Effect of Theaflavin-3,3’-digallate on Matrix Metalloproteinases in Mouse Chondrocytes

Masaya YAMAGUCHI, Shinichi IWAI, Kazuko TSUIHAMA, Yuri TOMITA and Katsuji OGUCHI

Abstract: Matrix metalloproteinases (MMPs) play an important role in cartilage degradation in rheumatoid arthritis (RA). The inhibition of MMP-3 mRNA expression, in particular, is an interesting therapeutic target in RA. The green tea polyphenol, epigallocatechin-3-gallate (EGCG), is known to inhibit MMP mRNA expression. However, the action of the black tea polyphenol, theaflavin-3,3’-digallate (TF3), on MMP mRNA expression in chondrocytes is not well understood. The ability for the polyphenols TF3 and EGCG to affect the mRNA expression of MMPs, members of the a disintegrin and metalloproteinase protein family (ADAMs), tissue inhibitors of metalloproteinases (TIMPs) or the 67 kDa laminin receptor (67LR) was tested in interleukin-1β-stimulated mouse chondrocytes in vitro. Mouse chondrocytes were obtained from neonatal ddy mice. After 1 week, TF3 or EGCG was added to the cultured mouse chondrocytes with or without IL-1β. TF3 suppressed interleukin-1β-induced increases in MMP-3, MMP-9 and MMP-13 mRNA expression to an equal or greater degree than EGCG, while ADAM-15 and 67LR mRNA expression did not change significantly. TF3 recovered interleukin-1β-induced suppression of TIMP-3 mRNA expression. However, ADAM-17 mRNA production was induced by TF3. This study suggests that TF3 may have a stronger effect than EGCG in controlling the degradation of cartilage in the inflamed joint, and has the therapeutic potential to prevent cartilage destruction via inhibition of MMPs.

Key words: theaflavin-3,3’-digallate (TF3), epigallocatechin-3-gallate (EGCG), matrix metalloproteinase (MMP), a disintegrin and metalloproteinases (ADAMs), chondrocyte

Introduction

Black tea contains catechins, theaflavin, and the theaflavin derivatives theaflavin-3-gallate, theaflavin-3’-gallate and theaflavin-3,3’-digallate (TF3)\(^1\). The theaflavin derivatives have two galloyl groups as a result of the polymerization of two epigallocatechin-3-gallate (EGCG) molecules. Catechins, especially EGCG, have antitumor activity via mechanisms including antioxidation, antiangiogenesis, and others\(^2\)\(^-\)\(^6\). Theaflavins in black tea and catechins in green tea have equally effective antioxidants\(^7\). Theaflavins also have an antitumor effect\(^8\),
and antiangiogenic activity of TF3 was demonstrated in vitro by its ability to inhibit MMPs\(^9\).

Cartilage is synthesized and maintained by chondrocytes, and is composed primarily of water, proteoglycan and collagen. The degradation of cartilage in vitro and in vivo is mediated by neutral endopeptidases from the metalloproteinase class of enzymes, which includes matrix metalloproteinases (MMPs) and ADAMs (a disintegrin and metalloproteinase protein family)\(^10\). The role of MMP-3 in rheumatoid arthritis (RA) has been highlighted from both pathophysiological studies and clinical studies\(^11\). Moreover, tissue inhibitors of metalloproteinases (TIMPs) tightly control the activities of the MMPs in a 1:1 ratio\(^12\).

Metalloproteinases play a crucial role in tissue remodeling, as well as in the destruction of cartilage and bone in an arthritic joint, due to their ability to degrade a wide variety of extracellular matrix components\(^13\). Their substrates include aggrecan, laminin, elastin and the collagens. MMPs also play a role in angiogenesis and inflammation\(^12,14\). Membrane Type-1 (MT1)-MMP is anchored to the cell surface via a transmembrane domain and also has a cytoplasmic tail. Its substrates include collagen, fibronectin, laminin, vitronectin and proteoglycan\(^14\). The production and release of MMPs is dependent on the cellular microenvironment, and is induced by several factors including the proinflammatory cytokine interleukin-1\(\beta\) (IL-1\(\beta\))\(^15\).

Metalloproteinases are inhibited by TIMPs, including TIMP-1, which also inhibits angiogenesis, and TIMP-3, which also inhibits some ADAMs, especially ADAM-17\(^14\). Members of the ADAM family have a disintegrin domain that can bind to integrins and prevent cell-cell interactions. They also possess a cysteine-rich, epidermal growth factor-like transmembrane domain and a cytoplasmic tail domain. The roles of ADAM-15 and ADAM-17 in cartilage have been described, and ADAM-17 is also known for its ability to release tumor necrosis factor (TNF)-\(\alpha\) from the cell surface\(^12\).

Green tea is a rich source of catechins, and several epidemiological and animal model studies have shown that green tea consumption is associated with health benefits including inhibition of inflammation. EGCG can influence a number of cellular mechanisms and inhibits the activities of the MMP family.

The 67 kDa laminin receptor (67LR) is a high affinity non-integrin laminin receptor\(^16\), and appears in cancer cells with high malignancy. The interaction of cancer cells with laminin has been implicated in tumor metastasis and invasiveness, and 67LR is believed to be involved in this process. It was recently reported that 67LR might be the receptor for EGCG\(^17\).

Unlike green tea polyphenols, few studies have reported the effects of black tea polyphenols, such as TF3, in chondrocytes. In the present study, the ability of TF3 and EGCG to affect mRNA expression of MMPs, ADAMs, TIMPs and 67LR in IL-1\(\beta\)-induced mouse chondrocytes was investigated.

**Materials and Methods**

**Reagents**

Mouse recombinant IL-1\(\beta\), EGCG and TF3 was obtained from Wako Pure Chemical Industries Ltd (Osaka).

**Isolation of Primary Chondrocytes**

Primary chondrocytes were isolated from neonatal ddY mice (less than postnatal day 7).
The anterior rib cage and sternum were removed and washed with sterile PBS. The costal cartilage was then incubated in a solution of collagenase D (3 mg/ml dissolved in serum-free Dulbecco’s modified Eagle’s medium) for 90 min at 37°C. The remaining sterna and costosternal junctions were further digested using a fresh collagenase D solution in Petri dishes in a 37°C incubator for 5 hours with intermittent shaking. The solution was centrifuged, and the cells were resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (penicillin, streptomycin, fosfomycin). The chondrocytes were seeded into 6-well plates, with approximately 1 x 10⁶ cells per well, and maintained at 37°C in a 95% air: 5% CO₂ humidified incubator⁹⁸.

**Preparation of total RNA**

After 1 week, TF3 (100 μM) or EGCG (100 μM) was added to the cultured mouse chondrocytes, with or without IL-1β (10 μg/L), for 20 hours before mRNA extraction. The cultured chondrocytes were assigned to five groups as follows: control (medium alone, n = 19); 10 μg/L IL-1β (n = 18); 10 μg/L IL-1β + 100 μM EGCG (n = 9); 10 μg/L IL-1β + 100 μM TF3 (n = 18); 100 μM EGCG (n = 5) or 100 μM TF3 (n = 13). After cells were collected, total RNA was isolated using an RNeasy Mini kit (Qiagen K.K., Tokyo) and quantified by measuring the optical density at 260 nm. Each RNA sample was diluted with RNase-free water and levels of mRNA normalized. Total RNA was reverse transcribed using an ExScript™ RT reagent kit (Takara Bio Inc., Shiga)⁹⁹,¹⁰⁰.

**Quantitative Real-Time PCR**

The real-time polymerase chain reaction was used to quantify mRNA levels of MMP-3, MMP-9, MMP-13, MT1-MMP, ADAM-15, ADAM-17, TIMP-1, TIMP-3 and 67LR in the mouse chondrocytes. Primers were purchased from Takara. Amplification of the corresponding cDNAs was performed on a LightCycler (Roche Diagnostics K.K., Tokyo) using SYBR® Premix Ex Taq™ (Perfect Real Time, Takara)⁹⁹,¹⁰⁰. Fluorescence data were analyzed using the LightCycler software (Roche). Table 1 shows the Takara ID number and the polymerase chain reaction program. Each primer pair generated a single product of the appropriate size when visualized by 2% agarose gel electrophoresis. The melting curve pattern confirmed a single melting peak. The mRNA levels were compared to the standard (18s rRNA)¹⁰¹, and relative expression ratios were subsequently calculated.

**Statistical analysis**

Data were analyzed using one-way analysis of variance (ANOVA) and included the Bonferroni correction. All data were expressed as mean ± S.E.M. Differences in P values of less than 0.05 were considered significant.

**Results**

Examination of the polygonal cobblestone morphology of mouse chondrocytes by light microscopy showed that chondrocytes of rounded morphology were slightly increased in size by IL-1β (Fig. 1B). However, the addition of TF3 appeared to prevent this change (Fig. 1C), with TF3 alone not affecting the morphology of the chondrocytes (Fig. 1D).

The expression of MMP mRNA, especially MMP-3, MMP-9 and MMP-13, was significantly increased by IL-1β (Fig. 2A, 2B, 2C), and TF3 suppressed this IL-1β-induced increase.
Table 1. Polymerase chain reaction (PCR) program

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*: Reference number

Fig. 1. Morphology of mouse chondrocytes cultured for 1 week and then treated with medium alone (control; panel A), IL-1β (panel B), IL-1β + TF3 (panel C) and TF3 alone (panel D). Chondrocytes are located side-by-side forming a polygonal cobblestone. Arrows indicate rounded chondrocyte cells. Magnification X100.

Abbreviations: IL-1β, interleukin-1β; TF3, theaflavin-3,3′-digallate

as well as, or better than, EGCG in the cultured chondrocytes. In the presence of IL-1β, mRNA expression of MMP-3 increased approximately 80-fold, but TF3 and EGCG reduced this increase by approximately 80% and 45%, respectively (Fig. 2A). Similarly, MMP-9 mRNA expression was increased 43-fold by IL-1β, but this increase was suppressed 46%
Effect of TF3 on MMPs in Chondrocytes

<table>
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| 35 | 40 | 40 | 40 | 38 |
| 60°C | 60°C | 62°C | 60°C | 63°C |

and 36% by TF3 and EGCG, respectively (Fig. 2B). MMP-13 mRNA expression was also increased 78-fold by interleukin-1β, and TF3 and EGCG reduced this increase by 39% and 32%, respectively (Fig. 2C). Levels of MMP mRNA expression were generally not changed by TF3 or EGCG alone (Fig. 2A, 2B, 2C).

The expression of MT1-MMP mRNA was not changed by IL-1β or EGCG, but was slightly increased by TF3 (Fig. 2D). None of the treatments affected ADAM-15 mRNA expression (Fig. 3A), although the level of ADAM-17 mRNA in the chondrocytes was increased by TF3 and by the combination of IL-1β and TF3 (Fig. 3B). The addition of IL-1β to cultured chondrocytes tended to increased TIMP-1 mRNA levels, but the level of TIMP-1 mRNA did not change significantly in the treatment groups (Fig. 4A). In contrast, IL-1β significantly decreased TIMP-3 mRNA expression by 14%, and TF3 or EGCG reversed this decrease by 93% or 56%, respectively. Interestingly, addition of either TF3 or EGCG to cultured chondrocytes increased TIMP-3 mRNA expression (Fig. 4B). The level of 67LR mRNA did not change in the treatment groups (Fig. 5).

Discussion

Matrix metalloproteinases are the enzymes responsible for the degradation of the collagen fibrils. They have, therefore, significant roles in the irreversible degradation of cartilage in RA. MMPs have an important role in osteoarthritis,22,23 and MMP-3 is a marker of joint degradation in clinical RA.11,24 In the current study, the in vitro addition of IL-1β to mouse chondrocytes induced an 80-fold up-regulation of MMP-3 mRNA compared to the control group. In addition, TF3 is a better inhibitor of MMP-3 mRNA expression in mouse inflammatory chondrocytes compared to EGCG. The expression of MMP-9 and MMP-13 mRNA were also inhibited more strongly by TF3 than EGCG.

Tea polyphenols are well known for their anti-oxidative ability.25 The galloyl group confers the anti-oxidative property of EGCG. However, the black tea polyphenol TF3, which has two galloyl groups, has a weaker anti-oxidative effect than EGCG but a similar anti-angiogenic effect.9 In the present study, TF3 suppressed MMP mRNA expression to a greater degree than EGCG in IL-1β-induced chondrocytes, suggesting that the effect of tea polyphenols on MMPs is not due to an anti-oxidative effect alone.

It is known that EGCG inhibits activation of the cytokine-activated c-jun amino-terminal
Fig. 2. The effect of EGCG and TF3 on mRNA expression of MMP-3 (panel A), MMP-9 (panel B), MMP-13 (panel C) and MT1-MMP (panel D) in mouse chondrocytes after 20 hours of treatment with medium alone (control, n=19); 10 μg/L IL-1β (n=18); 10 μg/L IL-1β + 100 μM EGCG (n=9); 10 μg/L IL-1β + 100 μM TF3 (n=18); 100 μM EGCG (n=5) or 100 μM TF3 (n=13). The graphs show the % increase in mRNA expression relative to the control value. Each column represents mean ± S.E.M.

* P<0.05, ** P<0.01 vs. control; * P<0.05, ** P<0.01 vs. IL-1β; † P<0.05, ‡ P<0.01 vs. IL-1β + EGCG; ‡‡ P<0.05 vs. IL-1β + TF3.

Abbreviations: IL-1β, interleukin-1β; TF3, theaflavin-3,3'-digallate; EGCG, epigallocatechin-3-gallate
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Fig. 3. The effect of EGCG and TF3 on mRNA expression of ADAM-15 (panel A) and ADAM-17 (panel B) in mouse chondrocytes after 20 hours of treatment with medium alone (control, n = 19); 10 μg/L IL-1β (n = 18); 10 μg/L IL-1β + 100 μM EGCG (n = 9); 10 μg/L IL-1β + 100 μM TF3 (n = 18); 100 μM EGCG (n = 5) or 100 μM TF3 (n = 13). The graphs show the % increase in mRNA expression relative to the control value. Each column represents mean ± S.E.M.

##P<0.01 vs. control ; **P<0.01 vs. IL-1β.

Abbreviations : IL-1β, interleukin-1β; TF3, theaflavin-3,3'-digallate ; EGCG, epigallocatechin-3-gallate

kinase and activator protein-1 (AP-1) pathways in human chondrocytes²⁵, and TF3 and EGCG also inhibit AP-1 and nuclear factor-κB (NF-κB) activation²⁷. MMP-3, MMP-9 and MMP-13 have promoter sites for AP-1 and NF-κB and/or are influenced by these transcription factors⁴. Thus, it is possible that TF3 and EGCG inhibit MMP mRNA expression via these transcription factors. However, TF3 and EGCG do not inhibit the signal transduction pathways equally. For example, EGCG disrupts the association of MEK1 [MAPK (mitogen-activated protein kinase) / ERK (extracellular signal-regulated kinase) kinase] with Raf-1, possibly by binding to the proline-rich sequences on MEK1, whereas TF3 promotes lysosome-mediated degradation of the Raf-1 protein⁸. These mechanisms lead to decreased phospho-MEK1 and phospho-ERK1/2 levels within the cell, which then inhibits AP-1²⁸. This may explain the differential effect of TF3 and EGCG on MMP mRNA expression observed in the present study.

The promoter of ADAM-15 does not contain the TATA or CAAT sequences²⁹. The promoter of ADAM-17 also does not contain the TATA sequence but has both the AP2 (activator protein 2) and SP1 (specificity protein 1) binding sites³⁰,³¹. Therefore, unlike the MMPs, the level of ADAM-15 and ADAM-17 mRNA did not change significantly following IL-1β treatment of the chondrocytes. Interestingly, TF3 induced ADAM-17 mRNA expression which could, therefore, contribute to joint degradation. However, this is unlikely since the enzymatic balance of the MMPs, ADAMs and TIMPs is tightly maintained in vivo³².
The TIMP family members are natural inhibitors of MMPs and tightly regulate their activity. In the present in vitro study, TF3 increased MT1-MMP and ADAM-17 mRNA expression, but TIMP-3 mRNA expression was returned to control levels. Moreover, a single administration of TF3 increased TIMP-3 mRNA expression two-fold compared with control. Among the TIMPs, TIMP-3 is the most effective inhibitor of MMP-13 and can also inhibit MMP-3, MMP-9 and ADAM-17. TIMP-1 and TIMP-3 overexpression by adenoviral gene transfer reduces the invasion and proliferation of rheumatoid synovial fibroblasts. The TIMP-1 gene has similar promoter sites to the MMP-9 gene, including sites for AP-1 and NF-κB. However, TF3 and EGCG did not affect TIMP-1 mRNA expression since the change in TIMP-1 mRNA levels in the present study was small.

It was recently reported that EGCG may prevent cancer through its interaction with 67LR. 67LR was associated with MMP-3, MT1-MMP and TIMP-1 when breast carcinoma cells were treated with peptide G laminin-1. In the present study, the expression of 67LR mRNA showed only small changes, as neither TF3 nor EGCG affected mRNA expression of 67LR. However, it is possible that TF3 and/or EGCG control MMPs, TIMPs and ADAMs through 67LR.

In this study using mouse chondrocytes, levels of MMP mRNA were up-regulated to widely differing levels by IL-1β. However, TF3 suppressed these increases equally, or to a greater degree, than EGCG. Additionally, compared to EGCG, TF3 increased mRNA
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Fig. 5. The effect of EGCG and TF3 on mRNA expression of MMP-3 67LR in mouse chondrocytes after 20 hours of treatment with medium alone (control, n=19); 10 μg/L IL-1β (n=18); 10 μg/L IL-1β + 100 μM EGCG (n=9); 10 μg/L IL-1β + 100 μM TF3 (n=18); 100 μM EGCG (n=5) or 100 μM TF3 (n=13). The graphs show the % increase in mRNA expression relative to the control value. Each column represents mean ± S.E.M.

Abbreviations: IL-1β, interleukin-1β; TF3, theaflavin-3,3′-digallate; EGCG, epigallocatechin-3-gallate

expression of MT1-MMP and ADAM-17 to a similar or greater degree, but these changes were much less when compared to the changes in MMPs. Moreover, TF3 induced mRNA expression of TIMP-3, a natural inhibitor of MMPs and ADAM-17. TIMP-3 might inhibit the release of TNF-α from cell membrane, which, in turn, inhibits ADAM-17 mRNA production. The results suggest that TF3 may have a stronger effect on controlling degradation in the inflamed joint cartilage than EGCG, implying that TF3 may be a potent anti-inflammatory compound with therapeutic potential.

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References


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