Effect of a Benzylidene Derivative, Novel Antirheumatic Agent, on the Production of Tissue Inhibitor of Metalloproteinases

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We investigated the effects of PAL (protocatechualdehyde), a metabolite of ACP (3,4-diacetoxy benzylidene diacetate) which is a candidate as a novel antirheumatic agent, on the production of a tissue inhibitor of metalloproteinases (TIMP) using a primary chondrocyte culture from bovine nasal septum cartilage.

The addition of human recombinant interleukin-1 α (hrIL-1 α) induced proteoglycan (PG) depletion from a chondrocyte matrix. PAL significantly reduced the induction of PG depletion. HrIL-1 α increased the casein-degrading activity, matrix metalloproteinase (MMP) activity, and the level of TIMP secretion in the culture media. PAL significantly reduced the casein-degrading activity and further increased TIMP secretion under hrIL-1 α stimulation. Phorbol myristate acetate (PMA) also increased the level of TIMP secretion, and staurosporine, a specific inhibitor of protein kinase C (PKC), reduced the TIMP secretion to the control level under hrIL-1 α or PMA stimulation. Furthermore, PAL showed a significant increase following treatment with a low concentration of PMA which alone did not increase TIMP production. These findings suggest that the activation of the PKC pathway plays an important role in TIMP production and that PAL increases the level of TIMP production through an additive or synergistic effect with the activation of the PKC pathway.

In conclusion, these findings demonstrate that the inhibition of the MMP activity and the increase of TIMP production by PAL contribute to the inhibition of PG depletion in a primary chondrocyte culture from bovine nasal septum cartilage.

Keywords TIMP; chondrocyte; interleukin-1; matrix metalloproteinase; proteoglycan; glycosaminoglycan

The benzylidene derivative, 3,4-diacetoxy benzylidene diacetate (ACP), is a candidate as a novel antirheumatic agent having pharmacological effects against chronic inflammatory models.¹⁾ ACP is a prodrug of protocatechualdehyde (PAL), a component of *Hatomugi*, *Coix Lacryma-jobi* LINNÉ, var. *ma-yuen* STAPE, which has various pharmacological effects.²⁻⁴⁾

Experiments using rabbit articular chondrocytes in a monolayer culture showed that PAL has favorable effects on chondrocyte metabolism. Primarily, PAL significantly inhibited proteoglycan (PG) degradation, which would have been induced by matrix metalloproteinases (MMPs). PAL reduced the production of MMPs such as collagenase and stromelysin.^{5,6)} These findings suggest that PAL reduces, at least in part, the matrix degradation *via* inhibition of MMPs production. However, the mechanism of action of PAL on PG degradation cannot be explained completely by the inhibition of MMPs production. Therefore, another mechanism contributing to the inhibitory effect on PG degradation has been considered.

The matrix degradation is controlled through the balance of MMPs and tissue inhibitor of metalloproteinases (TIMP) which is a natural inhibitor of MMPs. It has been proposed that TIMP is important in controlling the local activity of the enzymes to prevent resorption of the matrix in the arthritic joint, and the reported systemic administration of TIMP in collagen-induced arthritis in mice produced a decreased incidence of joint erosion.^{7,8)} From this point of view, we investigated the effects of PAL on TIMP production using a bovine nasal septum chondrocytes culture.

MATERIALS AND METHODS

Materials PAL was purified by recrystallizing crude PAL (Tokyo Kasei, Tokyo, Japan). 1,9-Dimethyl methylene blue was purchased from Polyscience, Inc. (Warrington, PA). Papain, chondroitin sulfate, collagenase, *N*-acetyl cysteine, EDTA, 4 α -phorbol-12,13-didecanoate (4 α -PDD), staurosporine, [¹⁴C]casein and phorbol myristate acetate (PMA) were from Sigma (St. Louis, MO); trypsin, penicillin, streptomycin, fungizone, fetal bovine serum (FBS), and Ham F-12 medium were from Gibco (Grand Island, NY); serum-free S-Clone medium was from Sanko Junyaku (Tokyo, Japan); transforming growth factor beta (TGF- β) was from Cosmo-Bio (Tokyo, Japan); human recombinant interleukin-1 α (hrIL-1 α) was from Boehringer Mannheim Yamanouchi (Osaka, Japan); teleocidin B-4 was from Funakoshi Co., Ltd. (Tokyo, Japan); *p*-aminophenylmercuric acetate (APMA) and casein were from Tokyo Kasei (Tokyo, Japan); trichloroacetic acid (TCA) was from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). All other reagents were of analytical grade.

Culture of Nasal Septum Chondrocytes The primary monolayer culture of chondrocytes was established from cells derived from pooled small segments of bovine nasal septum from a freshly slaughtered animal using the procedure described by Green.⁹⁾ Briefly, chondrocytes were dissociated by the digestion of finely diced cartilage with trypsin (0.2%), followed by collagenase (0.2%) digestion in Ham F-12 containing 10% FBS for 3 h. After being washed twice with the Ham F-12 medium, the cells were cultured on 24-well culture plates (Costar, Cam-

bridge, UK) at a concentration of 3×10^5 cells/ml in Ham F-12 supplemented with 10% FBS containing penicillin (100 U/ml), streptomycin (100 μ g/ml) and fungizone (250 ng/ml). After reaching confluency, the chondrocytes were used without subculture.

PG Depletion PG depletion was determined by the measurement of the glycosaminoglycan (GAG) content following digestion of the chondrocyte matrix with papain. The level of GAG was measured by spectrophotometry with a solution of 1,9-dimethylmethylene blue by the method of Farndale, using shark chondroitin sulfate as the standard.¹⁰ When the chondrocytes became confluent, the medium was changed to 800 μ l of fresh S-Clone media containing 0.1% human albumin. Two hours later, 100 μ l of various concentrations of test compounds or culture media, and 100 μ l of stimulants such as hrIL-1 α (200 U/ml), or PMA (1 μ g/ml) solution or culture media were added. Test compounds were dissolved in dimethyl sulfoxide (DMSO) to achieve 40 mM stock solutions, and were subsequently diluted with S-Clone media. The final concentration of DMSO was 0.25%. Forty-two hours later, culture media were processed to measure TIMP and casein-degrading activity. Cell layers were washed with a Ham medium and the remaining cell layer was digested with 1 ml papain solution (330 μ g/ml) in a neutral phosphate buffer (pH 6.5) together with 2 mM EDTA and 2 mM *N*-acetyl cysteine for 2 h at 65 °C. The amount of GAG in the papain solution was determined using the method described above.

Metalloproteinase Assay Casein-degrading activity was assayed using ¹⁴C-labeled β -casein as a substrate by the method of Golds.¹¹ An 80 μ l sample of cell culture media was activated with 20 μ l of 5 mM APMA solution for 1 h at 37 °C. Reaction mixtures containing 100 μ l [¹⁴C]casein, 400 μ l of assay buffer (100 mM Tris-HCl, pH 7.4; 0.4 M NaCl; 20 mM CaCl₂; 0.04% NaN₃), and a 100 μ l sample were incubated for 42 h at 37 °C. Reactions were stopped by the addition of 200 μ l of unlabeled casein and 360 μ l of 10% TCA. The mixtures were centrifuged at 4 °C for 15 min at 10000 rpm. Enzyme activity was defined as the level of TCA-soluble radioactivity. Five-hundred microliters of supernatant was collected, added to 10 ml of Aquasol-2, and the level of radioactivity was determined using a liquid scintillation counter.

Measurement of TIMP The level of TIMP was determined using a TIMP assay kit available for human and bovine TIMP (Fuji Pharmaceutical Co., Ltd. Toyama, Japan).¹²

Statistical Analysis The Student's *t*-test was used for the statistical analysis. Differences at a *p* value of less than 0.05 were considered to be statistically significant.

RESULTS

Effect of PAL on PG depletion The addition of hrIL-1 α at a concentration of 20 U/ml caused a 41% depletion in the PG content of the cell and matrix layer during 42 h of incubation, as compared with the PG content of the control. PAL inhibited PG depletion 30–50% (Table I).

Effect of PAL on Casein-Degrading Activity Induced by IL-1 α As shown in Table II, hrIL-1 α (20 U/ml)

TABLE I. Effects of PAL on Proteoglycan Depletion Induced by IL-1 α

GAG content (μ g/culture)	
Control	6.38 ± 0.25^b (100) ^a
IL-1 α (20 U/ml)	3.73 ± 0.13 (58.5)
+ PAL 10 μ M	3.92 ± 0.02 (61.5)
33 μ M	4.49 ± 0.08^c (70.3)
100 μ M	5.11 ± 0.12^c (80.1)

Each value represents the level of remaining GAG in the chondrocyte matrix, taken as the mean of three culture dishes $\pm$ S.E.M. a) Ratio of GAG content is expressed as a percentage of the level in the control culture. Significant difference from the IL-1-treated culture: b) *p* < 0.001, c) *p* < 0.01.

TABLE II. Effects of PAL on Casein-Degrading Activity Induced by IL-1 α

Casein-degrading activity (dpm $\times 10^{-3}$ /culture)	
Control	0.28 ± 0.13^b (2.51) ^a
IL-1 α (20 U/ml)	11.4 ± 0.26 (100)
+ PAL 10 μ M	8.00 ± 0.37^c (70.4)
33 μ M	6.24 ± 0.26^b (54.8)
100 μ M	6.54 ± 0.11^b (57.5)

Each value represents the level of casein-degrading activity in the chondrocyte culture, taken as the mean of three culture dishes $\pm$ S.E.M. a) Ratio of casein-degrading activity content is expressed as a percentage of the level in the IL-1-treated culture. Significant difference from the IL-1-treated culture: b) *p* < 0.001, c) *p* < 0.01.

TABLE III. Effects of PAL on TIMP Production

TIMP content (ng/culture)	
Control	22.7 ± 1.23 (100) ^a
+ PAL 10 μ M	27.1 ± 1.85 (119)
33 μ M	30.2 ± 2.38^b (133)
100 μ M	29.4 ± 0.61^c (130)

Each value represents the level of TIMP in the chondrocyte culture, taken as the mean of three culture dishes $\pm$ S.E.M. a) Ratio of TIMP content is expressed as a percentage of the level in the control culture. Significant difference from the control culture: b) *p* < 0.05, c) *p* < 0.01.

significantly increased the casein-degrading activity in the culture media compared to the control level. The addition of PAL at a concentration of 10 to 100 μ M inhibited the casein-degrading activity 30–40%.

Effect of PAL on TIMP Production Induced by IL-1 α The nasal septum chondrocytes showed TIMP secretion in the culture media without any stimulants, and PAL caused a 19–30% increase of TIMP secretion (Table III). TIMP secretion was significantly increased (74%) when exposed to 20 U/ml of hrIL-1 α after 42 h of culture, compared with the control level. However, no significant secretion was observed after the addition of TGF- β (10 ng/ml). (Control: 152 ng/culture, TGF- β : 136 ng/culture, data not shown). Under IL-1 α stimulation, the effect of PAL on TIMP secretion was examined. PAL caused a further 10–26% increase in TIMP secretion compared with that of the hrIL-1 α -treated culture in a dose-dependent manner. Staurosporine, a specific protein kinase C (PKC) inhibitor, reduced the level of TIMP secretion to the control level (Table IV). Unlike the TIMP level in the culture media, no remaining TIMP in the cell layer was observed following the freeze-thawing treatment (data

TABLE IV. Effects of PAL on TIMP Production Induced by IL-1 α

	TIMP content (ng/culture)
Control	40.0 $\pm$ 5.90 ^{b)} (100) ^{a)}
IL-1 α (20 U/ml)	69.9 $\pm$ 4.64 (174)
+ PAL 10 μ M	78.1 $\pm$ 7.78 (195)
33 μ M	84.2 $\pm$ 15.1 (210)
100 μ M	87.5 $\pm$ 2.84 ^{b)} (219)
Control	151 $\pm$ 8.19 (100)
IL-1 α (20 U/ml)	188 $\pm$ 16.7 (124)
+ Staurosporine 33 nM	138 $\pm$ 2.88 ^{c)} (91.8)

Each value represents the level of TIMP in the chondrocyte culture, taken as the mean of three culture dishes $\pm$ S.E.M. a) Ratio of TIMP content is expressed as a percentage of the level in the control culture. Significant difference from the IL-1-treated culture: b) $p < 0.01$, c) $p < 0.05$.

TABLE V. Effects of PKC Inhibitor on TIMP Production Induced by PMA

	TIMP content (ng/culture)
Control	153 $\pm$ 8.01 (100) ^{a)}
4 α -PDD (0.1 μ g/ml)	182 $\pm$ 9.61 (119)
Teleocidin B-4 (0.1 μ g/ml)	469 $\pm$ 4.78 ^{b)} (306)
PMA (0.1 μ g/ml)	457 $\pm$ 22.1 ^{b)} (299)
+ Staurosporine 33 nM	138 $\pm$ 6.63 ^{c)} (91)

Each value represents the level of TIMP in the chondrocyte culture, taken as the mean of three culture dishes $\pm$ S.E.M. a) Ratio of TIMP content is expressed as a percentage of the level in the control culture. b) Significant difference from the control culture, $p < 0.001$. c) Significant difference from the PMA-treated culture, $p < 0.001$.

TABLE VI. Effects of PAL on TIMP Production Induced by PMA

	TIMP content (ng/culture)
Control	19.5 $\pm$ 3.10 ^{b)} (100) ^{a)}
PMA (0.1 μ g/ml)	157 $\pm$ 1.81 (811)
+ PAL 10 μ M	138 $\pm$ 10.8 (712)
33 μ M	136 $\pm$ 10.4 (699)
100 μ M	147 $\pm$ 5.30 (753)
Control	153 $\pm$ 8.01 (100) ^{a)}
PMA (0.001 μ g/ml)	165 $\pm$ 7.79 (108)
+ PAL 100 μ M	435 $\pm$ 11.6 ^{b)} (284)

Each value represents the level of TIMP in the chondrocyte culture, taken as the mean of three culture dishes $\pm$ S.E.M. a) Ratio of TIMP content is expressed as a percentage of the level in the control culture. b) Significant difference from the PMA-treated culture, $p < 0.001$.

not shown).

Effect of PAL on TIMP Production Induced by PMA

The addition of PMA (0.1 μ g/ml) increased TIMP secretion markedly. Another PKC agonist, teleocidin B-4 (0.1 μ g/ml), also increased TIMP secretion, whereas the inactive phorbol ester 4 α -PDD (0.1 μ g/ml) had no effect. Staurosporine reduced the level of TIMP secretion to the control level (Table V).

The effect of PAL on TIMP secretion was examined under PMA stimulation. PAL had no effect on PMA stimulation at a concentration of 0.1 μ g/ml. However, PAL exhibited a synergistic effect on TIMP secretion in the presence of a low concentration of PMA (0.001 μ g/ml) which did not cause any increase alone (Table VI).

DISCUSSION

Nixon *et al.* and Couchman *et al.* reported that hrIL-1 α

or synovial culture media induced the depletion of PG from bovine nasal septum cartilage.^{13,14)} In the present study using bovine nasal septum chondrocyte cultures, PG was depleted by the addition of hrIL-1 α . PAL significantly reduced the depletion of PG under the condition of hrIL-1 α stimulation (Table I). The depletion of PG was considered to result from an imbalance in the synthesis and degradation of PG. In a previous study using a primary chondrocyte culture from rabbit articular cartilage, PAL inhibited the depletion of PG through the reduction of its degradation without affecting its synthesis. It was also demonstrated that the inhibition of MMPs production by PAL partially contributed to the suppression of PG degradation.^{5,6)} Secondly, we examined the effect of PAL on stromelysin activity in a culture media using casein-degrading activity. As shown in Table II, a small amount of casein-degrading activity was observed in the culture media under a non-stimulated condition, whereas the addition of hrIL-1 α significantly increased the casein-degrading activity. PAL significantly reduced the casein-degrading activity, suggesting that this effect contributed, at least in part, to the inhibition of PG depletion in this culture system. However, there seems to be another mechanism of action involved in the inhibition of PG depletion. Therefore, we investigated the secretion of TIMP, a natural inhibitor of MMPs, in the culture media.

It has been proposed that TIMP is important in controlling the local activity of the MMPs to prevent resorption of the matrix in arthritic joints, and stimuli such as blood mononuclear cell factors could cause a local excess of enzyme over inhibitor, thus allowing resorption to take place.⁷⁾ It was reported that TIMP has been extracted from bovine nasal septum cartilage by the HCl-guanidine method.¹⁵⁾ In the present study, TIMP secretion was also observed in the bovine nasal septum chondrocytes culture without any stimulation, unlike the secretion of casein-degrading activity. The range of TIMP secretion in the control cultures seemed to be variable, depending on the source of nasal septum cartilage (19.5–153 ng/ml). The addition of PAL significantly enhanced the TIMP secretion under a non-stimulated condition (Table III).

TIMP production has been reported to be regulated by various agents such as IL-1.^{16–18)} The most likely candidate is TGF- β , which has been shown to be capable of stimulating the production of both TIMP mRNA and the secretion of TIMP protein.^{19,20)} However, it was also reported that the addition of TGF- β alone did not induce the secretion of TIMP.²¹⁾ In the present study, while TGF- β alone did not increase TIMP secretion (data not shown), the addition of IL-1 (20 U/ml) increased TIMP secretion compared with that under a non-stimulated condition (Table IV). No detectable TIMP was observed in cell lysis following the freeze-thaw treatment under the non-stimulated or IL-1 stimulated condition, suggesting that most of the TIMP produced in the chondrocytes, just as well as MMPs, is secreted into the culture media (data not shown). While the addition of hrIL-1 α caused only a 1.7-fold increase in TIMP production as compared to that of the control, a 40-fold increase of casein-degrading

activity was observed. These findings suggest that the surplus production of MMPs increased the depletion of PG compared with that under a non-stimulated condition. PAL caused a significant and dose-dependent increase in TIMP production under IL-1 stimulation compared with that of the hrIL-1 α -treated culture. On the other hand, staurosporine reduced TIMP production, suggesting that IL-1 induced an increase of TIMP production through the modulation of the PKC pathway (Table IV). PMA (0.1 μ g/ml), a PKC activator, increased TIMP production. Another PKC activator, teleocidin B-4, also increased TIMP production, whereas an inactive phorbol ester, 4 α -PDD, did not. Staurosporine significantly reduced TIMP production, as in the case of hrIL-1 α stimulation (Table V). These findings suggest that PKC activation plays an important role in the increase of TIMP production. No further increase of TIMP level by PAL was observed with PMA (0.1 μ g/ml) treatment, whereas PAL showed a significant increase following treatment with a low concentration of PMA (0.001 μ g/ml) which alone did not increase TIMP production (Table VI). These findings suggest that PAL enhances TIMP production through an additive effect with hrIL-1 α stimulation or a synergistic effect with PMA stimulation. The signal transduction mechanism on TIMP production induced by hrIL-1 α seems to be more complicated compared with that of PMA stimulation.

Duncan *et al.* reported that razoxane, an EDTA derivative, inhibited collagenase production and stimulated TIMP production, followed by a reduction in IL-1-induced cartilage degradation.²²⁾ Furthermore, it has been reported that all-*trans*-retinoic acid regulates the production of MMPs and TIMP.²³⁻²⁵⁾ Our findings also suggest that the effects of PAL in suppressing the depletion of PG correlate with its ability to reduce MMPs production in the chondrocyte cultures, and PAL may offer an additional protective effect through the regulation of TIMP production. PAL caused neither the inhibition of non-specific protein synthesis nor the release of lactate dehydrogenase in chondrocyte cultures within the concentration range used in this study.⁶⁾ These findings demonstrate that PAL, an active metabolite of ACP, favorably modulates chondrocyte metabolism through the inhibition of MMPs production and the enhance-

ment of TIMP production without cytotoxicity.

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