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Effects of (-)-Epigallocatechin-3-gallate on Cyclooxygenase 2 and Matrix Metalloproteinase 3, 9, 13 Expression Induced by IL-1ß in Rat Meniscal Fibrochondrocytes

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ABSTRACT

The objective of this study was to examine the effect of (-)-epigallocatechin-3-gallate (EGCG) on cyclooxygenase 2 (COX-2) and matrix metalloproteinase (MMP) 3, 9, and 13 expressions induced by IL-1β in rat meniscal fibrochondrocytes. Rat normal meniscal fibrochondrocytes were enzymatically isolated from meniscal tissue from the hind leg knee joint. Cells were pretreated with EGCG at a concentration of 50 μM to examine its effect in the presence of IL-1β insult. Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR were used to assess the COX-2 and MMP3, 9, and 13 gene expressions, respectively. COX-2 gene upregulation induced by IL-1β in primary cultures of rat meniscal fibrochondrocytes was significantly suppressed by EGCG ($p < 0.05$). MMP 3, 9, and 13 gene expressions were also significantly induced by IL-1β stimulation and significantly inhibited by EGCG treatment ($p < 0.05$). These results indicated that EGCG may be of value in the treatment of meniscal inflammation associated with IL-1β secretion.

Key words: epigallocatechin-3-gallate, cyclooxygenase 2, matrix metalloproteinase, meniscus, fibrochondrocyte, IL-1β

INTRODUCTION

The knee joint menisci are able to distribute loads to maintain joint stabilization and lubrication[(1)]. However, they are frequently injured during trauma, which is characterized by the occurrence of tears and/or ragged or peripheral serrated splitting[(2,3)]. Also, degenerative tears can commonly occur in older persons and in association with osteoarthritis (OA) of the knee. It has been well recognized that high interleukin-1 beta (IL-1β) expression is present on the surface of osteoarthritic cartilage and synovial membranes at all stages of the disease[(4-6)]. Expression of IL-1β may lead to induction of cyclooxygenase 2 (COX-2) and subsequent prostaglandin E2 (PGE_2) production, which may subsequently modulate matrix degradation of the human knee joint cartilage[(7)].

Matrix metalloproteinases (MMPs) are a large family of zinc-dependent proteinases involved in the degradation and remodeling of the extracellular matrix. Under normal physiological conditions, the activities of these enzymes are involved in normal cell functions and regulate host defense mechanisms. These enzymes are tightly regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs)[(8)].MMPs have also been reported to be associated with tumorigenic processes that promote tumor invasion and metastasis[(9)]. Aberrant MMP activities due to an imbalance in the levels of MMPs and TIMPs have been implicated in the pathogenesis of a variety of diseases such as OA and rheumatoid arthritis (RA)[(10)]. Early work has demonstrated the presence of multiple MMP family members in disease-state joints, and recent advances in the development of new knockout mice and disease models have allowed investigators to directly test their roles in arthritis[(11)].

MMPs have been found to be elevated in animal models and in cartilage tissue and synovial fluid from OA and RA patients. MMP-3 and MMP-9 are initially upregulated in the synovium, which may play a pivotal role in the pathogenesis of cartilage degeneration[(12-14)]. MMP13 catalyzes the cleavage of type II collagen, the main structural protein in articular hyaline cartilage[(15)], and this is the rate-limiting step in the process of collagen degradation.

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MMP inhibitors (MMPIs) are expected to be useful for the treatment of diseases such as OA, RA, and cancer. A vast number of molecules such as doxycycline, CMT-7 (a modified tetracycline), and Ro 32-3555 have been developed as MMPIs in recent years[16,17]. The (-)-Epigallocatechin-3-gallate (EGCG) is extracted from the leaves of the *Camellia sinensis* plant as its most abundant catechin, and the beneficial effects of this purified compound have been tested in various diseases. EGCG may prevent UVB-induced skin damage, isoproterenol-induced myocardial infarction, and lipopolysaccharide-induced oxidative stress by its function as an antioxidant[18-20]. EGCG also inhibits the proliferation of human prostate and breast cancer cells[21,22]. EGCG has been shown to inhibit the IL-1β-induced expression and activity of cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) in OA chondrocytes isolated from degenerated hyaline cartilage[23]. Its effects on other arthritic conditions have also been examined[24,25]. However, its effect on meniscal fibrochondrocytes has not yet been investigated.

In the present study we established primary cultures of meniscal fibrochondrocytes and examined their responses to IL-1β insult[26,27]. We then tested the effect of EGCG on MMP3, 9, and 13 gene expressions induced by IL-1β. EGCG demonstrated an inhibitory effect on COX-2 and MMP3, 9, and 13 gene expressions. These data suggest that EGCG may be of value in the treatment of meniscal inflammation associated with IL-1β secretion.

MATERIALS AND METHODS

I. *Materials*

The (-)-Epigallocatechin gallate (EGCG) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). A stock solution at a concentration of 10 mM was prepared in deionized water (50 mg of EGCG powder in 10.9 mL of water). IL-1β, a powder product, was purchased from R&D Company (Minneapolis, MN, USA). An IL-1β stock solution at a concentration of 5 μg/mL was prepared in sterile phosphate-buffered saline (PBS).

II. *Meniscal Fibrochondrocytes Isolation and Culture*

Male Sprague Dawley (SD) rats (8-week-old, 280-300 g) were obtained from BioLASCO Taiwan Co., Ltd (Taiwan). All experiments were approved by the local Institutional Review Board and performed in adherence to the Laboratory Animals Guidelines (Council of Agriculture, Executive Yuan, R.O.C.) for the treatment of laboratory animals. Meniscus tissue is fibrocartilage and predominantly contains fibrochondrocytes and a few small, peripheral blood vessels. The inner third of the meniscus tissue is devoid of blood vessels. The inner third of the meniscus tissue was aseptically removed from normal SD rats, cut into small fragments, and incubated with antimicrobial solution (500 IU/mL penicillin/streptomycin, Gibco™ Invitrogen, Burlington, Canada) for 1 h and then washed with PBS before digestion with 3 mg/mL type H collagenase (Sigma, St. Louis, MO, USA) at 37°C for 12 h. The samples were checked for digestion by examination under a light microscope. The cell suspension was collected using a sterile pastette. After centrifugation at 1500 rpm for 10 min, the supernatant was discarded and the pellet resuspended in 10 mL DMEM-F12 medium supplemented with 10% FBS (Sigma, St. Louis, MO, USA), 100 IU/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). Cells were seeded in a complete medium at a density of 1×10^5 cell/mL in 60 mm petri dishes (Orange Scientific, Braine-I'Alleud, Belgium) and then cultured in a humidified 5% CO_2 atmosphere at 37°C. Cells between passages 2 and 3 were used.

III. *Experimental Protocol for EGCG Test*

Cells were cultured for 2 weeks in 60 mm tissue culture Petri dishes. Dishes of meniscal fibrochondrocytes were placed in serum-free media overnight, then incubated with 10 ng/mL of IL-1β at 37°C for 0 (control) and 6 h. Cells were pretreated with EGCG for 12 h at a concentration of 50 μM, as described previously for RA synovial fibroblasts[28].

IV. *Immunocytochemistry*

After a 24 h culture of meniscal fibrochondrocytes, cells on the dish were washed with ice-cold PBS twice and then fixed using 2 mL of a 1: 1 methanol/acetone mixture per dish for 5 min at -20°C. Cells were then stained by immunocytochemistry. Immunodetection for CD34 and type II collagen was performed with a standard avidin-biotin-peroxidase complex detection kit (DakoCytomation, Glostrup, Denmark). Dishes were washed twice with PBS and blocked by incubation with 200 μL 1% non-immune horse serum (Vector Laboratories Inc., Burlingame, California, USA) in 1% BSA in antibody diluent (DakoCytomation) for 30 min at room temperature. The solution was poured off and the cells incubated sequentially with CD34 (1: 100) (DakoCytomation, Germany) and type II collagen (1: 200) specific antibody (Sigma) for 60 min, biotinylated secondary antibody (1: 200) for 45 min, and HRP-conjugated streptavidin for 20 min. Between each incubation cells were washed with TBST (12.5 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) three times. For the negative controls, the primary antibody was omitted. The chromogen 3-amino-9-ethylcarbazole (AEC) was then added for 15 min, and then the cells were counterstained with Mayer's hematoxylin. The cells were mounted with a cover slip and visualized under a light microscope.

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V. *Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*

Total RNA was isolated from cells using an easy-BLUETM Total RNA Extraction Kit (iNtRON, Biotechnology, Gyeonggi-do, Korea). For first strand cDNA synthesis, 3 μg of total RNA was used in a single-round RT reaction (total volume 20 μL) containing 0.75 μg oligo(dT)$_{14}$ primer, 1 mM dNTPs, 1x first strand buffer, 0.4 mM DTT, 40 units RNaseOut recombinant ribonuclease inhibitor, and 200 units of superscript II reverse transcriptase (Invitrogen). The RT reaction was performed at 42°C for 2 h, followed by 95°C for 5 min. The PCR was run using 0.9 μL of the RT reaction mixture as template, 0.4 mM of gene specific primers, 1x PCR buffer, 0.25 mM dNTPs, and 1.5 units of *Taq* DNA polymerase (BioMan, Taipei, Taiwan). The amplification was carried out at 94°C for 2 min, then for 35 cycles at 94°C for 45 sec, 56°C for 1 min, and 72°C for 45 sec followed by a final extension at 72°C for 10 min. The PCR products were size-fractionated by 1.5% agarose gel electrophoresis, and DNA bands were visualized by staining the gel with 0.1 μg/mL ethidium bromide. The bands were analyzed using a gel documentation system (Bio-Profil, Bio-1D version 99, Viogene, CA, USA). The primers used were as follows: COX-2 5'-GTCTCTCATCTGCAATAATGTG-3' (sense) and 5'-ATCTGTGTGGGTACAAATTTG-3' (antisense) (Gene bank accession number S67722.1; PCR product 801 bp); GAPDH 5'-CCCATCACCATCTTCCAGGAG-3' (sense) and 5'-GTTGTCATGGATGACCTTGGCC-3' (antisense) (Gene bank accession number X02231; PCR product 284 bp).

VI. *Real-time Quantitative PCR*

In order to quantitatively analyze the regulatory effects of IL-1β and/or EGCG on the expression of MMP3, 9, and 13 in meniscal fibrochondrocytes, real-time RT-PCR was performed using a Biorad iCycler iQ (Biorad, Hercules, CA, USA) utilizing specific primers (Table 1). Two microliters of template cDNA was amplified with SYBR Green Supermix (Biorad Hercules, CA, USA) in a 25 μL reaction containing SYBR Green Supermix, 0.3 mM of each primer, and de-ionized water. The mixture was initially heated at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 10 sec. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Following amplification, melt curve protocols were performed to ensure that primer-dimers or non-specific products had been eliminated or minimized. To analyze the data obtained by real-time PCR, the comparative threshold cycle (C_T) method was applied. Briefly, the amount of a target, normalized to GAPDH and relative to a calibrator, is determined by $2^{-\Delta\Delta}C_T$, where $^{\Delta\Delta}C_T = \Delta C_T$ (sample) $- \Delta C_T$ (calibrator), and ΔC_T is the C_T of the target gene subtracted from the C_T of GAPDH[29].

VII. *Statistical Analysis*

The values from RT-PCR were expressed as the ratio of the band intensity of the target gene to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Variance and *p* values were analyzed by Alphaimager 1220 V5.5 (Alpha Innotech Corporation, San Leandro, CA, USA). Student's test was used for statistical comparison between groups. The results were considered significant at a value of $p < 0.05$.

RESULTS

I. *Characterization of Meniscal Fibrochondrocytes*

Although the meniscal fibrochondrocytes were isolated from the inner third of the meniscus tissue, the isolated tissues were further characterized to make sure they contained no other cellular components. The cultured meniscus cells were characterized by

Table 1. Oligonucleotides primers for real-time quantitative PCR

Gene	Sequence (5'-3')	Size (bp)	Accession no.
MMP3	Forward: TGGGAAGCCAGTGGAAATG Reverse: CCATGCAATGGGTAGGATGAG	81	NM_133523
MMP9	Forward: TGCTCCTGGCTCTAGGCTAC Reverse: TTGGAGGTTTTCAGGTCTCG	88	NM_031055
MMP13	Forward: CTGACC TGG GATTTCCAAAA Reverse: ACACGTGGTTCCCTGAGAAG	90	XM_343345
GAPDH	Forward: CTCAACTACATGGTCTACATGTTCCA Reverse: CTTCCCATTCTCAGCCTTGACT	81	X02231

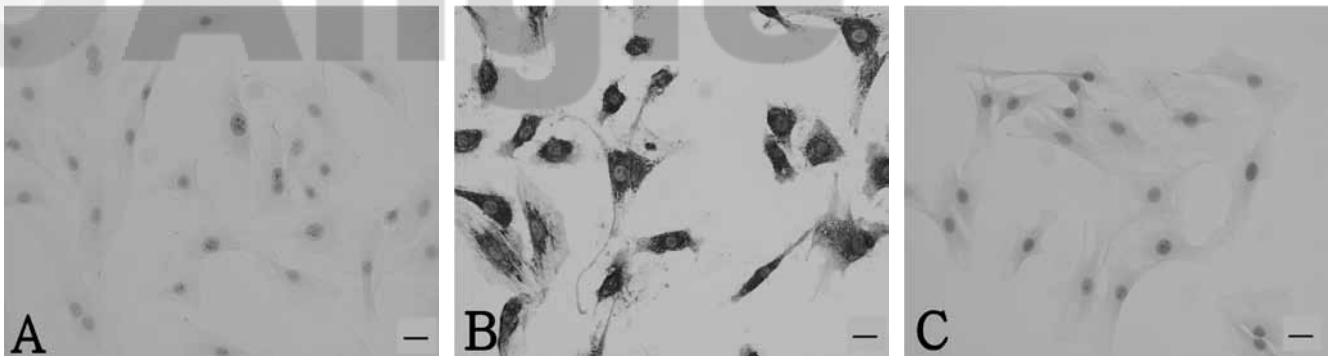

Figure 1. Meniscal fibrochondrocytes was characterized by immunocytochemistry.
(A) Negative control with omitted primary antibody showed no signal. (B) Primary cultures of meniscus cells demonstrated expression of type II collagen. (C) The fibrochondrocytes did not express CD34 (200x). Scale bar 20 μm.

immunocytochemistry with antibodies to detect the expression of type II collagen (produced by fibrochondrocytes) and CD34 (endothelial cell marker expressed by blood vessels). Meniscus cells in vitro (100%) demonstrated a polygonal shape and expression of type II collagen. The cultured cells did not express CD34 (Figure 1A, 1B and 1C). The miniscal fibrochondrocytes were then used for the following experiments.

II. *COX-2 Induction in Meniscal Fibrochondrocytes*

Cyclooxygenase 2 induction by IL-1β in meniscus chondrocytes was examined, and then the effect of EGCG at a concentration of 50 μM was investigated. The concentration of EGCG used showed no significant effect on cell viability. IL-1β at a concentration of 10 ng/mL significantly induced COX-2 gene expression. The ratio of COX-2 / GAPDH was control 0.0972 ± 0.0433 *versus* IL-1β stimulation 0.6920 ± 0.1103 ($p = 0.00828$). Induction of COX-2 was significantly inhibited by pretreatment with EGCG (COX-2 / GAPDH: 0.1012 ± 0.0315, $p = 0.04079$). Treatment with EGCG alone showed no significant change in COX-2 gene expression (COX-2 / GAPDH: 0.0192 ± 0.09807, $p = 0.09807$) (Figure 2). These data suggested that EGCG could suppress the COX-2 expression inducted by IL-1β stimulation in meniscal fibrochondrocytes.

III. *Inhibition of MMP3, 9, and 13 Expression by EGCG*

Six normal rat meniscal fibrochondrocytes were isolated and cultured. IL-1β significantly induced MMP3, 9, and 13 gene expressions in meniscal fibrochondrocytes. The increases were 102.4 ± 28.7 ($p = 0.00586$), 156.9 ± 89.2 ($p = 0.01739$), and 144.2 ± 57.6 fold ($p = 0.00511$) for MMP3, MMP9, and MMP13, respectively. The upregulations of MMP3, 9, and 13 were significantly inhibited by EGCG. Treatment with EGCG alone did not significantly cause any change in MMP 3, 9, and 13 expressions as compared with the control (Figure 3A, 3B, and 3C). These data suggested that EGCG could suppress MMP 3, 9, and 13 expressions induced by IL-1β stimulation in meniscal fibrochondrocytes.

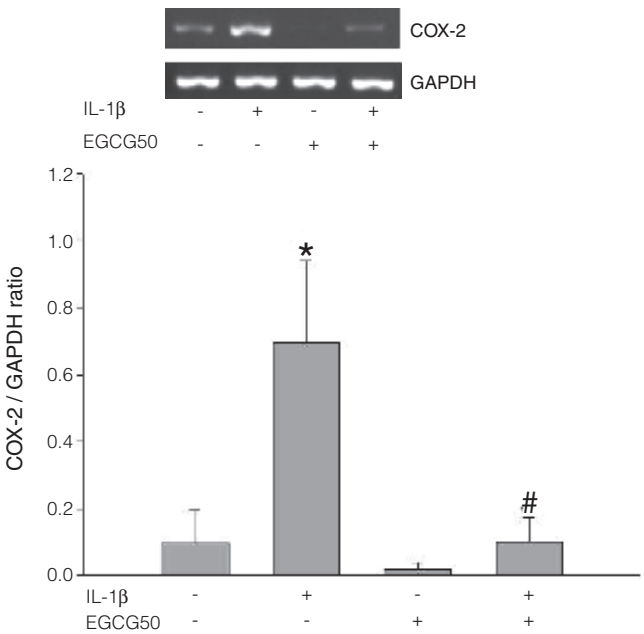

Figure 2. COX-2 induction by IL-1β was suppressed by EGCG at a concentration of 50 μM (EGCG50). COX-2 expression by meniscal fibrochondrocytes was assessed after 6 h co-culture of cells with IL-1β at a concentration of 10 ng/mL. Upper panel: a representative agarose gel demonstrated COX-2 mRNA expression as assessed by reverse transcription-polymerase chain reaction (RT-PCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as internal control. Lower panel: the graph showed the results of densitometric analysis of DNA bands expressed as the mean ± standard deviation (S.D.) of the COX-2 / GAPDH ratio (n = 6). $^{*,\#}p < 0.05$.

DISUSSION

The present study has demonstrated that COX-2 and

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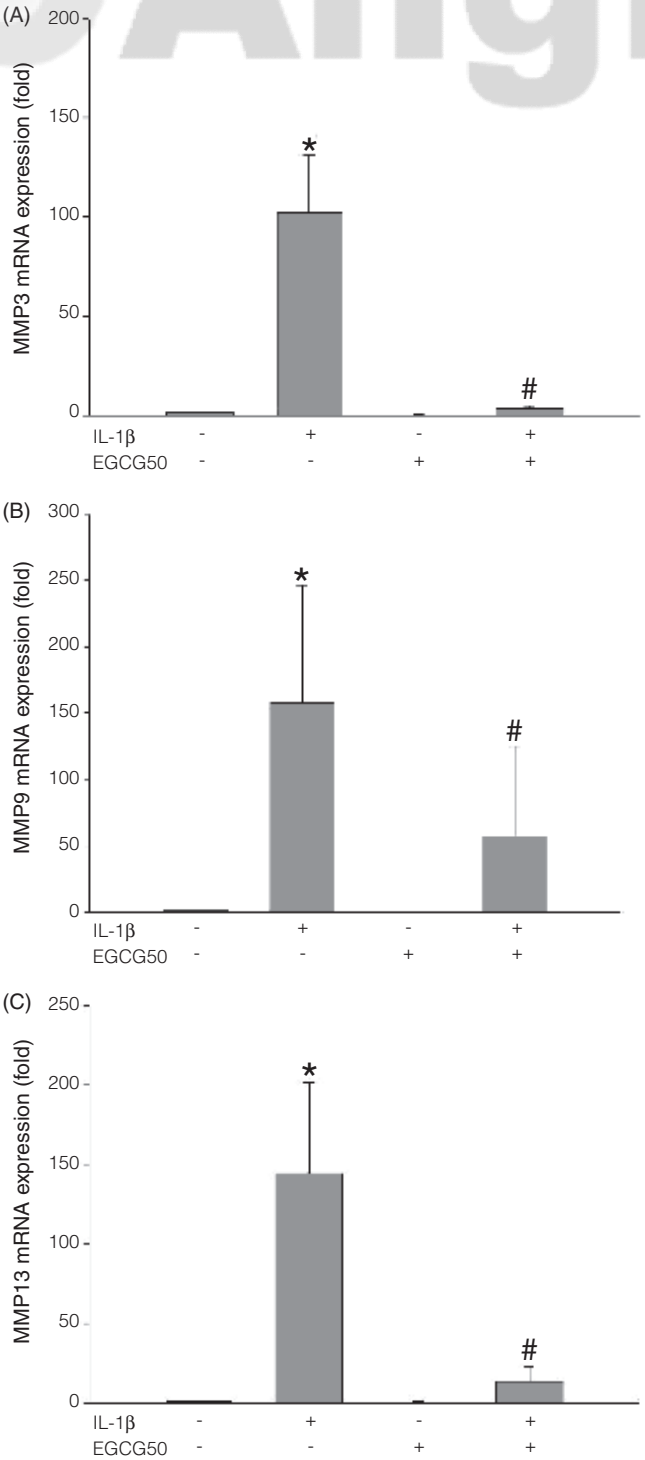

Figure 3. Expression of MMP3, 9, and 13 induced by IL-1β stimulation was suppressed by EGCG at a concentration of 50 μM (EGCG50)**.** MMP3 (A), MMP9 (B), and MMP13 (C) were significantly induced by IL-1β and suppressed by EGCG (n = 6). $^{*,\#}p < 0.05$.

MMP3, 9, and 13 gene expressions are induced by IL-1β stimulation. This upregulation is suppressed by pretreatment with EGCG in meniscal fibrochondrocytes. The high levels of cyclooxygenase / prostaglandin E synthase (COX / PGES) activity in IL-1β-treated meniscus explanted from osteoarthritic patients have been demonstrated[30]. Consistent with our data, IL-1β can induce COX-2 expression in normal meniscal fibrochondrocytes *in vitro*. After establishment of this *in vitro* model we examined the effect of EGCG on the gene expression of the matrix degradation enzymes, MMP3, 9, and 13, which are involved in the pathogenesis of meniscus degeneration.

MMP may be involved in cancer invasion and metastasis[31,32]. EGCG, a green tea polyphenol with chemopreventive properties, has been shown to be an inhibitor of MMPs in human colorectal cancer cells[33], bronchus tumor cells[34], and oral cancer cells[35]. The inhibition pathway of MMP2 and MMP9 in prostate tumor cells by EGCG is mediated via inhibition of phosphorylation of the extracellular regulated kinase (ERK1/2) and p38 molecules, and inhibition of activation of transcription factors c-jun and nuclear factor-kappa B (NF-kB)[36]. In addition to the above anti-neoplastic effects, EGCG also shows benefits in other systems. EGCG suppresses MMP9 activation and reduces neuronal death after transient global cerebral ischemia in the mouse brain[37]. EGCG can rapidly and substantially hamper UV-B irradiation - induced activation of extracellular matrix degradation[38,39]. EGCG reduces the IL-1β stimulated expression of both collagenase and stromelysin, an effect which may be mediated by inhibition of the JNK/SAPK pathway in human tendon fibroblasts[40]. EGCG markedly attenuates the activation of hepatic stellate cells, as well as their MMP2 activity, to prevent hepatic fibrosis[41,42].

In the articular systems, the effects of EGCG on RA synovial fibroblasts and OA articular chondrocytes from hyaline cartilage have also been tested. In synovial fibroblasts, EGCG suppresses TNF-α - induced production of MMP1 and MMP3, which is accompanied by inhibition of the mitogen activated protein kinase (MAPK) and activator protein-1 (AP-1) pathways[43]. EGCG may cause a complete block of IL-1β - induced production of RANTES, ENA-78, and GRO-α[28]. In articular chondrocytes, EGCG inhibits the IL-1β induced mRNA and protein expression of MMP1 and MMP13[44]. The effect of EGCG on MMP gene suppression is consistent with our model. Nevertheless, MMP3, 9, and 13 are involved in meniscal fibrochondrocytes. Inhibition of MMP by the broad-spectrum MMP inhibitor, GM6001, can enhance *in vitro* repair of the meniscus[45]. EGCG has demonstrated its effects in the modulation of MMP activity, including the neoplastic systems, non-neoplastic systems, and arthritic systems. In our meniscus models, we also identify for the first time that IL-1β induces MMP3, 9, 13 upregulations in rat normal meniscus cells and this MMP upregulation was also inhibited by EGCG. EGCG may be of value in the treatment of arthritis. The detailed mechanisms and signalling pathways involved in this process need to be further elucidated.

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