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Esculetin suppresses proteoglycan metabolism by inhibiting the production of matrix metalloproteinases in rabbit chondrocytes

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Abstract

The possible mechanism of the chondroprotective effect of 6,7-dihydroxycoumarin (esculetin) was investigated using primary cultures of rabbit articular chondrocytes. Esculetin (EST) significantly suppressed the proteoglycan depletion and the release of pulse-labeled [^{35}S]proteoglycan from the matrix layer of rabbit chondrocytes treated with recombinant human interleukin-1α. The matrix metalloproteinase inhibitor, 1,10-phenanthroline, also blocked the proteoglycan depletion and [^{35}S]proteoglycan release. From these results, it is likely that recombinant human interleukin-1α-induced proteoglycan depletion is mediated by matrix metalloproteinases. Although esculetin did not directly inhibit collagenolytic activity in the culture media, it significantly suppressed the production of pro-matrix metalloproteinase-1/interstitial procollagenase and pro-matrix metalloproteinase-3/prostromelysin 1, accompanied by a decrease in the steady-state levels of their mRNAs. These results suggest that esculetin is a therapeutically effective candidate for inhibition of cartilage destruction in osteoarthritis and rheumatoid arthritis.

Keywords: Esculetin; 6,7-Dihydroxycoumarin; Proteoglycan; Chondrocyte; Osteoarthritis; Matrix metalloproteinase

1. Introduction

Osteoarthritis is a multifunctional disease in which several complex mechanisms lead to a progressive destruction of joints. The primary changes in the articular cartilage, such as matrix degradation, seem to be induced by both extrinsic and intrinsic factors. Mechanical disturbances may also contribute to the development of osteoarthritis (Sokoloff, 1987). Matrix metalloproteinases are considered to play critical roles in the degradation of extracellular matrix components. Interstitial collagenase/matrix metalloproteinase-1 specifically degrades native types I, II and III collagen (Welgus et al., 1981), and stromelysin 1/matrix metalloproteinase-3 digests proteoglycans and types IX and X collagen (Galloway et al., 1983; Wu et al., 1991). Matrix metalloproteinase-3 also acts as a specific activator of pro-matrix metalloproteinase-1 and progelatinase B/pro-matrix metalloproteinase-9 (Murphy et al., 1988; Ogata et al., 1992). In addition to these two enzymes, matrix metalloproteinase-9 and matrix metalloproteinase-13/collagenase 3 are suggested to play a critical role in the pathological degradation of cartilage (Mohtai et al., 1993; Mitchell et al., 1996). Furthermore, inflammatory cytokines, such as interleukin 1 and tumor necrosis factor α induce the production of pro-matrix metalloproteinase-1, pro-matrix metalloproteinase-3 and pro-matrix metalloproteinase-9 in various cell lines (Gowen et al., 1984; Dayer et al., 1985). These observations suggest that inhibitors of matrix metalloproteinases would be a new type of chondroprotective drug for the treatment of osteoarthritis. From this point of view, we have investigated the effect of various natural compounds, such as flavone, coumarin and cinnamic acid derivatives, etc., on matrix metabolism to find lead compounds that have chondroprotective properties since the effects of these compounds on chondrocyte metabolism have not been fully investigated yet. Among them, esculetin (EST), which is a coumarin derivative, showed interesting pharmacological activities on chondrocyte metabolism.

Esculetin, 6,7-dihydroxycoumarin, is a component of *Artemisia scoparia* and *Fraxinus japonica Blume* and has

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been reported to exert interesting biological and biochemical activities, such as inhibition of platelet 12-lipoxygenase and 5-lipoxygenase of polymorphonuclear leukocytes, anti-inflammatory effects in the Croton oil ear test, antiproliferative effects on 7, 12-dimethylbenz[α]anthracene-induced mammary carcinoma in rat and scavenging of O^{2-} (Loggia et al., 1988; Tubaro et al., 1988; Huang et al., 1993; Noguchi et al., 1993; Martín-Aragón et al., 1996). We have found that esculetin has suppressive activity on matrix metalloproteinase production and proteoglycan degradation. In addition, esculetin down-regulates the production of pro-matrix metalloproteinase-1 and pro-matrix metalloproteinase-3, accompanied by a reduction in their mRNA expression.

2. Materials and methods

2.1. Materials

Esculetin was purchased from Aldrich Chemical (Milwaukee, WI, USA). 1,9-Dimethylmethylene blue was from Poly-Science, (Warrington, PA, USA); papain, shark chondroitin sulfate, *N*-acetyl cysteine, lactalbumin enzymatic hydrolysate, 1,10-phenanthroline and cycloheximide were from Sigma (St. Louis, MO, USA); $Na_2[^{35}S]O_4$ and [^{14}C]amino acid mixture were from Amersham (Bucks, UK); penicillin G, streptomycin, fungizone, fetal bovine serum and Ham/F-12 medium were from Life Technologies (Grand Island, NY, USA); S-Clone medium was from Sanko Junyaku (Tokyo, Japan); cetylpyridinium chloride and 4-aminophenylmercuric acetate were from Tokyo Kasei (Tokyo, Japan) and recombinant human interleukin-1α ($\geq 1 \times 10^8$ units/mg) was from Genzyme (Cambridge, MA, USA). Sheep anti-(rabbit pro-matrix metalloproteinase-1 or pro-matrix metalloproteinase-3) serum, human pro-matrix metalloproteinase-1 cDNA (1.6 kb) and human pro-matrix metalloproteinase-3 cDNA (1.4 kb) were gifts from Dr. Hideaki Nagase (University of Kansas Medical Center, KS, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was from Clontech Laboratories (Palo Alto, CA, USA). Dulbecco's modified Eagle's medium (DMEM) was from Dainippon Pharmaceutical (Osaka, Japan). The other chemicals were all of reagent grade.

2.2. Cell culture of rabbit articular chondrocytes

Primary monolayer cultures of articular chondrocytes were established from articular cartilage of the knee and shoulder joints of male New Zealand White rabbits weighing 1.2 kg. Cells isolated by treatment with bacterial collagenase were cultured in 24-multiwell culture plates (Becton Dickson, Singapore) at a density of $1–3 \times 10^5$ cells/ml in Ham/F-12 supplemented with 10% (v/v) fetal bovine serum containing penicillin G (100 μg/ml), streptomycin (100 μg/ml) and fungizone (250 ng/ml). Confluent chondrocytes of primary culture were used for all experiments.

2.3. Proteoglycan depletion

When chondrocytes in 24-multiwell plates reached confluence, the culture medium was changed to 900 μl of fresh S-Clone media/0.1% (w/v) human albumin containing various concentrations of test compounds. Two hours later, 100 μl of recombinant human interleukin-1α (200 units/2 ng protein/ml) solution or the culture medium was added. Test compounds were dissolved in dimethylsulfoxide (DMSO) as a 40-mM stock solution and were subsequently diluted with S-clone medium. The final concentration of DMSO was 0.25% (v/v) and the same amount of vehicle was added to the control cultures. Forty-two hours later, the cell layers were washed once with Ham/F-12 medium and digested with 330 μg/ml of papain in neutral phosphate buffer (pH 6.5) containing 2 mM EDTA and 2 mM *N*-acetyl cysteine, for 4 h at 65°C. Proteoglycan depletion is expressed as the glycosaminoglycan content in the cell layer. The level of glycosaminoglycan was measured by spectrophotometry with a solution of 1,9-dimethylmethylene blue by the method of Farndale et al. (1982). The amount of glycosaminoglycan was calculated from a standard curve obtained concomitantly for authentic shark chondroitin sulfate.

2.4. [^{35}S]proteoglycan release

Confluent chondrocytes in 24-multiwell plates were pulse-labeled with 74 kBq (2 μCi) of $Na_2[^{35}S]SO_4$ in Ham/F-12 medium supplemented with 10% (v/v) fetal bovine serum. After 24 h, free $[^{35}S]SO_4^{2-}$ was removed and the cell layer was washed several times with Ham/F-12 medium. Then, 900 μl of fresh S-clone medium/0.1% (w/v) human albumin containing test compounds prepared as described above was added. Two hours later, 100 μl of recombinant human interleukin-1α (1000 units/ml) or culture media was added. Forty-two hours later, [^{35}S]proteoglycan release was determined by measuring the amount of [^{35}S]sulfated glycosaminoglycan precipitated following a 4-h incubation at 37°C with cetylpyridinium chloride, as described by Solursh and Meier (1972).

2.5. [^{35}S] proteoglycan synthesis

Confluent chondrocytes in 24-multiwell plates were incubated in 1.4 ml of fresh S-clone medium/0.1% (w/v) human albumin and 200 μl of test compounds prepared as described above was added. Two hours later, 200 μl of 74 kBq (2 μl) of $Na_2[^{35}S]SO_4$ and 200 μl of recombinant human interleukin-1α (200 units/ml) or culture medium was added. Twenty-four hours later, the supernatant was removed and the amount of [^{35}S]sulfated glycosamino-

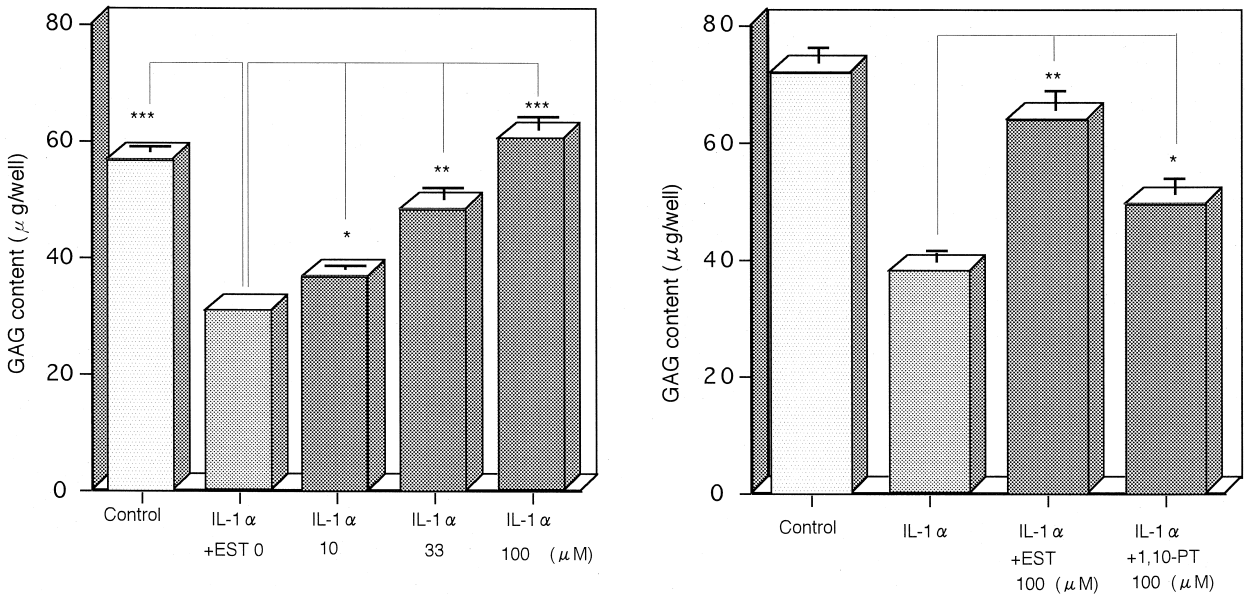

Fig. 1. Effect of esculetin (EST) and 1,10-phenanthroline (1,10-PT) on the proteoglycan depletion induced by recombinant human interleukin-1α (IL-1α). The proteoglycan depletion was measured in the absence or the presence of test compounds under recombinant human interleukin-1α (20 units/ml) stimulation and is expressed as the glycosaminoglycan (GAG) content, using authentic shark chondroitin sulfate as described in Section 2. Three independent experiments with cells of different origin were highly reproducible, and typical data are shown. Each bar represents the mean ± S.E.M. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, when compared to the value for control or recombinant human interleukin-1α-treated control cells.

glycan was measured using the same method described as for proteoglycan release. Alternatively, the cell layers were immediately digested with papain solution and then the amount of [^{35}S]sulfated glycosaminoglycan was measured using the same method described for proteoglycan release. The sum of [^{35}S]sulfated glycosaminoglycan in the cell layer and the culture medium was counted. The results are expressed as the ratio of synthesized proteoglycan (%).

2.6. Measurement of collagenolytic activity

Collagenolytic activity in the culture medium was measured using fluorescein isothiocyanate (FITC)-conjugated type-I collagen as substrate, according to the method of Nagai et al. (1984). To determine the total collagenolytic activity, the culture medium was treated with 1 mM 4-aminophenylmercuric acetate for 2 h at 37°C. One unit of collagenolytic activity is defined as the amount of enzyme which degrades 1 μl substrate/min at 37°C.

2.7. Treatment of chondrocytes for the production of pro-matrix metalloproteinases and Western blot analysis

Confluent chondrocytes in 24-multiwell plates were treated with various concentrations of esculetin in a total of 900 μl of fresh DMEM/0.2% (w/v) lactalbumin enzymatic hydrolysate. Two hours later, 20 units of recombinant human interleukin-1α dissolved in 100 μl of the above culture medium or vehicle culture medium was added to the culture. After a 42-h treatment, the conditioned culture medium was harvested and stored at −20°C until use.

Western blot analysis for pro-matrix metalloproteinases was carried out as follows. Each sample from triplicate wells was concentrated by precipitating proteins with 3.3% (w/v) trichloroacetic acid and subjected to sodium dode-

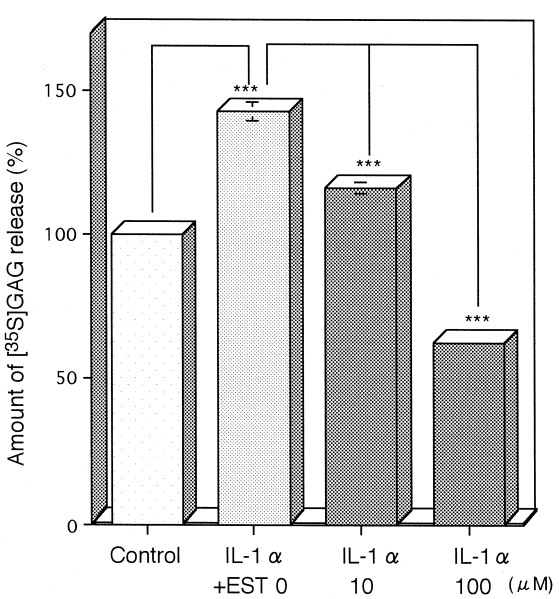

Fig. 2. Effect of esculetin (EST) on the [^{35}S]proteoglycan release induced by interleukin-1α (IL-1α). [^{35}S]Proteoglycan ([^{35}S]GAG) release was measured in the absence or the presence of test compounds under recombinant human interleukin-1α (100 units/ml) stimulation and is expressed as the amount of [^{35}S]glycosaminoglycan released (%), as described in Section 2. Each bar represents the mean ± S.E.M. ($n = 3$). $^{***}P < 0.001$, when compared to the value for control or recombinant human interleukin-1α-treated control cells.

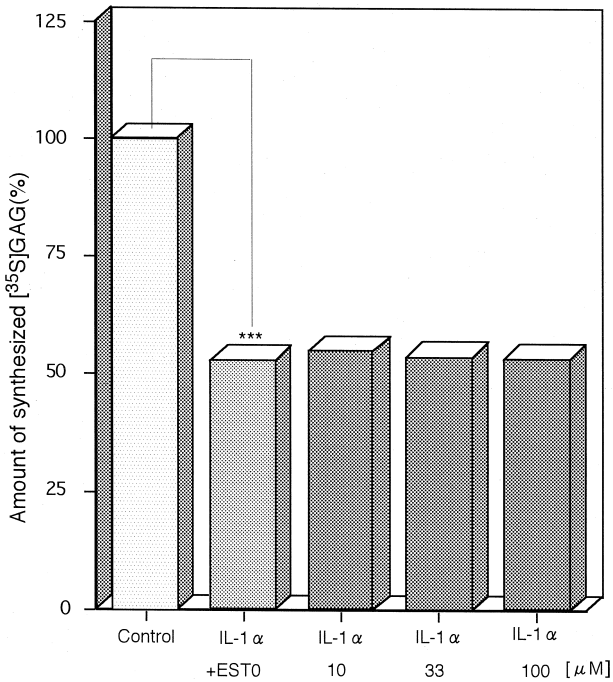

Fig. 3. Effect of esculetin (EST) on the suppression of [^{35}S]proteoglycan synthesis induced by interleukin-1α (IL-1α). The [^{35}S]proteoglycan ([^{35}S]GAG) synthesis was measured in the absence or the presence of test compounds under recombinant human interleukin-1α (20 units/ml) stimulation and is expressed as the amount of [^{35}S]glycosaminoglycan synthesized (%), as described in Section 2. Each bar represents the mean ± S.E.M. ($n = 4$). $^{***}P < 0.001$, when compared to the control value.

cyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 11.5% (w/v) acrylamide gel under reducing conditions (Laemmli, 1970). After electrophoresis, proteins in the gel were electrotransferred onto a nitrocellulose filter. The filter was incubated with sheep anti-(rabbit pro-matrix metalloproteinase-1 or pro-matrix metalloproteinase-3) antibody, which was then complexed with horseradish peroxidase-conjugated donkey anti-(sheep immunoglobulin G (H + L)) immunoglobulin G. Immunoreactive pro-matrix metalloproteinases-1 and -3 were indirectly visualized using ECL-Western blotting detection reagents according to the manufacturer's instructions (Amersham Life Science, Tokyo, Japan).

2.8. RNA isolation and Northern blot analysis

Total cytoplasmic RNA of cultured rabbit chondrocytes was isolated by using a commercial RNA extraction reagent (Isogen; Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The isolated RNA (20 μg), which was denatured with formaldehyde and formamide, was run on a 1% (w/v) formaldehyde-agarose gel and then transferred onto a nylon membrane. The membrane was hybridized with [^{32}P]labeled random primed cDNA of human pro-matrix metalloproteinase-1, pro-matrix metalloproteinase-3 or GAPDH at 42°C in a hybridization solution: 5 × SSC (1 × SSC (Standard Saline Citrate), 0.15 M NaCl/0.015 M sodium citrate, pH 7.5)/1 × Denhardt's solution/50 mM sodium phosphate (pH 6.5)/0.1% (w/v) SDS/50% (v/v) formamide and 200 μg/ml of heat-denatured salmon sperm DNA. After hybridization, the membrane was washed with 0.1 × SSC/0.1% (w/v) SDS for 10 min at room temperature and exposed to Konica X-ray film/medical at −80°C.

2.9. Measurement of protein synthesis and cell viability

Confluent chondrocytes in 24-multiwell plates were treated with esculetin and cycloheximide in 900 μl of S-Clone media containing 0.1% (w/v) human albumin. Then, 37 kBq (1 μl) of [^{14}C]amino acid mixture was added to label the proteins for 18 h. The radioactivity in acid-precipitable proteins was measured by the method of Pohjanpelto and Hölttä (1990).

Cell viability was also monitored by measuring the release of cytoplasmic lactate dehydrogenase (LDH) into the culture medium, using a diagnostic reagent system (Chugai Pharmaceutical, Tokyo, Japan).

2.10. Statistical analysis

The data are expressed as means ± S.E.M. P values less than 0.05 were considered to be significant (unpaired Student's t-test).

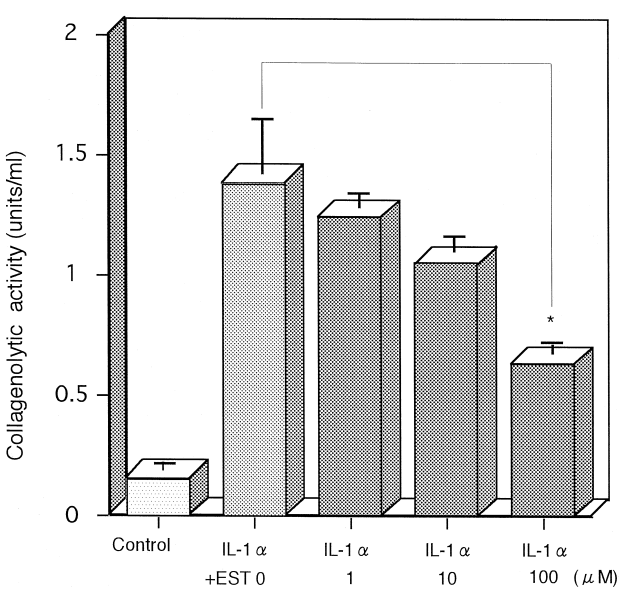

Fig. 4. Effect of esculetin (EST) on the collagenolytic activity in the culture medium induced by interleukin-1α (IL-1α). Collagenolytic activity was measured in the absence or the presence of test compounds under recombinant human interleukin-1α (20 units/ml) stimulation and is expressed as the total collagenolytic activity, as described in Section 2. Each bar represents the mean ± S.E.M. ($n = 3$). $^{*}P < 0.05$, when compared to the value for the recombinant human interleukin-1α treated control cells.

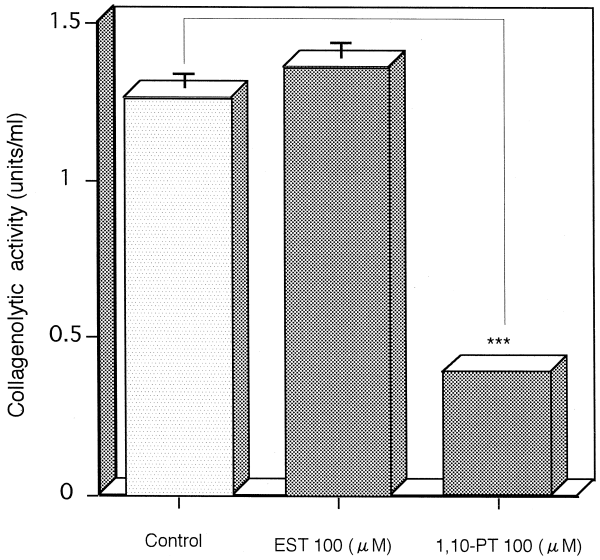

Fig. 5. Effect of esculetin (EST) on the collagenolytic activity of activated matrix metalloproteinases. The culture medium of recombinant human interleukin-1α treated chondrocytes was used as a source of enzyme and was first treated with 1 mM 4-aminophenylmercuric acetate at 37°C for 2 h to activate pro-matrix metalloproteinases. Then an aliquot of the medium was incubated with EST (100 μM) or 1,10-phenanthroline (1,10-PT) (100 μM) for 4 h and the residual collagenolytic activity was determined as described in Section 2. Each bar represents the mean ± S.E.M. of duplicate experiments. $^{***}P < 0.001$, when compared to the control value.

3. Results

3.1. Effect of esculetin on the interleukin-1α mediated depletion of proteoglycan, [^{35}S]proteoglycan release and synthesis in rabbit chondrocytes

When the confluent rabbit chondrocytes were treated with recombinant human interleukin-1α for 42 h, the glycosaminoglycan content of the matrix layer decreased significantly to about 50% of the control value (Fig. 1). Esculetin interfered with the recombinant human interleukin-1α mediated depletion of proteoglycan in a dose-dependent manner (Fig. 1); 100 μl esculetin almost abolished the proteoglycan depletion. Furthermore, co-treatment of articular chondrocytes with recombinant human interleukin-1α and 1,10-phenanthroline (100 μM), a known inhibitor of matrix metalloproteinases (Braunhut and Moses, 1994), also suppressed the depletion of proteoglycan from chondrocytes.

We further examined the effects of esculetin on the release of pulse-labeled [^{35}S]proteoglycan from rabbit chondrocytes. When the chondrocytes were treated with 100 units/ml of recombinant human interleukin-1α for 42 h, the release of [^{35}S]proteoglycan increased 1.4-fold as compared with that of untreated control cells (Fig. 2). In contrast, ESt interfered with the recombinant human interleukin-1α induced release of [^{35}S]proteoglycan from the

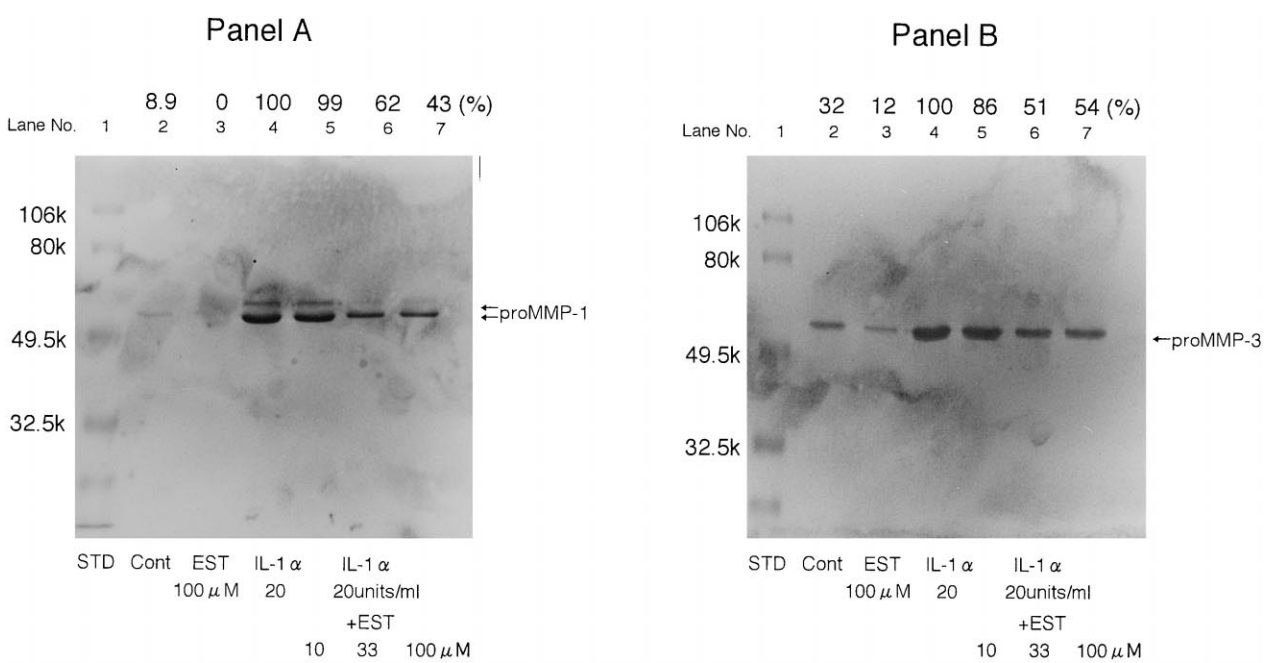

Fig. 6. Effect of esculetin (EST) on the production of pro-matrix metalloproteinase-1 (proMMP-1) and pro-matrix metalloproteinase-3 (proMMP-3) by chondrocytes in culture. The amount of proMMP-1 and proMMP-3 was measured by Western blotting as described in Section 2. The relative amounts of proMMP-1 and proMMP-3 were quantified by densitometric scanning, taking the recombinant human interleukin-1α (IL-1α)-treated cells as 100%. Lane 1, protein size marker (STD); lane 2, control cells; lane 3, cells treated with EST (100 μM); lane 4, cells treated with recombinant human IL-1α (20 units/ml) and lanes 5–7, cells treated with recombinant human IL-1α (20 units/ml) and EST 10, 33 and 100 μM, respectively.

cells in a dose-dependent manner. However, esculetin did not have a significant effect on the suppression of [^{35}S]proteoglycan synthesis under recombinant human interleukin-1α stimulation (Fig. 3). 1,10-Phenanthroline also inhibited effectively [^{35}S]proteoglycan release in recombinant human interleukin-1α treated chondrocytes (data not shown). These observations suggest that matrix metalloproteinases are likely to participate in the depletion and release of proteoglycan from interleukin-1α treated chondrocytes.

3.2. Effect of esculetin on collagenolytic activity

To elucidate whether esculetin affects the activity of matrix metalloproteinase in the culture medium, we investigated the effect of esculetin on recombinant human interleukin-1α induced collagenolytic activity in rabbit articular chondrocytes. When confluent rabbit chondrocytes were treated with recombinant human interleukin-1α (20 units/ml) for 48 h, collagenolytic activity was significantly increased: about a 9-fold increase was observed as compared with that of untreated cells (Fig. 4). Esculetin was found to suppress the recombinant human interleukin-1α augmented collagenolytic activity in a dose-dependent manner with a maximal 61% inhibition being observed with 100 μM esculetin. In addition, it was investigated whether esculetin had a direct inhibitory effect against collagenolytic activity. The culture medium of recombinant human interleukin-1α treated chondrocytes was used as a source of enzyme and was treated with 1 mM 4-aminophenylmercuric acetate at 37°C for 2 h to activate pro-matrix metalloproteinases. As shown in Fig. 5, esculetin did not directly modulate the apparent collagenolytic activity whereas 1,10-phenanthroline inhibited it almost completely, suggesting that esculetin diminishes the apparent collagenolytic activity by suppressing the production of interstitial collagenase/matrix metalloproteinase-1 in rabbit articular chondrocytes.

3.3. Esculetin suppresses the interleukin-1α mediated production of pro-matrix metalloproteinase-1 and pro-matrix metalloproteinase-3 in rabbit chondrocytes

Western blot analysis showed that rabbit chondrocytes spontaneously produced and released pro-matrix metalloproteinase-1 (Fig. 6, panel A) and pro-matrix metalloproteinase-3 (Fig. 6, panel B) into the culture medium and that recombinant human interleukin-1α enhanced the production of both pro-matrix metalloproteinases. Similar to its effect on the collagenolytic activity in the culture medium, esculetin suppressed the recombinant human interleukin-1α induced production of pro-matrix metalloproteinase-1 and pro-matrix metalloproteinase-3 in a dose-dependent manner.

We investigated whether the suppressive effect of esculetin on the production of pro-matrix metalloproteinases was due to the modification of their gene expression. As shown in Fig. 7, recombinant human interleukin-1α significantly enhanced the steady-state levels of both pro-matrix metalloproteinase-1 (2.3 kb) and pro-matrix metalloproteinase-3 (2.3 kb) mRNAs in rabbit chondrocytes. esculetin was found to suppress recombinant human interleukin-1α induced gene expression: a maximal 70% inhibition of both transcripts was observed after treatment with 100 μl esculetin. These results indicated that the decreased production of pro-matrix metalloproteinases-1 and -3 by esculetin was due to the suppression of their gene expression in rabbit chondrocytes.

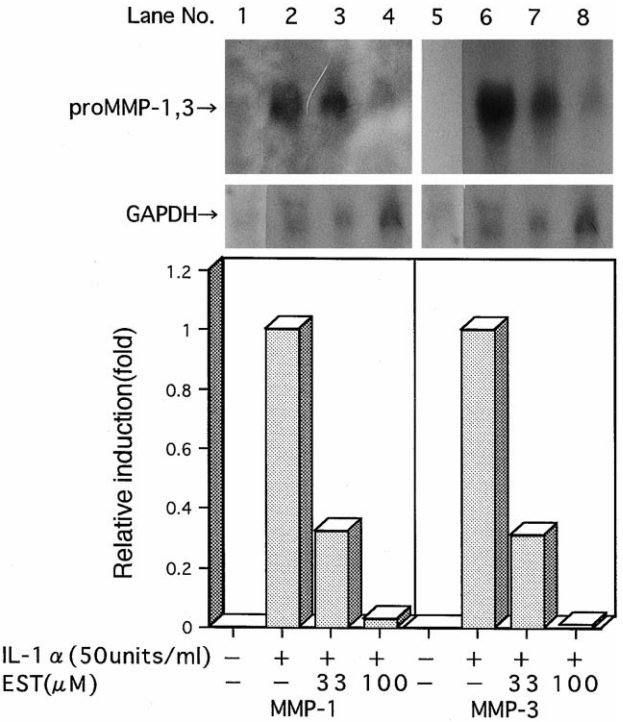

Fig. 7. Suppression by esculetin (EST) of recombinant human interleukin-1α (IL-1α)-induced expression of pro-matrix metalloproteinase-1 (pro-MMP-1) and pro-matrix metalloproteinase-3 (proMMP-3) mRNA in rabbit chondrocytes. The amount of proMMP-1 mRNA and proMMP-3 mRNA was measured by Northern blotting as described in Section 2. Two independent experiments using cells of different origins were highly reproducible, and representative data are shown. The relative amounts of mRNA standardized by GAPDH mRNA were quantified by densitometric scanning, taking the value of recombinant human IL-1α-treated cells as 1. Lane 1, control cells; lane 2, cells treated with recombinant human IL-1α (50 units/ml) and lanes 3–4 and 7–8, cells treated with recombinant human IL-1α (50 units/ml) and EST 33 and 100 μM, respectively.

3.4. Cytotoxicity of esculetin for rabbit chondrocytes

To clarify whether esculetin suppresses cellular functions including the production of pro-matrix metalloproteinases, the cytotoxicity of esculetin to rabbit chondrocytes was examined by measuring protein synthesis and the release of LDH. As shown in Fig. 8A, [^{14}C]amino acid incorporation into acid-precipitated proteins was slightly increased in the esculetin-treated cells whereas it was completely inhibited by cycloheximide. In addition, as shown in Fig. 8B, EST did not augment the release of

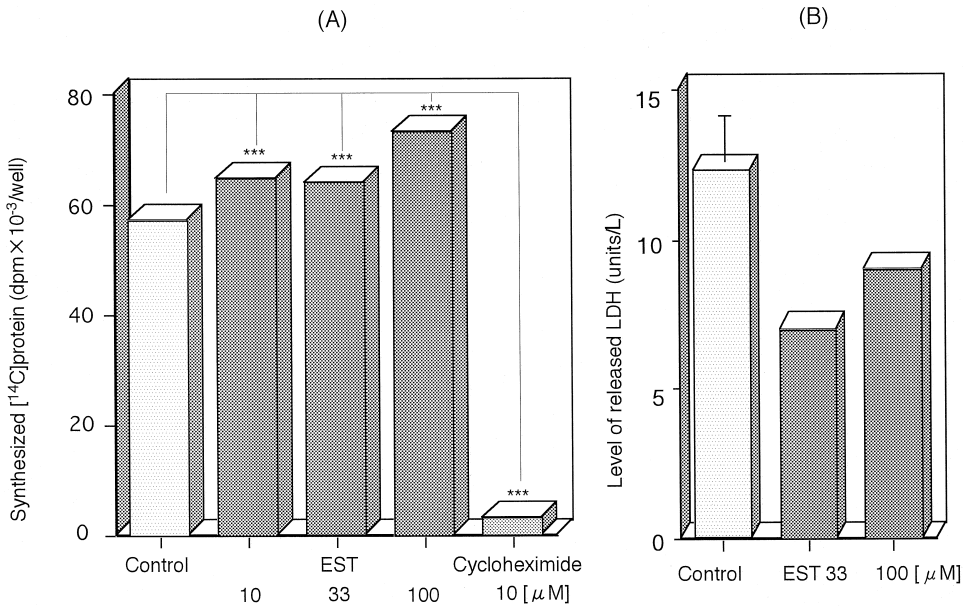

Fig. 8. Effect of esculetin (EST) on the protein synthesis and viability of rabbit chondrocytes. Protein synthesis (A) was measured by the incorporation of [^{14}C]amino acid mixture into acid-precipitable proteins of chondrocytes treated with EST (10, 33 and 100 μM) or without EST. Each bar represents the mean ± S.E.M. ($n = 3$). Cell viability (B) was determined by measuring the level of LDH in the culture media from rabbit chondrocytes treated with recombinant human interleukin-1α (IL-1α) 20 units/ml) and/or EST (33 and 100 μM, respectively). Each bar represents the mean ± S.E.M. ($n = 3$). $^{***}P < 0.001$, when compared to the control value.

LDH from rabbit chondrocytes. These results suggested that esculetin does not alter cell viability under these experimental conditions and specifically down-regulates the production of both pro-matrix metalloproteinases-1 and -3 in rabbit chondrocytes.

4. Discussion

Changes in the properties of cartilage in joints are substantially involved in osteoarthritis. Pain and functional disability, which are the most general clinical features of diseases, are commonly used to monitor osteoarthritis in daily rheumatological practice. These symptoms can be therapeutically improved with drugs such as analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs) and anti-depressive agents. However, since NSAIDs or lipoxygenase inhibitors do not effectively block the destruction of the cartilage matrix, the development of a new class of drug is a high priority (Shinmei et al., 1988; Rainsford, 1989). Recent studies have revealed the involvement of various cytokines and matrix metalloproteinases in the destruction of articular cartilage in diseases such as rheumatoid arthritis and osteoarthritis, which has led to the development of new candidate drugs strong enough to inhibit the actions of interleukin-1 and matrix metalloproteinases. An effective drug has to reach the stage of therapeutic use, although many drugs for the treatment of osteoarthritis and tumors are at a developmental stage (Morphy et al., 1995; Caron et al., 1996; Smith et al., 1998).

In general, the erosion of cartilage in osteoarthritis is initially caused by a decrease in the proteoglycan content, followed by destruction of collagen fibers. Therefore, we focused on the decrease of proteoglycan in the matrix of primary chondrocyte cultures to investigate a new type of agent with chondroprotective properties. It was demonstrated that esculetin significantly suppressed the proteoglycan depletion in response to recombinant human interleukin-1α in a dose-dependent manner. This decrease in proteoglycan content is thought to result from an imbalance between the synthesis and degradation of proteoglycan. We observed that esculetin did not modulate proteoglycan synthesis in rabbit chondrocytes but rather reduced the [^{35}S]proteoglycan release induced by recombinant human interleukin-1α. In this case, esculetin inhibited the release of [^{35}S]proteoglycan below the basal level. This is likely due to the inhibition of spontaneous proteoglycan release by esculetin through the suppression of the spontaneous production of pro-matrix metalloproteinase-3, as shown in Fig. 6.

We also investigated whether the suppressive action of esculetin was due to cytotoxicity under our experimental conditions. Cycloheximide significantly reduced protein synthesis whereas esculetin did not suppress but slightly augmented it. Furthermore, the release of LDH was not augmented by the treatment of chondrocytes with esculetin and the intracellular level of LDH was not decreased (data not shown), indicating that the suppressive action of esculetin on the proteoglycan depletion and release was not the result of a cytotoxic action.

Arner et al. (1987) reported that cycloheximide blocked recombinant human interleukin-1α-stimulated proteoglycan release which suggests that some proteinases are required for the degradation of proteoglycan in cartilage. It is well known that many proteinases, such as matrix metalloproteinases, cysteine proteinases and serine proteinases, and oxygen radicals, which are found in connective tissues and inflammatory cells, can degrade cartilage matrix components (Welgus et al., 1981; Galloway et al., 1983; Van Noorden et al., 1988; Gavrilovic and Murphy, 1989; Wu et al., 1991). A recent study of human osteoarthritis cartilage indicated that the destruction of cartilage extracellular matrix is likely mediated by an increase in matrix metalloproteinase activity. Of the matrix metalloproteinases, the activities of matrix metalloproteinase-1 and matrix metalloproteinase-3 are significantly increased in osteoarthritis, and the increased activity of matrix metalloproteinases in knee synovial fluid and osteoarthritis cartilage was found to be due to a relative decrease in the level of their physiological inhibitors (Tissue inhibitor of matrix metalloproteinase; TIMP) and/or increase in the level of their physiological activators (Pelletier et al., 1983; Dean et al., 1989; Lohmander et al., 1993). We demonstrated that 1,10-phenanthroline inhibited the recombinant human interleukin-1α-mediated-proteoglycan depletion and [^{35}S]proteoglycan release in rabbit chondrocytes, suggesting that the production of matrix metalloproteinases closely contributes to the degradation and depletion of proteoglycan in these primary chondrocyte cultures. From this point of view, we focused on the effect of esculetin on the production of matrix metalloproteinases-1 and -3 and demonstrated that the recombinant human interleukin-1α mediated production and gene expression of pro-matrix metalloproteinases-1 and -3 were significantly suppressed by esculetin in a dose-dependent manner. However, esculetin did not directly inhibit the collagenolytic activity whereas l, 10-phenanthroline did. Thus, these results suggest that esculetin transcriptionally regulates the production of both pro-matrix metalloproteinase-1 and pro-matrix metalloproteinase-3, unlike the direct synthetic matrix metalloproteinase inhibitors which have been under investigation as therapeutic agents for cancer and osteoarthritis.

We have recently synthesized a novel coumarin derivative, 6-[[2-(acetylamino)-2-deoxy-β D-glucopyranosyl]-oxy]-7-hydroxycoumarin (CPA-926), as a prodrug of esculetin. This derivative exerts potent inhibitory activity against the degradation of cartilage in a lateral meniscectomized rabbit osteoarthritis model following oral administration (Yamada et al., in press). It is likely that the in vivo chondroprotective effect of CPA-926 is mediated by its metabolite, esculetin, by inhibition of pro-matrix metalloproteinase production by chondrocytes, although further experiments are needed to verify this.

In conclusion, we have demonstrated that esculetin effectively inhibits the proteoglycan release induced by recombinant human interleukin-1α and transcriptionally down-regulates the production of pro-matrix metalloproteinases-1 and -3 without being cytotoxic to other cell functions, leading to the suppression of proteoglycan depletion in rabbit chondrocytes. Therefore, esculetin could be a useful therapeutic candidate for cartilage diseases such as osteoarthritis.

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