Kimata, K. and Nagase, H. FEBS Lett. 244, 473–476.
[8] Wu, J.J., Lark, M.W., Chun, L.E. and Eyre, D.R. (1991) J. Biol. Chem. 266, 5625–5628.
[9] Mohtai, M., Smith, R.L., Schurman, D.J., Tsuji, Y., Torti, F.M., Hutchinson, N.I., Stetler-Stevenson, W.G. and Goldberg, G.I. (1993) J. Clin. Invest. 92, 179–185.
[10] Murphy, G., Cockett, M.I., Ward, R.V. and Docherty, A.J.P. (1991) Biochem. J. 277, 277–279.
[11] Chang,J., Gilman, S.C. and Lewis, A.J. (1986) J. Immunol. 136, 1283–1287.
[12] Dayer, J.M., Golgring, S.R., Robinson, D.R. and Krane, S.M. (1979) Biochim. Biophys. Acta 586, 87–105.
[13] Case, J.P., Lafyatis, R., Kumkumian, G.K., Remmars, E.F. and Wilder, R.L. (1990) J. Immunol. 145, 3755–3761.
[14] Salvatori, R., Duidon Jr., P.T., Rapuano, B.E. and Bockman, R.S. (1992) Endocrinology 131, 21–28.
[15] Lefebvre, V., Peeters-Joris, C. and Vaes, G. (1991) Biochim. Biophys. Acta 1094, 8–18.
[16] Ogata, Y., Pratta, M.A., Nagase, H. and Arner, E.C. (1992) Exp. Cell Res. 201, 245–249.
[17] Takahashi, S., Ito, A., Nagino, M., Mori, Y., Xie, B. and Nagase, H. (1991) J. Biol. Chem. 266, 19894–19899.
[18] Green Jr., W.T. (1971) Clin. Orthop. Rel. Res. 75, 248–260.
[19] Vater, C.A., Nagase, H. and Harris Jr., E.D. (1983) J. Biol. Chem. 258, 9374–9382.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Ito, A. and Nagase, H. (1988) Arch. Biochem. Biophys. 267, 211–216
[22] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[23] Ito, A. and Mori, Y. (1990) Res. Commun. Chem. Pathol. Pharmacol. 70, 131–142.
[24] Malemud, C.J., Moskowitz, R.W. and Hassid, A. (1981) Biochim. Biophys. Acta 663, 480–490.
[25] Goodwin, J.S. (1991) J. Rheumatol. 18 (Suppl.), 26–29.
[26] Zurier, R.B. and Ballas, M. (1973) Arthritis Rheum. 16, 251–258.
[27] Kunkel, S.L. and Chensue, S.W. (1985) Biochem. Biophys. Res. Commun. 128, 892–897.
[28] Muir, H., Carney, S.L. and Hall, L.G. (1988) Drugs 35(Suppl.), 15–23.
[29] Brandt, K.D. (1987) Am. J. Med. 83 (Suppl.) 29–33.
[30] Dingle, J.T. (1991) J. Rheumatol. 18 (Suppl.), 30–37.
[31] de Vries, B.J., van den Berg, W.B., Vitters, E. and van de Putte, L.B.A. (1988) Drugs 35 (Suppl.), 24–32.
[32] Gitter, B.D., Labus, J.M., Lees, S.L. and Scheetz, M.E. (1989) Immunology 66, 196–200.
[33] Rashad, S., Revell, P., Hemingway, A., Low, F., Rainford, K. and Walker, F. (1989) Lancet ii, 519–522.