Abstract. Tumor necrosis factor (TNF)-α and a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4) are important in osteoarthritis (OA) cartilage degradation. In the present study, we explored the interaction between the two proteins by examining the effect of TNF-α on ADAMTS-4 expression and activity in osteoarthritic chondrocytes. Human osteoarthritic chondrocytes were treated with TNF-α in different concentrations (5, 15, 30, 45 and 60 ng/ml) for different lengths of time (1, 6, 12, 18 and 24 h) with or without the TNF receptor 1 (TNFR1) inhibitor SPD304 or different kinase inhibitors. TNF-α increased the ADAMTS-4 mRNA level in a statistically significant dose- and time-dependent manner within 18 h, which was reflected in the dose-dependent induction of the ADAMTS-4 promoter activity, ADAMTS-4 protein expression and ADAMTS-4 activity. SPD304 (50 μM) and p38 mitogen-activated protein kinase (MAPK) siRNA and inhibitor PD169316 (25 μM) completely eradicated the promoting effect of TNF-α on ADAMTS-4 expression and activity. TNF-α induces ADAMTS-4 expression and activity in human osteoarthritic chondrocytes at the transcriptional level via TNFR1 by a p38 MAPK-dependent mechanism. To the best of our knowledge, this is the first evidence of crosstalk between TNF-α and ADAMTS-4 in relation to OA cartilage degradation, which adds novel insight into the pathophysiology of OA and cartilage degradation.

Introduction

Osteoarthritis (OA) is the most common degenerative disease affecting articular cartilage and is characterized by disrupted cartilage extracellular matrix homeostasis, ultimately resulting in the loss of cartilage without effective replacement (1-4). OA is caused in part by the exposure of chondrocytes to inflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α), which stimulate chondrocyte responses that promote catabolism of type II collagen and proteoglycans, thereby compromising cartilage extracellular matrix integrity and tissue homeostasis in OA (2). TNF-α is crucial in cartilage degradation. It promotes the expression of cytokines and chemokines in synovial cells and chondrocytes, thereby maintaining the renewal of local inflammatory mediators (5). The presence of TNF-α correlates with a general loss of cartilage matrix molecules, including type II collagen and aggrecan (5). TNF-α is produced by numerous cell types, including macrophages, lymphocytes, fibroblasts and keratinocytes in response to inflammation, infection and other environmental stresses (6). TNF-α acts by binding to its receptors, TNFR1 (p55) and TNFR2 (p75) on the cell surface. The majority of cells express TNFR1, which is believed to be the major mediator of the cytotoxicity of TNF-α (6). A previous study demonstrated that p55 TNF-α receptor expression is significantly increased in OA chondrocytes ex vivo. Enhanced expression of p55 may contribute to OA cartilage degradation (7).

The pathogenesis of OA is poorly understood, however a major feature is the loss of the two most important components of cartilage extracellular matrix: Type II collagen and aggrecan (8). Aggrecanases represent a class of proteinases belonging to the a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS) family. Previous studies have demonstrated that ADAMTS 1, 4, 5, 8, 9 and 15 possess aggrecanase activity (9,10). Song et al demonstrated that the knockdown of aggrecanase-1 (ADAMTS-4), aggrecanase-2 (ADAMTS-5) or both attenuates the degradation of aggrecan in human cartilage stimulated by TNF-α and oncostatin M (11). It has been stated that ADAMTS-4 is selectively overexpressed in human OA cartilage and is positively correlated with the degree of cartilage destruction, whereas ADAMTS-5 is similarly expressed in normal and OA cartilages (12). The results suggest that ADAMTS-4 is a major aggrecanase in human OA cartilage and its induction is involved in the pathogenesis of OA.

TNF-α and ADAMTS-4 are thought to be important in OA cartilage degradation. For the first time, to the best of
our knowledge, we explored the interaction between the two proteins by examining the effect of TNF-α on ADAMTS-4 expression and activity in human osteoarthritic chondrocytes.

Materials and methods

Reagents. Recombinant human TNF-α, TNFR1 inhibitor SPD304, phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002, protein kinase C inhibitor Go6983, mitogen-activated protein kinase (MAPK) inhibitor PD98059 and p38 MAPK inhibitor PD169316 were purchased from Sigma (St. Louis, MO, USA). TRizol reagent for RNA isolation and the SYBR Green Master mix were purchased from Invitrogen (Carlsbad, CA, USA) and Applied Biosystems (Foster City, CA, USA), respectively. Anti-ADAMTS-4 (PA1-1749A) antibody was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Anti-phospho-p38 (Thr180/Tyr182; no. 9212) antibody, anti-p38 (no. 8690) antibody and SignalSilence® p38 mitogen-activated protein kinase (MAPK) siRNA (no. 6564) were purchased from Cell Signaling Technology (Danvers, MA, USA). The SensoLyte® 520 aggrecanase-1 assay kit (no. 72114) was purchased from AnaSpec Inc. (Fremont, CA, USA). Human ADAMTS-4 promoter-luciferase reporter (with pairwise comparisons using Renilla reniformis luciferase (at one-fifth molar ratio to test plasmids) was co-transfected with test plasmids in each transfection as an internal control for data normalization. Luciferase assays were performed with a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Each experiment was repeated three times in duplicates. Untreated human osteoarthritic chondrocytes were used as a control.

Luciferase reporter assay. Human osteoarthritic chondrocytes were transfected with human ADAMTS-4 promoter-luciferase reporter plasmids using Lipofectamine 2000 transfection reagent (Invitrogen) and then treated with TNF-α (30 or 60 ng/ml) for 18 h. Plasmid PRL-CMV encoding Renilla reniformis luciferase (at one-fifth molar ratio to test plasmids) was demonstrated with an ECL kit (GE Healthcare, Pittsburgh, PA, USA) and applied using secondary antibodies with horse-radish peroxidase conjugate (1:5,000, 1 h). Peroxidase was revealed with an ECL kit (GE Healthcare, Pittsburgh, PA, USA). Proteins were quantified prior to being loaded onto the gel.

ADAMTS-4 activity assay and western blot analysis. ADAMTS-4 activities in cell culture supernatants were determined using a SensoLyte® 520 aggrecanase-1 assay kit (AnaSpec Inc.) according to the manufacturer's instructions. In western blot analyses, human osteoarthritic chondrocytes were lysed in 250 µl of 2X SDS loading buffer (62.5 mm TrisHCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol) and incubated at 95°C for 10 min. An equal amount of proteins (100 µg) for each sample were separated by 8-15% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride microporous membrane (Millipore, Billerica, MA, USA). Membranes were incubated for 1 h with a 1:1,000 dilution of primary antibody and then washed and revealed using secondary antibodies with horse-radish peroxidase conjugate (1:5,000, 1 h). Peroxidase was revealed with an ECL kit (GE Healthcare, Pittsburgh, PA, USA). Proteins were quantified prior to being loaded onto the gel.

Statistical analysis. Statistical analyses were performed with SPSS for Windows 10.0 (IBM, Chicago, IL, USA). Data values were expressed as the means ± SD. Comparisons of means among multiple groups were performed with one-way ANOVA followed by post hoc pairwise comparisons using Tukey’s tests. P<0.05 was considered to indicate a statistically significant difference.

Results

TNF-α increases ADAMTS-4 mRNA expression. Cultured human osteoarthritic chondrocytes were treated with TNF-α in different concentrations (5, 15, 30, 45, 60 ng/ml) for different lengths of time (1, 6, 12, 18 and 24 h). ADAMTS-4 mRNA levels were examined using real-time quantitative RT-PCR. The ADAMTS-4 mRNA level of treated cells was shown as fold changes to that of untreated control cells (designated as 1). Each experiment was repeated three times in triplicates. Results are expressed as the mean ± SD.

Luciferase reporter assay. Human osteoarthritic chondrocytes were transfected with human ADAMTS-4 promoter-luciferase reporter plasmids using Lipofectamine 2000 transfection reagent (Invitrogen) and then treated with TNF-α (30 or 60 ng/ml) for 18 h. Plasmid PRL-CMV encoding Renilla reniformis luciferase (at one-fifth molar ratio to test plasmids) was co-transfected with test plasmids in each transfection as an internal control for data normalization. Luciferase assays were performed with a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Each experiment was repeated three times in duplicates. Untreated human osteoarthritic chondrocytes were used as a control.

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human chondrocytes and its effect on ADAMTS-5 expression was inconsistent in normal and osteoarthritic human chondrocytes (data not shown).

**Table I. Relative ADAMTS-4 mRNA levels in human osteoarthritic chondrocytes in the presence of TNF-α with or without TNF receptor inhibitor.**

<table>
<thead>
<tr>
<th>TNF-α (ng/ml)</th>
<th>1 h</th>
<th>6 h</th>
<th>12 h</th>
<th>18 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.01±0.04</td>
<td>1.03±0.03</td>
<td>1.04±0.05</td>
<td>1.04±0.05</td>
<td>1.06±0.07</td>
</tr>
<tr>
<td>15</td>
<td>1.04±0.05</td>
<td>1.27±0.07</td>
<td>1.83±0.10</td>
<td>2.69±0.17</td>
<td>2.82±0.18</td>
</tr>
<tr>
<td>30</td>
<td>1.07±0.05</td>
<td>1.86±0.12</td>
<td>2.71±0.14</td>
<td>3.15±0.20</td>
<td>3.28±0.19</td>
</tr>
<tr>
<td>45</td>
<td>1.07±0.06</td>
<td>2.76±0.19</td>
<td>3.05±0.13</td>
<td>3.66±0.21</td>
<td>3.83±0.25</td>
</tr>
<tr>
<td>60</td>
<td>1.08±0.09</td>
<td>2.88±0.22</td>
<td>3.24±0.23</td>
<td>3.79±0.26</td>
<td>3.92±0.25</td>
</tr>
<tr>
<td>60 + SPD304 (50 µm)</td>
<td>0.94±0.11</td>
<td>0.99±0.05</td>
<td>0.97±0.07</td>
<td>1.03±0.07</td>
<td>1.05±0.06</td>
</tr>
</tbody>
</table>

ADAMTS-4 mRNA level of treated human osteoarthritic chondrocytes was shown as fold changes to that of untreated control cells (designated as 1). *P<0.05 compared with TNF-α treatment at 5 ng/ml or TNF-α (60 ng/ml) + SPD304 (50 µm); †P<0.05 compared with TNF-α treatment at 15 ng/ml; ‡P<0.05 compared with TNF-α treatment at 30 ng/ml; §P<0.05 compared with 1 h of TNF-α treatment at each concentration; □P<0.05 compared with 6 h of TNF-α treatment at each concentration and △P<0.05 compared with 12 h of TNF-α treatment at each concentration. TNF-α, tumor necrosis factor-α; ADAMTS-4, a disintegrin and metalloproteinase with thrombospondin motifs 4.

**Table II. Relative ADAMTS-4 mRNA levels in human osteoarthritic chondrocytes in the presence of TNF-α with or without kinase inhibitors or siRNA.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative ADAMTS-4 mRNA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.77±0.24*</td>
</tr>
<tr>
<td>+PD169316 (25 µm)</td>
<td>1.05±0.09</td>
</tr>
<tr>
<td>+Go6983 (250 nm)</td>
<td>3.68±0.21*</td>
</tr>
<tr>
<td>+PD098059 (25 µm)</td>
<td>3.72±0.26*</td>
</tr>
<tr>
<td>+LY294002 (50 µm)</td>
<td>3.65±0.29*</td>
</tr>
<tr>
<td>+p38 MAPK siRNA</td>
<td>0.97±0.15</td>
</tr>
</tbody>
</table>

Human osteoarthritic chondrocytes were pretreated with the kinase inhibitor for 30 min and then incubated with the kinase inhibitor and TNF-α (60 ng/ml) for 18 h. All kinase inhibitors were dissolved in DMSO (final concentration of DMSO 0.05%). Human osteoarthritic chondrocytes treated with TNF-α (60 ng/ml) + DMSO (0.05%) was used as the control. The ADAMTS-4 mRNA level of treated cells was shown as fold changes to that of control cells treated with 0.05% DMSO (designated as 1). *P<0.05 compared with TNF-α (60 ng/ml) + PD169316 (25 µm) or TNF-α (60 ng/ml) + p38 MAPK siRNA. MAPK, mitogen-activated protein kinase; TNF-α, tumor necrosis factor α; DMSO, dimethyl sulfoxide; ADAMTS-4, a disintegrin and metalloproteinase with thrombospondin motifs 4.

**Discussion**

Cartilage degradation in OA constitutes a major structural change in the joint, which may severely impair its function and cause pain and disability (16). Among the inflammatory mediators associated with OA, TNF-α is an established key mediator for cartilage (5). ADAMTS-4 is believed to be important in the degradation of aggrecan during the progression of joint diseases (17). In the present study, we provided, to the best of our knowledge, the first evidence for a regulatory effect of TNF-α on ADAMTS-4 expression and activity in human osteoarthritic chondrocytes.
TNF-α is produced in a variety of cell types, including macrophages, lymphocytes, fibroblasts and keratinocytes in response to inflammation, infection and other environmental stresses (6). TNF-α in the concentration range of 1-100 ng/ml has been used in chondrocytes in vitro (18). In the present study, we used 5-60 ng/ml of TNF-α to determine whether TNF-α is able to regulate ADAMTS-4 expression in osteoarthritic chondrocytes. Within this testing concentration range, while TNF-α at 5 ng/ml had no significant effect on ADAMTS-4 expression, a statistically significant dose-dependent effect of TNF-α on ADAMTS-4 expression was observed in the concentration range of 5-45 ng/ml and the effect reached a plateau in the range of 45-60 ng/ml. TNF-α at a concentration as low as 15 ng/ml increased ADAMTS-4 expression by >2.5 fold within 18 h, suggesting that TNF-α is a strong positive regulator of ADAMTS-4 expression.

The depletion of aggregan in articular cartilage is an early event in the pathogenesis of OA. ADAMTS-4 and ADAMTS-5 are believed to be important in the degradation of aggregan (17). Recent studies suggest that ADAMTS-4 may be the principal aggreganase of aggregan degradation in human OA as it is selectively overexpressed in human OA cartilage and is positively correlated with the degree of cartilage destruction, whereas ADAMTS-5 is similarly expressed.
in normal and OA cartilages (12,19). Indeed, we demonstrated that TNF-α had no induction effect on ADAMTS-4 expression in normal human chondrocytes. In addition, the effect of TNF-α on ADAMTS-5 expression was inconsistent in normal and osteoarthritic human chondrocytes. Thus, we only selected ADAMTS-4 as the target of the present study.

The main extracellular matrix macromolecules of the articular cartilage are type II collagen and aggrecan and the pathogenesis of OA involves the degradation of aggrecan and type II collagen (20,21). The presence of TNF-α reportedly correlates with the loss of type II collagen and aggrecan in OA cartilage due to increased production of matrix metalloproteinases (5). Aggrecan has a protective effect against collagen degradation. Mechanistically, type II collagen is exposed due to the degradation of aggrecan and the exposed collagen becomes an easy target for enzymatic degradation by collagenase (20,21). Since our findings demonstrate that ADAMTS-4 is a downstream target of TNF-α signaling in human osteoarthritic chondrocytes, the loss of aggrecan and type II collagen caused by TNF-α in OA cartilage is at least partially mediated by ADAMTS-4, besides matrix metalloproteinases. Further studies are needed to explore this issue in vivo. Our results demonstrated that TNF-α enhanced the ADAMTS-4 promoter activity and increased the ADAMTS-4 mRNA level, suggesting that TNF-α induced ADAMTS-4 expression at the transcriptional level. The underlying transcriptional regulatory mechanisms aim to be elaborated in our future studies.

In conclusion, we demonstrated that TNF-α induces ADAMTS-4 expression and activity in human osteoarthritic chondrocytes at the transcriptional level via TNFR1 by a p38 MAPK-dependent mechanism. To the best of our knowledge, this is the first evidence of crosstalk between TNF-α and ADAMTS-4 in relation to OA cartilage degradation, which
adds novel insight into the pathophysiology of OA and cartilage degradation.

References