

# Comparative Effects of 2 Antioxidants, Selenomethionine and Epigallocatechin-Gallate, on Catabolic and Anabolic Gene Expression of Articular Chondrocytes

RINA ANDRIAMANALIJAONA, CATHERINE BAUGÉ, EMMANUELLE RENARD, FLORENCE LEGENDRE, MAHA RAOUDI, MADELEINE KYPRIOTOU, KARIM BOUMEDIENE, HUGUES GATTO, PATRICIA MONGINOX, and JEAN-PIERRE PUJOL

**ABSTRACT. Objective.** To determine the effects of selenomethionine (Se-met) and epigallocatechin-gallate (EGCg) on gene expression, activation of mitogen-activating kinases, and DNA binding of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and apolipoprotein-1 (AP-1) in articular chondrocytes.

**Methods.** Chondrocytes, cultured in low-oxygen tension, were pretreated with L-selenomethionine or EGCg for 24 h, followed by interleukin 1 (IL-1 $\beta$ ) for 1 h (nuclear and cytoplasmic extracts) or 24 h (RNA extraction). Reverse transcription-polymerase chain reaction was performed to determine mRNA levels of matrix metalloproteinases (MMP-1, -3, -13), aggrecanases (-1, -2), IL-1 $\beta$ , inducible nitric oxide synthase, cyclooxygenases (-1, -2), type II collagen and aggrecan, and transforming growth factor- $\beta$  (TGF- $\beta$ 1, -2, -3) and their receptors I and II. Activity of mitogen-activating protein kinases (MAPK) was assayed by Western blot and AP-1/NF- $\kappa$ B DNA binding by electrophoretic mobility shift assay.

**Results.** Pretreatment with 0.5  $\mu$ M Se-met prevented IL-1 $\beta$ -induced MMP-1 and aggrecanase-1 expression, and reduced the cytokine inhibitory effect on type II collagen, aggrecan core protein, and TGF- $\beta$  receptor II (TGF- $\beta$ R2) mRNA levels. EGCg was more efficient in modulating the effects of IL-1 $\beta$  on the genes studied. Whereas EGCg inhibited the IL-1 $\beta$ -activated MAPK, NF- $\kappa$ B, and AP-1, Se-met stimulated that signaling pathway. This could account for the differential effects exerted by these antioxidants on chondrocytes.

**Conclusion.** Our data provide insights into the mechanisms whereby EGCg and selenium modulate chondrocyte metabolism. Despite their differential mechanisms of action, the 2 compounds may exert global beneficial effects on articular cartilage. (J Rheumatol 2005;32:1958–67)

## Key Indexing Terms:

SELENIUM

ANTIOXIDANTS

CHONDROCYTES

EPIGALLOCATECHIN-GALLATE

OSTEOARTHRITIS

Joint diseases are characterized by dramatic degradation of articular cartilage, caused by overexpression of matrix metalloproteinases (MMP), and reduced anabolic activity of chondrocytes<sup>1,2</sup>. Disruption of the balanced network of cytokines/growth factors that normally maintains cartilage metabolism is thought to induce breakdown of extracellular matrix. Interleukin-1 $\beta$  (IL-1 $\beta$ ) plays a major role in joint diseases<sup>3</sup>, causing overproduction of prostaglandin E<sub>2</sub>, reactive oxygen species (ROS), nitric oxide, and MMP, which all contribute to the degradative process of cartilage<sup>4,5</sup>. IL-1 $\beta$

also reduces levels of inhibitors of MMP (tissue inhibitors of MMP, TIMP) in arthritic joints<sup>6</sup> and decreases biosynthesis of type II collagen and aggrecan<sup>7,8</sup>, thus limiting the repair potential of cartilage. These effects have been confirmed by *in vivo* studies showing that intraarticular administration of IL-1 $\beta$  into animal joints caused proteoglycan loss from the cartilage<sup>9</sup>, whereas injection of IL-1 receptor antagonist (IL-1ra) was found to protect cartilage in arthritic joints<sup>10</sup>. Conversely, the anabolic activity of chondrocytes is sustained by growth factors such as insulin-like growth factor-I (IGF-I), fibroblast growth factor-2 (FGF-2), transforming growth factor- $\beta$  (TGF- $\beta$ ), and bone morphogenetic proteins (BMP). For example, TGF- $\beta$ 1 exerts opposite effects to IL-1 $\beta$  and downregulates the expression of IL-1r<sup>11</sup> in cultured articular chondrocytes, suggesting that it may be crucial for cartilage homeostasis. The expression of TGF- $\beta$  receptor II (TGF- $\beta$ R2) in articular cartilage is dramatically depressed in the rabbit model of osteoarthritis (OA)<sup>12</sup>, and transgenic mice expressing an inactive TGF- $\beta$ R2 develop joint lesions similar to human symptoms of OA<sup>13</sup>. Thus, osteoarthritic cartilage breakdown involves not only IL-1 $\beta$  overexpres-

From the Laboratory of Connective Tissue Biochemistry, Faculty of Medicine, Caen; and Virbac Laboratories, Carros, France.

Supported by Virbac Laboratories, Carros, France.

R. Andriamanalijaona, PhD; C. Baugé, Graduate; E. Renard, Graduate; F. Legendre, PhD; M. Raoudi, PhD; M. Kypriotou, PhD; K. Boumediene, PhD; J.-P. Pujol, PhD, Laboratory of Connective Tissue Biochemistry, Faculty of Medicine, Caen; H. Gatto, PhD; P. Monginoux, PhD, Virbac Laboratories.

Address reprint requests to Dr. J.-P. Pujol, Laboratory of Connective Tissue Biochemistry, Faculty of Medicine, 14032 Caen Cedex, France. E-mail: pujol@ibfa.unicaen.fr

Accepted for publication May 30, 2005.

sion, but also reduced responsiveness of chondrocytes to TGF- $\beta$ 1<sup>14</sup>.

IL-1 $\beta$  can stimulate chondrocytes and synoviocytes to produce ROS, including superoxide radical (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), nitric oxide (NO), and hydroxyl radical (OH)<sup>15</sup>. These highly reactive molecules regulate gene expression, transcription factor activation<sup>16</sup>, cellular proliferation<sup>17</sup>, and apoptosis<sup>15</sup>. In affected joints, ROS are produced mainly by polymorphonuclear leukocytes and macrophages, but also by chondrocytes<sup>18</sup>. IL-1 has been found to induce release of several ROS from chondrocytes, including H<sub>2</sub>O<sub>2</sub><sup>19</sup>, superoxide anion (O<sub>2</sub><sup>-</sup>)<sup>20</sup>, and NO<sup>21</sup>. ROS enhance the catabolism of collagen, proteoglycans, and hyaluronan, and reduce the synthesis of these macromolecules<sup>22,23</sup>. Moreover, ROS take part in signaling pathways, such as those elicited by IL-1<sup>21</sup>. Thus, it is of interest to search for antioxidative molecules that exert anticatabolic effects on chondrocytes, and could display chondroprotective action.

Dietary manipulation or supplementation with vitamins, minerals, and trace elements has gained some interest in the treatment of joint diseases. Selenium has shown antiinflammatory and immunomodulatory properties in animals<sup>24-26</sup>, later confirmed in humans<sup>27</sup>. Its role is attributed to the presence of selenocysteine at the catalytic sites of glutathione peroxidase, which reduces hydrogen peroxide and organic peroxides<sup>28</sup>. Thus, selenium contributes to cellular defence, protecting membrane lipids and possibly proteins and nucleic acids from oxidant damage. The pattern of several trace elements is altered in rheumatoid arthritis (RA), possibly in relation to the inflammatory response<sup>29,30</sup>. Low selenium values are found in patients with RA and juvenile chronic arthritis<sup>31,32</sup>. However, the role of selenium in OA and its potential effect on articular chondrocyte metabolism are not well understood. Selenium deficiency can induce degenerative processes in articular joints<sup>33</sup>, whereas dietary vitamins and selenium diminish the development of mechanically induced OA and increase the expression of antioxidative enzymes in the knee joint of STR/IN mice<sup>34</sup>. Selenium from varying chemical entities is absorbed by different intestinal mechanisms and both the storage in diverse organs and the extent of incorporation into selenoproteins depend on the chemical form of selenium<sup>35,36</sup>. We used L-selenomethionine as a form that can easily be absorbed and has shown efficacy in other domains, such as skin damage<sup>37</sup>.

Polyphenolic fractions from green tea, which is rich in antioxidants, possess antiinflammatory as well as anticarcinogenic properties<sup>38</sup>. They contain several catechins, including epigallocatechin-gallate (EGCg), (-)-epigallocatechin (EGC), (-)-epicatechin-gallate (ECg), (-)-epicatechin (EC), and (+)-catechin (C)<sup>38</sup>, EGCg being the major component. Green tea compounds have been shown to reduce inflammation in a murine model of inflammatory arthritis<sup>39</sup> and to inhibit cartilage degradation *in vitro*<sup>40</sup>. However, no

study has investigated both the catabolic and anabolic gene expressions by articular chondrocytes.

We investigated the respective effects of the 2 antioxidants L-selenomethionine and epigallocatechin-gallate on expression of catabolic and anabolic genes in bovine articular chondrocytes.

## MATERIALS AND METHODS

**Reagents.** Mouse anti-phospho-MAPK and rabbit anti-MAPK antibodies (ERK1/2-CT) were from Upstate Biotechnology (Lake Placid, NY, USA), and rabbit anti-p38 antibodies were provided by Cell Signaling Technology (Beverly, MA, USA). Peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for detection, with an ECL+Plus kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The probes, apolipoprotein-1 (AP-1) (5'-CGC TTG ATG AGT CAG CCC GGA A-3') and NF- $\kappa$ B (5m'-AGT TGA GGG GAC TTT CCC AGG C-3'), were supplied by Invitrogen (San Diego, CA, USA). L-selenomethionine and EGCg were supplied by Sigma-Aldrich Co. (St. Quentin Fallavier, France). Human IL-1 $\beta$  was provided by Dr. S. Sato, Kyowa Hakko Co., Ltd., Tokyo, Japan.

**Culture and treatment of articular chondrocytes.** Chondrocytes were enzymatically isolated from metacarpophalangeal joints of calves<sup>41</sup>. The cells were seeded in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS; Invitrogen). They were cultured in 5% O<sub>2</sub> in a sealed chamber, as described<sup>42</sup>. Confluent cells were pretreated with L-selenomethionine (0.5 or 1.0  $\mu$ M) or EGCg (20 or 50  $\mu$ M) for 24 h, followed by IL-1 $\beta$  (10 ng/ml) for a further 1 h (nuclear and cytoplasmic extracts) or 24 h (RNA extraction). The concentrations of compounds were selected according to the information available in the literature on blood levels and the concentrations used in previous reports of *in vitro* experiments.

**Cytoplasmic and nuclear extracts.** Extracts were prepared as described<sup>43</sup>. The protein amount was determined by the Bradford colorimetric procedure (Bio-Rad, Hercules, CA, USA).

**Western blot analysis.** Cellular lysates (15  $\mu$ g protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis<sup>44</sup>, transferred to PVDF membranes (NEN Life Sciences, Zaventem, Belgium), then treated as described<sup>45</sup>.

**Electrophoretic mobility shift assays (EMSA).** EMSA were performed as described<sup>43</sup>.

**RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR).** RNA was extracted as described<sup>46</sup>. Samples of 10  $\mu$ g were loaded on agarose gel (MOPS-formaldehyde) to check the integrity of RNA samples and the potential presence of DNA. Conditions for reverse transcription and real-time PCR (ABI Prism 7000 SDS; Applied Biosystems, Foster City, CA, USA) were as described<sup>43</sup>. Reactions were performed in triplicate and relative expression of each gene was obtained by normalizing to 18S RNA as a house-keeping gene. Relative quantity of cDNA was calculated from the number of cycles corresponding to 100% polymerization efficiency, using the 2<sup>- $\Delta\Delta$ CT</sup> method<sup>47</sup>. Primer sequences were as follows: the sequences are indicated from 5' to 3'. Aggrecanase 1: sense CCG CTT CAT CAC TGA CTT CCT, antisense GGA GCC TCC GGC TTG TCT; aggrecanase 2: sense AGC GCT TAA TGT CTT CCA TCC T, antisense GTG GCT GAG GTG CAT TTG G; 18S: sense CGG CTA CCA CAT CCA AGGAA, antisense GCT GGA ATT ACC GCG GCT; aggrecan: sense TCG AGG ACA CGC AGG CC, antisense TCG AGG GTG TAG CGT GTA GAG A; type II collagen: sense GCA TTG CCT ACC TGG ACG AA, antisense CGT TGG AGC CCT GGA TGA; IL-1 $\beta$ : sense TCT CCG ACC ACC ACT ACA GCA A, antisense GGG GAA CTG GGC AGA CTC A; cyclooxygenase-1 (COX-1): sense TCA GCA CCC AGC AAA TCC T, antisense GTG ATC TGG ATG TCA GCA CG; COX-2: sense GCA CCA ATC TGA TGT TTG CAT TC, antisense GGT CCT CGT TCA AAA TCT GTC TTG; inducible nitric oxide synthase (iNOS): sense GGC CCA GGA AAT GTT

CGA A, antisense ACC TGA TGT TGC CGT TGT TG; MMP-1: sense GAC CAG CAA TTT CCA AGA TTA TAA CTT, antisense CCA AGG GAA TGG CCA AA; MMP-3: sense TAC GGG TCT CCC CCA GTT TC, antisense GGT TCG GGA GGC ACA GAT T; MMP-13: sense TTC TTC TGG CGG CTG CAT, antisense GGA AGT TCT GGC CCA AAC G; TGF- $\beta$ R1: sense TTA AAA GGC GCA ACC AAG AAC, antisense GTG GTG ATG AGC CCT TCG AT; TGF- $\beta$ R2: sense GGA GCG GAA GAC GGA GTT G, antisense GAC ATG CCG CGT CAG GTA CT; TGF- $\beta$ B1: sense CAT CTG GAG CCT GGA TAC ACA GT, antisense GAA GCG CCC GGG TTG T; TGF- $\beta$ B2: sense CTG TGT GCT GAG CGC TTT T, antisense CGA GTG TGC TGC AGG TAG TCA; TGF- $\beta$ B3: sense CAA TTA CTG CTT CCG CAA CTT G, antisense GAT CCT GTC GGA AGT CAA TGT AGA.

*Statistical analysis.* Three different experiments were performed. The values are means  $\pm$  standard deviation and the significance of differences was calculated with Student's t test.

## RESULTS

*Effects of Se-met and EGCg on MMP and aggrecanase mRNA levels.* Se-met and EGCg exerted differential effects on the expression of various degradative enzymes by articular chondrocytes cultured in the presence or absence of IL-1 $\beta$  (Figure 1). Se-met did not significantly alter the basal levels of MMP-1, MMP-3, and MMP-13 mRNA, whereas it decreased the amount of aggrecanase-1 at the concentration of 1  $\mu$ M and that of aggrecanase-2 at both 0.5 and 1.0  $\mu$ M. Pretreatment with 0.5  $\mu$ M Se-met effectively prevented IL-1 $\beta$ -induced MMP-1 and aggrecanase-1 expression, but only slightly reduced the level of MMP-13. In contrast, Se-met at 1.0  $\mu$ M did not inhibit IL-1 $\beta$ -enhanced levels of MMP and aggrecanase mRNA, and was even found to further increase the cytokine effect (MMP-3, MMP-13, aggrecanase-1). It must be noted that the expression of aggrecanase-2 appeared to be differently controlled by IL-1 $\beta$  and Se-met than by aggrecanase-1. Indeed, IL-1 decreased the level of the enzyme mRNA by half and 1.0  $\mu$ M Se-met increased this level compared to the control.

We observed 20  $\mu$ M EGCg had no effect on basal enzyme mRNA levels, whereas 50  $\mu$ M generally enhanced those levels, except for aggrecanase-2. However, in contrast with Se-met, EGCg clearly prevented IL-1 $\beta$  stimulation of MMP and aggrecanase mRNA levels, particularly at 50  $\mu$ M. This inhibitory effect was significant for MMP-1, MMP-3, MMP-13, and aggrecanases-1 and -2. Nevertheless, a difference was again found between the profiles of aggrecanase-1 and -2 mRNA, since an enhancing effect was seen for aggrecanase-2 mRNA in chondrocytes treated with 20  $\mu$ M EGCg and IL-1 $\beta$ , compared to IL-1 $\beta$  alone (Figure 1E).

*Effects of Se-met and EGCg on the expression of IL-1 $\beta$ , iNOS, and COX-1/COX-2.* We examined whether Se-met and EGCg influenced the expression of inflammatory genes implicated in joint diseases, including IL-1 $\beta$ , iNOS, COX-1, and COX-2 (Figure 2). As expected, IL-1 $\beta$  induced a stimulatory effect on the mRNA levels of these genes, excepted for COX-1, which was not altered or even downregulated (Figure 2C). Addition of Se-met to the cultures did not influence basal mRNA levels or IL-1 $\beta$ -induced changes.

EGCg alone was found to enhance IL-1 $\beta$  mRNA when used at 50  $\mu$ M. However, this concentration prevented autoinduction of IL-1 $\beta$ , restoring the control value (Figure 2A). Concentrations of 20 and 50  $\mu$ M EGCg decreased the IL-1 $\beta$ -induced stimulation of iNOS in a dose-dependent manner (Figure 2B). COX-2, whose mRNA was significantly enhanced by IL-1 $\beta$ , was downregulated only at a concentration of 50  $\mu$ M EGCg (Figure 2D).

*Effects of Se-met and EGCg on expression of type II collagen and aggrecan core protein.* Since IL-1 $\beta$  downregulates type II collagen and aggrecan synthesis in chondrocytes<sup>7,8</sup>, we investigated the effects of Se-met and EGCg on the levels of type II collagen and aggrecan core protein mRNA produced in IL-1 $\beta$ -treated chondrocytes. We found 0.5  $\mu$ M Se-met pretreatment prevented IL-1 $\beta$  downregulation of type II collagen and core protein (Figure 3A). This effect was reduced for 1  $\mu$ M Se-met, suggesting that concentrations > 0.5  $\mu$ M are less efficient on these genes and probably act through a different pathway.

Similarly, 20  $\mu$ M EGCg was capable of preventing IL-1 $\beta$  downregulation of type II collagen and aggrecan core protein expression, whereas 50  $\mu$ M was without effect (Figure 3A, 3B).

*Effects of Se-met and EGCg on the TGF- $\beta$  system.* Given that TGF- $\beta$  plays a crucial role in cartilage homeostasis<sup>11-14</sup>, it was of interest to determine the effects of antioxidants on the expression of the main TGF- $\beta$  isoforms and their receptors I and II. Se-met alone did not significantly influence the basal levels of TGF- $\beta$ 1 mRNA, but decreased levels of TGF- $\beta$ 2, TGF- $\beta$ 3, T $\beta$ R1, and T $\beta$ R II (Figure 4). IL-1 $\beta$  exerted a depressive effect on all these genes, except TGF- $\beta$ 1. Interestingly, 1  $\mu$ M Se-met was found to partially or totally prevent the cytokine-induced downregulation of both receptor genes (Figure 4D, 4E) and to enhance the expression of TGF- $\beta$ 1 (Figure 4A). Further, in the case of TGF- $\beta$ R2, this was observed for 0.5  $\mu$ M Se-met concentration (Figure 4E).

EGCg alone did not influence the basal expression of TGF- $\beta$ 1 and their receptors. A significant stimulatory effect of 20  $\mu$ M EGCg was observed on TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ R1, and TGF- $\beta$ R2 expression in the presence of IL-1, whereas 50  $\mu$ M EGCg was generally not effective.

*Effects of Se-met and EGCg on MAP kinases (Erk1/Erk2, p38 kinase) and NF- $\kappa$ B and AP-1 activity.* To determine whether the preceding effects could be associated with modulation of downstream pathways following IL-1 receptor occupation, we analyzed the activation state of MAP kinases (Erk1/Erk2, p38 kinase) and binding activity of the transcription factors NF- $\kappa$ B and AP-1. Incubation with 0.5  $\mu$ M Se-met did not alter the basal activity of Erk1, Erk2, and p38, whereas it slightly strengthened the IL-1 $\beta$ -induced activation of Erk1 and Erk2 (Figure 5A, 5B). In contrast, 50  $\mu$ M EGCg exerted marked inhibition on both basal and IL-1 $\beta$ -stimulated MAPK (Figure 5A, 5B). Se-met was also found to strongly induce NF- $\kappa$ B DNA binding to a greater extent

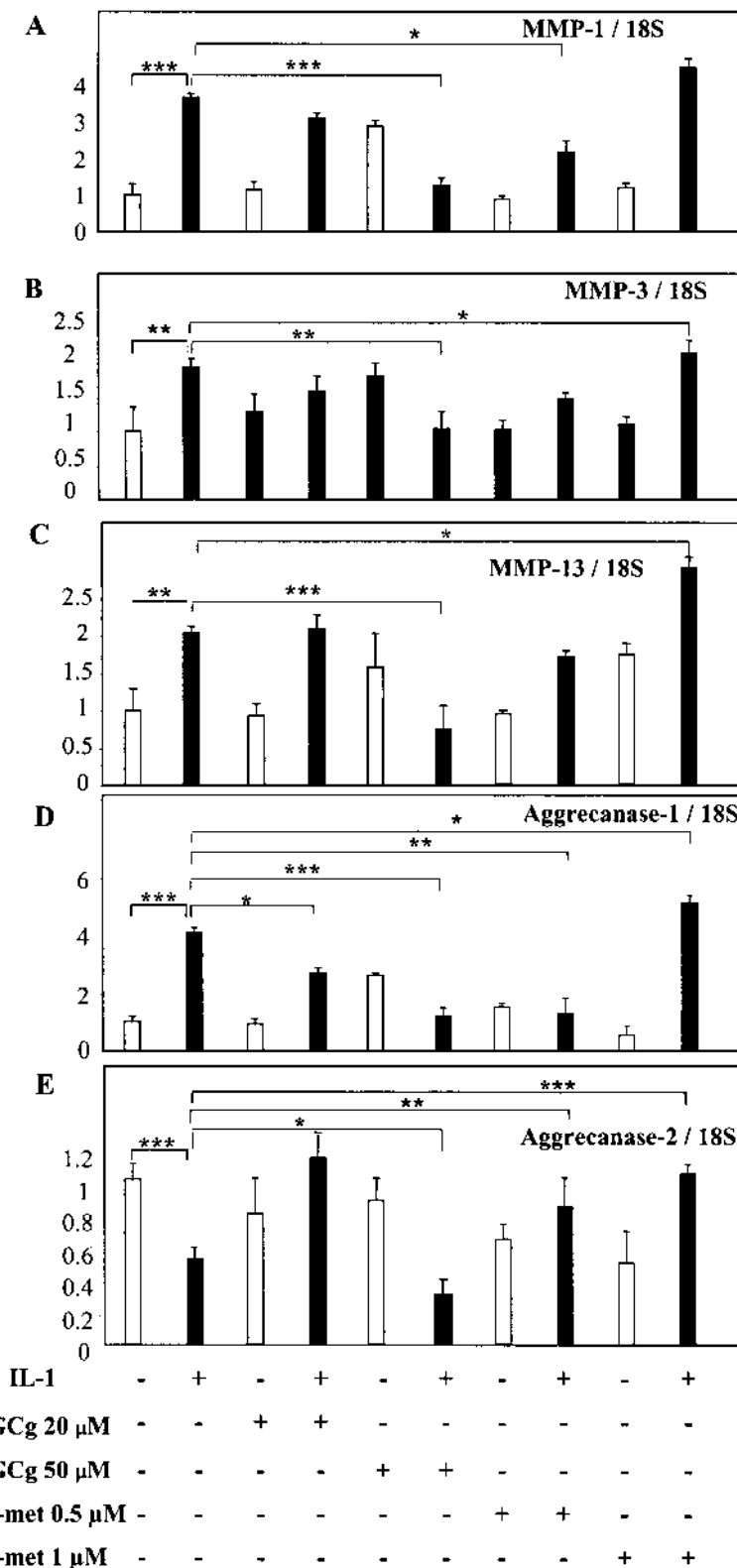


Figure 1. Effect of L-selenomethionine and EGCg on MMP and aggrecanase mRNA expression under IL-1 $\beta$  treatment. Confluent bovine articular chondrocytes were pretreated for 24 h with L-selenomethionine (SM, 0.5 or 1.0  $\mu$ M) or with EGCg (20 or 50  $\mu$ M). Then 10 ng/ml IL-1 $\beta$  was added for 24 h. Total RNA was extracted and used in quantitative PCR to determine steady-state levels of MMP-1 (A), MMP-3 (B), MMP-13 (C), aggrecanase-1 (D) and aggrecanase-2 (E) mRNA using specific primers. Data were normalized with 18S RNA and expressed as means  $\pm$  SD of 3 experiments. Statistical significance of difference between control and treated cells was determined by Student t test (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001).

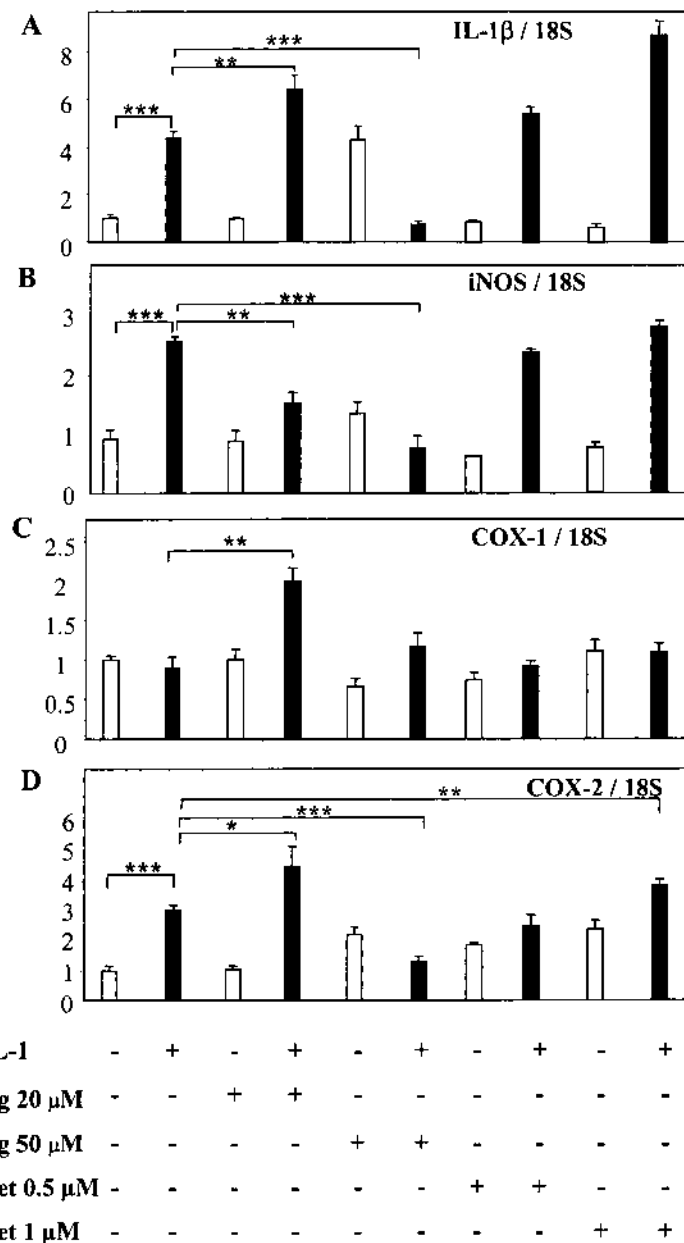


Figure 2. Effect of L-selenomethionine and EGCg on inflammation gene expression under IL-1 $\beta$  treatment. Confluent bovine articular chondrocytes were pre-treated for 24 h with L-selenomethionine (0.5 or 1.0  $\mu$ M) or with EGCg (20 or 50  $\mu$ M). Then 10 ng/ml IL-1 $\beta$  was added for 24 h. Total RNA was extracted and used in quantitative PCR to determine steady-state levels of IL-1 $\beta$  (A), iNOS (B), COX-1 (C), and COX-2 (D) mRNA using specific primers. Data were normalized with 18S RNA and expressed as means  $\pm$  SD of 3 experiments. Statistical significance of difference between control and treated cells was determined by Student t test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

than IL-1 $\beta$  (Figure 5C). In the presence of the cytokine, Se-met caused an even greater effect, compared to IL-1 $\beta$  alone. Similar action was exerted by Se-met on AP-1 DNA binding (Figure 5D). On the other hand, reduced activation of NF- $\kappa$ B and AP-1 was observed for EGCg on both basal and IL-1 $\beta$ -induced levels (Figure 5C, 5D).

## DISCUSSION

Our study shows that the antioxidants selenomethionine and epigallocatechin-gallate both can modulate the transcription

of several genes implicated in the metabolism of extracellular matrix and its pathological dysfunction. However, Se-met and EGCg exert no similar effects, and the signaling pathways underlying these actions are likely to be different. We found 0.5  $\mu$ M Se-met was capable of preventing IL-1-stimulated MMP-1 and aggrecanase-1 expression, whereas higher concentration (1  $\mu$ M) strengthened the effect of the cytokine. In contrast, the green tea polyphenol EGCg generally downregulated MMP expression in a dose-dependent manner. Surprisingly, IL-1 was found to decrease the

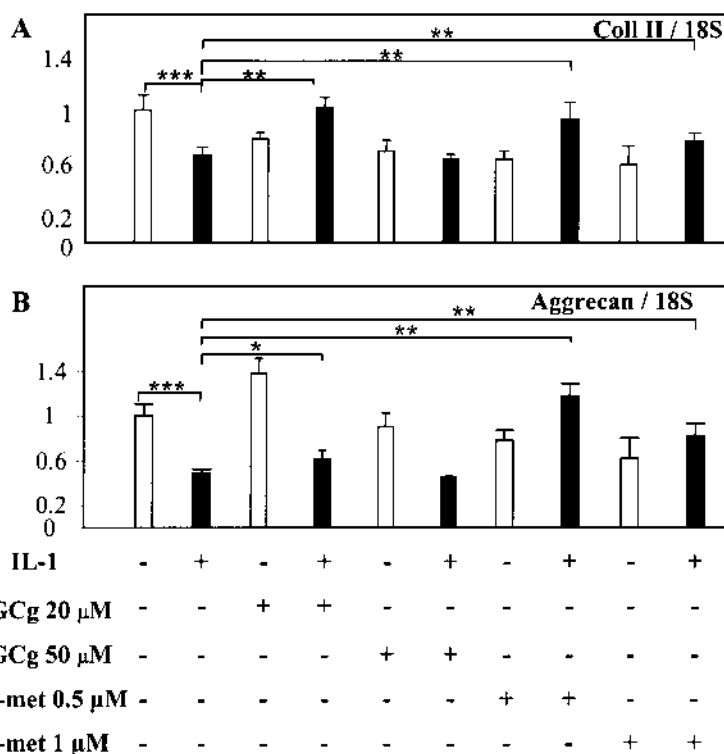


Figure 3. Effect of L-selenomethionine and EGCg on cartilage phenotypic markers of mRNA expression under IL-1 $\beta$  treatment. Confluent bovine articular chondrocytes were pretreated for 24 h with L-selenomethionine (0.5 or 1.0  $\mu$ M) or with EGCg (20 or 50  $\mu$ M). Then 10 ng/ml IL-1 $\beta$  was added for 24 h. Total RNA was extracted and used in quantitative PCR to determine steady-state levels of type II collagen (A) and aggrecan core protein (B) mRNA using specific primers. Data were normalized with 18S RNA and expressed as means  $\pm$  SD of 3 experiments. Statistical significance of difference between control and treated cells was determined by Student t test. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

expression of aggrecanase-2, in contrast with its stimulation of aggrecanase-1. In the absence of information on the aggrecanase gene promoter, it is difficult to suggest some molecular mechanism that could explain this differential behavior. Se-met was also without significant effect on the expression of proinflammatory genes, such as IL-1 $\beta$ , iNOS, and COX1/COX2, in contrast to EGCg, which inhibited most of them. Finally, the most interesting effect of Se-met was the prevention of IL-1-decreasing action on type II collagen, aggrecan core protein expression, and TGF- $\beta$ RII expression, suggesting that Se-met could contribute to the anabolic activity of chondrocytes and maintain their homeostasis. Nevertheless, it is difficult from these *in vitro* findings to determine the exact role of selenium in the pathophysiology of articular cartilage, which remains controversial. While selenium deficiency has been reported to cause degenerative process in articular joints<sup>33</sup> and supplementation with vitamins and selenium was shown to improve OA in an animal model<sup>34</sup>, no significant effect of the compound could be observed by other investigators<sup>48</sup>. In this regard, it must be noted that one of the difficulties of such *in vitro* study is that the effect of Se-met was often quite different according to the concentration (0.5  $\mu$ M versus 1.0  $\mu$ M), and that the concentration that was effective in one category of

genes was not the same with others. Further, to be efficient in gene expression, Se-met might require longer incubation time than the period we used. This seems plausible, since the role of selenium is generally explained by its incorporation into selenoproteins such as glutathione peroxidase<sup>28</sup>.

Among the major catechins present in green tea, EGCg is the most abundant and the one that has been the most extensively studied. Numerous biological effects have been reported, including anticarcinogenic activity, antiinflammatory effects, and alleviation of cardiovascular disease<sup>38</sup>. We show that EGCg prevents IL-1-upregulated expression of MMP-1, MMP-3, MMP-13, and aggrecanase-1 genes, which are enzymes suspected to degrade the cartilage matrix. These findings are in agreement with reports on cartilage explants<sup>40</sup> or isolated chondrocytes<sup>49</sup>. Interestingly, 20  $\mu$ M EGCg was also capable of opposing the IL-1 reduction of type II collagen and aggrecan core protein expression, 2 specific markers of cartilage matrix. Our results further show that IL-1 $\beta$  and iNOS expressions were inhibited by EGCg. This effect is probably due to decreased levels of NF- $\kappa$ B DNA binding to its consensus site, since expression of iNOS and IL-1 $\beta$  is dependent on the activation of NF- $\kappa$ B<sup>50</sup>. We have previously shown that erosion of cartilage in OA is associated with a progressive alteration of the TGF- $\beta$

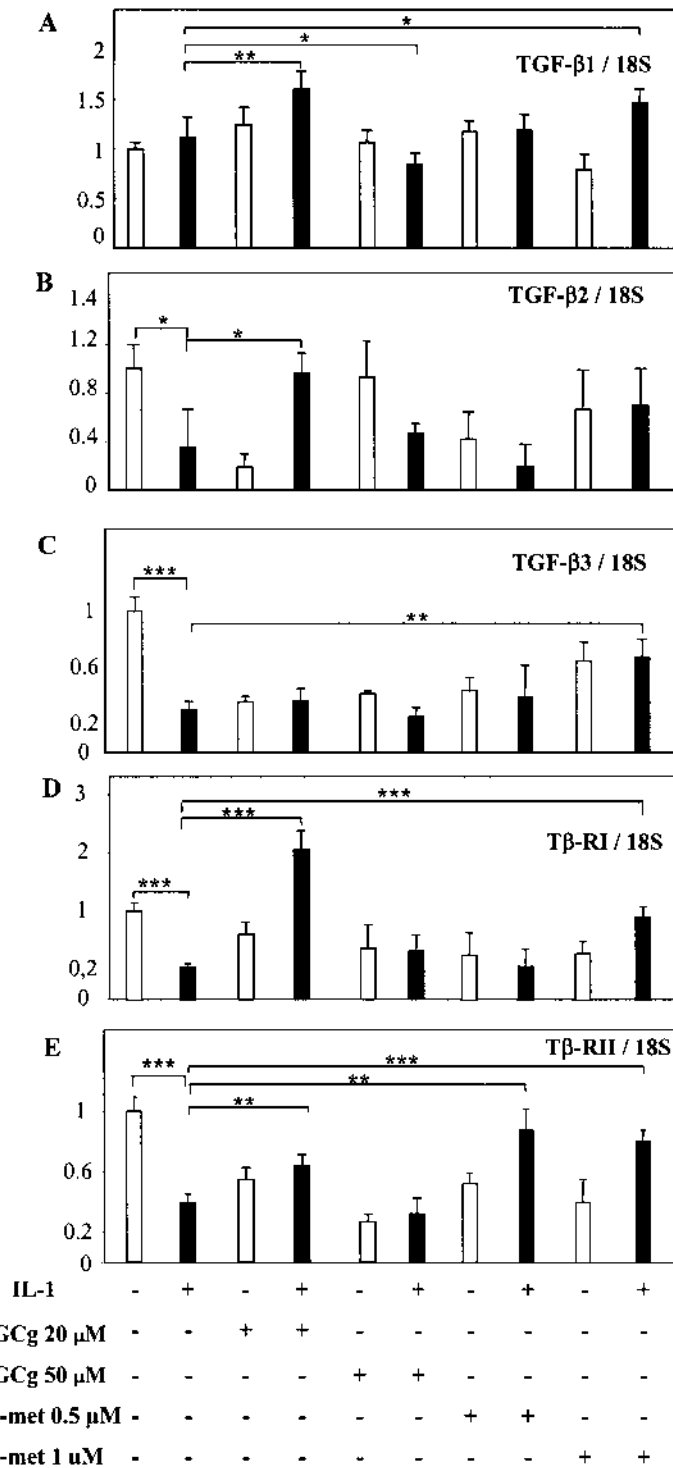
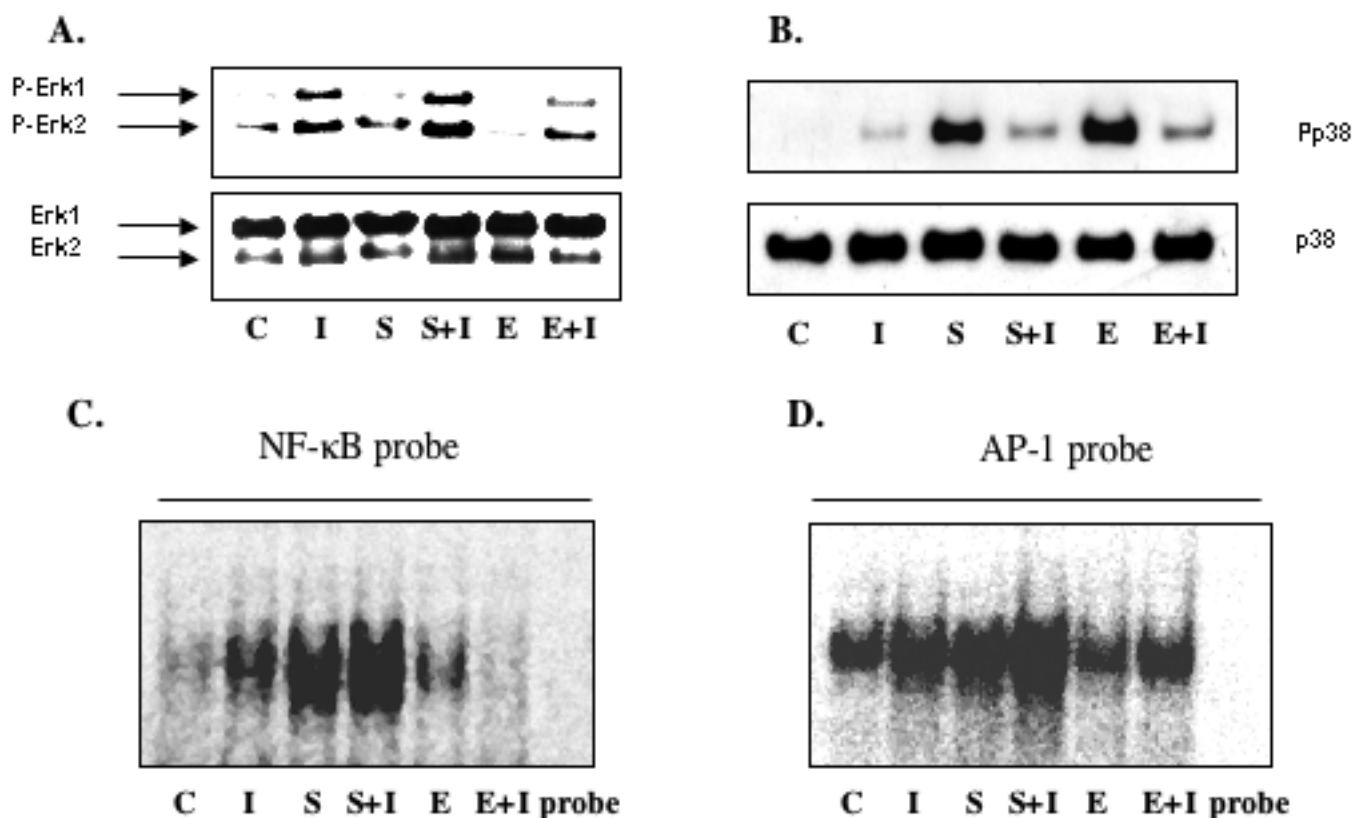


Figure 4. Effect of L-selenomethionine and EGCg on TGF-β family mRNA expression under IL-1β treatment. Confluent bovine articular chondrocytes were pretreated for 24 h with L-selenomethionine (0.5 or 1.0 μM) or with EGCg (20 or 50 μM). Then 10 ng/ml IL-1β was added for 24 h. Total RNA was extracted and used in quantitative PCR to determine steady-state levels of TGF-β1 (A), TGF-β2 (B), TGF-β3 (C), TGF-βRI (D), and TGF-βRII (E) mRNA using specific primers. Data were normalized with 18S RNA and expressed as means ± SD of 3 experiments. Statistical significance of difference between control and treated cells was determined by Student t test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



**Figure 5.** Effect of L-selenomethionine and EGCg on Erk1/2, p38 MAPK activity, and NF- $\kappa$ B and AP-1 DNA binding under IL-1 $\beta$  treatment. Confluent bovine articular chondrocytes were pretreated for 24 h with L-selenomethionine (0.5  $\mu$ M) or with EGCg (50  $\mu$ M). Then 10 ng/ml IL-1 $\beta$  was added for 1 h. Cytoplasmic extracts were prepared and used for Western blot analysis, using anti-phospho-Erk1/2 and anti-Erk1/2 (A), anti-phospho-p38 and anti-p38 (B). The protein-antibody complexes were visualized by chemiluminescence, using horseradish peroxidase-conjugated secondary antibody. Nuclear extracts were prepared as described and used in EMSA to determine DNA binding activity of NF- $\kappa$ B (C) and AP-1 (D), using consensus sequences  $^{32}$ P-radiolabeled as probes. Results shown are representative of 3 independent experiments. Erk1/2: extracellular signal regulated protein kinase 1/2; C: control; I: IL-1 $\beta$ ; S: L-selenomethionine; E: Epigallocatechin-gallate.

system, including reduced expression of TGF- $\beta$  isoforms and receptors (mainly TGF- $\beta$ RII)<sup>12</sup>. This process could result from enhanced IL-1 expression in OA, since one *in vitro* study showed that the cytokine exerts inhibitory effects on the TGF- $\beta$  system<sup>43</sup>. Our results show for the first time that EGCg can prevent the reduction of TGF- $\beta$ 2 expression, and particularly that of TGF- $\beta$ R1 and TGF- $\beta$ R2. Together, these data suggest that EGCg may be of potential benefit in the treatment of both degenerative and inflammatory joint diseases.

We searched for the signaling pathways that could be responsible for the effects of Se-met and EGCg on gene expression. Our results suggest that EGCg inhibited the gene expression of MMP and aggrecanases by suppressing the phosphorylation of extracellular signal-regulated kinases (Erk1/Erk2) and p38 kinase. These kinases are known to regulate MMP expression<sup>51</sup>. Further, EGCg was found to inhibit the DNA binding of both NF- $\kappa$ B and AP-1, which are required for IL-1 induction of the transcription of several MMP. However, the activity of NF- $\kappa$ B was more sensitive

to the effect of EGCg, as shown by the dramatic decrease of DNA binding compared to that of AP-1. Reduced NF- $\kappa$ B activity may also explain the downregulation of iNOS and IL-1 $\beta$  expression by EGCg. To what extent the MAPK signaling pathway and the transcription factors NF- $\kappa$ B/AP-1 could also be responsible for the effect of EGCg on other genes, including type II collagen and aggrecan core protein, remains to be investigated. Other additional mechanisms might be involved in that regulation.

Unlike EGCg, Se-met did not inhibit the MAPK system or the DNA binding of NF- $\kappa$ B and AP-1, but surprisingly it reinforced the IL-1-stimulating effect as well as the basal DNA binding of the transcription factors. Despite the scarcity of studies dealing with selenium's effect on cell signaling, it has been reported that phosphorylation of Erk1/Erk2 MAP kinases was elevated in hepatocytes treated with selenate<sup>52</sup>. The mechanism whereby 0.5  $\mu$ M Se-met downregulates IL-1-induced activation of MMP-1, MMP-3, MMP-13, and aggrecanase-1 remains to be elucidated.

Since reactive oxygen species participate in the IL-1

stimulating effect on MMP expression<sup>18</sup>, we may suggest as a hypothesis that Se-met acts as a ROS scavenger, directly or via the activity of glutathione peroxidase. Regarding the stimulating effect of Se-met on type II collagen and aggrecan core protein expression, this could be the result of a non-specific metabolic action of the compound. Indeed, selenium in selenate form has been reported to enhance the glycolytic pathway, indicated by increased activities of hexokinase and fructokinase in the liver<sup>53</sup>. To the degree that Se-met could act in the same way as selenate, this would lead to an augmentation of metabolic energy in chondrocytes, which are hypoxic cells mainly relying on glycolysis-derived ATP.

One limitation of this study is that determination of steady-state levels of mRNA does not necessarily reflect protein expression and activity. However, despite this limitation, mRNA measurements are potentially a good indicator of important mediators of the process under investigation and a simple procedure to estimate the expression and activity of several genes at the same time.

Our study shows that the 2 antioxidants Se-met and EGCg exert differential effects on the metabolism of articular chondrocytes in the presence of IL-1 $\beta$ . They also modulate the MAP kinase pathway and both NF- $\kappa$ B and AP-1 transcription factors in opposite ways. EGCg may be an effective therapeutic agent for inhibiting IL-1 $\beta$ -induced cartilage degradation in joint diseases. By its stimulating effect on expression of cartilage-specific matrix genes and TGF- $\beta$  receptor II, Se-met could also be an additional treatment in OA. However, further research is necessary to elucidate the possible benefit of selenium supplementation in joint diseases.

## REFERENCES

1. Pelletier J, Di Battista J, Lajeunesse D. Biochemical factors in joint articular tissue degradation in osteoarthritis. In: Reginster J-Y, Pelletier J-P, Martel-Pelletier J, Henrotin Y, editors. *Osteoarthritis: Clinical and experimental aspects*. Berlin; Springer-Verlag 1999:156-87.
2. Aigner T, Vornehm SI, Zeiler G, Dudhia J, von der Mark K, Bayliss MT. Suppression of cartilage matrix gene expression in upper zone chondrocytes of osteoarthritic cartilage. *Arthritis Rheum* 1997;40:562-9.
3. Pujol J-P, Loyau G. Interleukin-1 and osteoarthritis. *Life Sci* 1987;41:1187-98.
4. Aigner T, Kurz B, Fukui N, Sandell L. Roles of chondrocytes in the pathogenesis of osteoarthritis. *Curr Opin Rheumatol* 2002;14:578-84.
5. Goldring MB. The role of the chondrocyte in osteoarthritis. *Arthritis Rheum* 2000;43:1916-26.
6. Dean DD, Martel-Pelletier J, Pelletier JP, Howell DS, Woessner JF. Evidence for metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. *J Clin Invest* 1989;84:678-85.
7. Chadichristos C, Ghayor C, Kypriotou M, et al. Sp3/Sp1 ratio mediates IL-1 $\beta$  inhibition of COL2A1 gene. *J Biol Chem* 2003;278:39762-72.
8. Benton HP, Tyler JA. Inhibition of cartilage proteoglycan synthesis

- by interleukin-1. *Biochem Biophys Res Commun* 1988;154:421-8.
9. Goldring MB. The role of cytokines as inflammatory mediators in osteoarthritis: lessons from animal models. *Connect Tissue Res* 1999;40:1-11.
10. Fernandes J, Tardiff G, Martel-Pelletier J, et al. In vivo transfer of interleukin-1 receptor antagonist gene in osteoarthritic rabbit knee joints: prevention of osteoarthritic progression. *Am J Pathol* 1999;154:1159-69.
11. Redini F, Mauviel A, Pronost S, Loyau G, Pujol J-P. Transforming growth factor  $\beta$  exerts opposite effects from interleukin-1 $\beta$  on cultured rabbit articular chondrocytes through reduction of interleukin-1 receptor expression. *Arthritis Rheum* 1993;36:44-50.
12. Boumediene K, Conrozier T, Mathieu P, et al. Decrease of cartilage transforming growth factor- $\beta$  receptor II expression in the rabbit experimental osteoarthritis — potential role in cartilage breakdown. *Osteoarthritis Cartilage* 1998;6:146-9.
13. Serra R, Johnson M, Filvaroff EH, et al. Expression of a truncated, kinase defective TGF- $\beta$  type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. *J Cell Biol* 1997;139:541-52.
14. Pujol J-P. TGF- $\beta$  and osteoarthritis: In vivo veritas? *Osteoarthritis Cartilage* 1999;7:439-40.
15. Bauer G. Reactive oxygen and nitrogen species: efficient selective and interactive signals during intercellular induction of apoptosis. *Anticancer Res* 2000;20:4115-40.
16. Sun Y, Oberley LW. Redox regulation of transcriptional activators. *Free Rad Biol Med* 1996;21:335-48.
17. Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Rad Biol Med* 1995;18:775-94.
18. Henrotin YE, Bruckner P, Pujol J-P. The role of reactive oxygen species in homeostasis and degradation of cartilage. *Osteoarthritis Cartilage* 2003;11:747-55.
19. Tiku ML, Liesch JB, Robertson FM. Production of hydrogen peroxide by articular chondrocytes. Enhancement by cytokines. *J Immunol* 1990;145:690-6.
20. Hiran TS, Moulton PJ, Hancock JT. Detection of superoxide and NADPH oxidase in porcine articular chondrocytes. *Free Radic Biol Med* 1997;23:736-43.
21. Hayashi T, Abe E, Yamate T, Taguchi Y, Jasin HE. Nitric oxide production by superficial and deep articular chondrocytes. *Arthritis Rheum* 1997;40:261-9.
22. Oh M, Fukuda K, Asada S, Yasuda Y, Tanaka S. Concurrent generation of nitric oxide and superoxide inhibits proteoglycan synthesis in bovine articular chondrocytes: Involvement of peroxinitrite. *J Rheumatol* 1998;25:2169-74.
23. Tiku ML, Gupta S, Desmukh DR. Aggrecan degradation in chondrocytes is mediated by reactive oxygen species and protected by antioxidants. *Free Rad Res* 1999;30:395-405.
24. Neve J. Physiological and nutritional importance of selenium. *Experientia* 1991;47:187-93.
25. Kirimidjan-Schumacher L, Slotsky G. Selenium and immune responses. *Environ Res* 1987;42:277-303.
26. Peretz A, Nève J, Famaey JP. Selenium in rheumatic diseases. *Semin Arthritis Rheum* 1991;20:1-18.
27. Peretz A, Nève J, Desmedt J, Duchateau J, Dramaix M, Famaey JP. Lymphocyte response is enhanced by supplementation of elderly subjects with selenium-enriched yeast. *Am J Clin Nutr* 1991;52:1323-8.
28. Sunde RA, Hoekstra WG. Structure, synthesis and function of glutathione peroxidase. *Nutr Rev* 1980;38:265-73.
29. Balogh Z, El-Ghobarev AF, Fell GS, Brown DH, Dunlop J, Dick WC. Plasma zinc and its relationship to clinical symptoms and drug treatment in rheumatoid arthritis. *Ann Rheum Dis* 1980;39:329-32.
30. Brown DH, Buchanan WW, El-Ghobarev AF, Smith WE, Teape J. Serum copper and its relationship to clinical symptoms in

- rheumatoid arthritis. *Ann Rheum Dis* 1979;38:174-6.
31. Aaseth J, Munthe E, Forre, O, Steinnes E. Trace elements in serum and urine of patients with rheumatoid arthritis. *Scand J Rheumatol* 1978;7:237-40.
  32. Tarp U, Overvad K, Hansen JC, Thorling EB. Low selenium level in severe rheumatoid arthritis. *Scand J Rheumatol* 1985;14:97-101.
  33. Peng A, Yang CL. Examination of the roles of selenium in the Kashin-Beck disease: cartilage cells and model studies. *Biol Trace Elem Res* 1991;28:1-9.
  34. Kurz B, Jost B, Schünke M. Dietary vitamins and selenium diminish the development of mechanically induced osteoarthritis and increase the expression of antioxidative enzymes in the knee joint of STR/IN mice. *Osteoarthritis Cartilage* 2002;10:119-26.
  35. Wolfram S, Berger B, Grenacher B, Scharrer E. Transport of selenoamino acids and their analogues across the intestinal brush border membrane. *J Nutr* 1989;119:706-12.
  36. Behne D, Kyriakopoulos A, Scheid S, Gessner H. Effects of chemical form and dosage on the incorporation of selenium into tissue proteins. *J Nutr* 1991;121:806-14.
  37. Burke KE, Combs GF Jr, Gross EG, Bhuyan KC, Abu-Libdeh H. The effects of topical and oral L-selenomethionine on pigmentation and skin cancer induced by ultraviolet irradiation in Skh:2 hairless mice. *Nutr Cancer* 2000;38:87-97.
  38. Katiyar SK, Mukhtar H. Tea antioxidants in cancer chemoprevention. *J Cell Biochem Suppl* 1997;27:59-67.
  39. Haqqi TM, Anthony DD, Gupta S, et al. Prevention of collagen-induced arthritis in mice by a polyphenolic fraction from green tea. *Proc Natl Acad Sci USA* 1999;96:4524-9.
  40. Adcocks C, Collin P, Buttle DJ. Catechins from green tea (*Camelia sinensis*) inhibit bovine and human cartilage proteoglycan and type II collagen degradation in vitro. *J Nutr* 2002;132:341-6.
  41. Benya PD, Padilla SR, Nimni ME. The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not type II. Verification by cyanogen bromide peptide analysis. *Biochemistry* 1977;16:865-72.
  42. Martin G, Andriamanalijaona R, Grässel R, et al. Effect of hypoxia and reoxygenation on gene expression and response to interleukin-1 in cultured articular chondrocytes. *Arthritis Rheum* 2004;50:3549-60.
  43. Andriamanalijaona R, Felisaz N, Kim SJ, et al. Mediation of interleukin-1 $\beta$ -induced transforming growth factor  $\beta$ 1 expression by activator protein 4 transcription factor in primary cultures of bovine articular chondrocytes: possible cooperation with activator protein 1. *Arthritis Rheum* 2003;48:1569-81.
  44. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
  45. Legendre F, Dudhia J, Pujol JP, Bogdanowicz P. JAK/STAT but not ERK1/ERK2 pathway mediates interleukin (IL)-6/soluble IL-6R downregulation of type II collagen, aggrecan core, and link protein transcription in articular chondrocytes. Association with a down-regulation of SOX 9 expression. *J Biol Chem* 2003;278:2903-12.
  46. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
  47. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 2001;25:402-8.
  48. Tarp U, Overvad K, Thorling EB, Graudal H, Hansen JC. Selenium treatment in rheumatoid arthritis. *Scand J Rheumatol* 1985;14:364-8.
  49. Ahmed S, Wang N, Lalonde M, Goldberg VM, Haqqi TM. Green tea polyphenol epigallocatechin-3-gallate (EGCG) differentially inhibits interleukin-1 $\beta$ -induced expression of metalloproteinase-1 and -13 in human chondrocytes. *J Pharmacol Exp Ther* 2004;308:767-73.
  50. Singh R, Ahmed S, Islam N, Goldberg VM, Haqqi TM. Epigallocatechin-3-gallate inhibits interleukin-1 $\beta$ -induced expression of nitric oxide synthase and production of nitric oxide in human chondrocytes. *Arthritis Rheum* 2002;46:2079-86.
  51. Gum R, Wang H, Lengyel E, Juarez J, Boyd D. Regulation of 92 kDa type collagenase expression by the jun-aminoterminal kinase- and the extracellular signal-regulated kinase-dependent signaling cascades. *Oncogene* 1997;14:1481-93.
  52. Stapleton SR, Garlock GL, Foellmi-Adams L, Kletzien RE. Selenium potent stimulator of tyrosyl phosphorylation and activator of MAP kinase. *Biochim Biophys Acta* 1997;1355:259-60.
  53. Mueller AS, Pallauf J, Rafael J. The chemical form of selenium affects insulinomimetic properties of the trace element: investigations in type II diabetic dbdb mice. *J Nutr Biochem* 2003;14:637-47.