Levels of matrix metalloproteinase (MMP)-1 in paired sera and synovial fluids of juvenile idiopathic arthritis patients: relationship to inflammatory activity, MMP-3 and tissue inhibitor of metalloproteinases-1 in a longitudinal study


Objectives. To measure levels of the collagenases matrix metalloproteinase (MMP)-1 and -13 in the synovial fluid (SF) and serum of patients with juvenile idiopathic arthritis (JIA), and to correlate these measurements with inflammatory activity, levels of the collagenase activator MMP-3 and the tissue inhibitor of metalloproteinases-1 (TIMP-1).

Methods. Levels of MMP-1, -3, -13 and TIMP-1 were measured in paired SF and serum from 82 JIA patients using enzyme-linked immunosorbent assay and compared between subtypes and patients of different ages and disease durations. These levels were also correlated to the active joint count (AJC) and standard measures of inflammatory activity and therapeutic response, including erythrocyte sedimentation rate (ESR) and platelet count (PLT).

Results. MMP-1 was detected in JIA SF and correlated with PLT. MMP-3 levels were high in SF and detectable in serum where they correlated with PLT, ESR and AJC. MMP-13, however, was not detected in SF or serum. No differences were observed between patients grouped by subtype, age or disease duration. MMP-3 contributed the majority of total MMP in SF samples resulting in excess MMP levels over TIMP-1.

Conclusions. MMP-1 is up-regulated in SF concordant with inflammatory activity in JIA. This was true for patients in all JIA subtypes and age groups, suggesting that the capability for degradation of type II collagen is present in early disease, and throughout the disease course. MMP-3 may be important in the activation of collagenases and the saturation of exogenous inhibitors. Serum MMP-3 may therefore be a useful, measurable and specific marker of active disease in JIA.

KEY WORDS: MMP, TIMP, Collagenase, Stromelysin, JIA.
secreted as a pro-form, which requires activation by cleavage of a 10 kDa pro-region. This occurs predominantly through proteolytic cleavage by other MMPs or serine proteases [22]. MMP-3 in particular is known to have a number of MMP substrates, and crucially is an activator of collagenases [23]. It is detected at very high concentrations even in serum, and may be predictive of joint damage in RA patients [24, 25]. Levels of MMP-3 were found to be high in the SF of JIA patients, and were associated with disease activity [26]; serum levels presumably reflected enzyme produced within the joint and migrating into the circulation [26, 27].

Several inhibitors of MMPs have been characterized. The large, broad-spectrum protease inhibitor α2-macroglobulin is considered to be the main inhibitor of MMPs in serum and possibly in SF, but the major tissue inhibitors of active MMPs are thought to be the tissue inhibitor of metalloproteinases (TIMP) family [28–30]. TIMPs belong to a four-member family of small, tight-binding MMP inhibitors and are commonly up-regulated during chronic arthritis, including JIA [26, 27]; however, it is thought that these inhibitory mechanisms are overwhelmed by local MMP production leading to joint damage [11].

We utilized a retrospective sample bank of paired SF and serum samples taken from JIA patients attending the Regional Paediatric Rheumatology service in Newcastle, where there is a continuity of care service into adult rheumatology. These samples were used to study collagenase levels, assessing a longitudinal population of patients from early diagnosis to adults with several decades of active disease to establish whether collagenases could contribute to joint damage early in the disease course. We further compared levels of MMP-1, MMP-3 and TIMP-1, and assessed their association with inflammatory activity to determine whether they may be candidates as specific markers of joint destruction, and hence potential targets for therapeutic intervention in JIA.

**Patients and methods**

**Patients**

The study was performed on JIA patients who donated serum and/or SF to a sample bank at the Freeman Hospital (FRH), Newcastle-upon-Tyne between 1996 and 2003. This included patients with early onset disease up to adults with disease of several decades tracked through the transitional services at the FRH. A total of 82 patients were selected, all diagnosed using the International League Against Rheumatism (ILAR) Durban criteria [1, 2]; 39 persistent oligoarticular JIA, 13 extended oligoarticular JIA, 14 polyarticular JIA (including two rheumatoid factor (RF) positive), eight systemic JIA, four psoriatic JIA and four enthesitis-related JIA. Seventy-three patients donated at least one serum sample, and 65 at least one SF sample. Samples were obtained during routine joint aspiration for active disease, and centrifuged at 1300 g—10 min for serum and 30 min for SF. The supernatent was then divided into aliquots and stored at −80°C until use.

**Disease activity analysis**

Retrospective analysis of patient case notes was performed to establish standard clinical and laboratory indicators of disease activity. The clinically active joint count (AJC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), platelet count (PLT), total white cell count (WCC) and neutrophil count (NEC) were obtained for as many sampling points as possible. A total of 101 SF samples and 103 serum samples were available with corresponding data, an average (range) of 1.2 (1–6) samples per patient. A total of 89 paired sera and SF taken at the same time point were available. The medication regimen, NSAID (non-steroidal anti-inflammatory drug), DMARD (disease modifying anti-rheumatic drug) and corticosteroid treatment at each sampling point, was also recorded.

**Enzyme-linked immunosorbent assay (ELISA) reagents**

MMP-3 levels were measured using a commercial ELISA kindly donated by the Daiichi Fine Chemical Company, Japan. This ELISA has a limit of detection of 12.5 ng/ml, and was performed according to the manufacturer’s instructions. MMP-1 and TIMP-1 were measured in the samples using ELISA methods previously described [31–33], with a slight modification—an reagent was included to avoid any possible interference by RF in patient serum [34]. This reagent, from Chemicon (Australia), is included with the samples at a dilution of 1:40. These ELISA assays have a linear range of 5–50 ng/ml. The monoclonal capture antibodies were used at 2 and 5 μg/ml for MMP-1 and TIMP-1, respectively, and the rabbit polyclonal detection antibodies at 0.25 μg/ml and 0.17 μg/ml, respectively. MMP-13 was measured using an ELISA developed in-house using antibodies kindly donated by the Daiichi Fine Chemical Company. The capture antibody Mab#61 and biotinylated detection antibody Mab#46 were used at 1.25 and 0.125 μg/ml, respectively. Recombinant human pro-MMP-13 standard was generated from a transformed insect cell line, and the assay had a linear range of 0.6–20 ng/ml. All of the ELISA methods used to detect MMP were able to recognize both pro- and active-MMP, and the TIMP-1 ELISA also recognized MMP-TIMP-1 complexes. A standard method was used for all assays, as described previously [31–33]. Samples were assayed in duplicate, and all measurements performed at the same time. The coefficient of variation of the duplicates was <12% for all samples.

**Statistical analysis**

Spearman’s rank correlation analysis of the sample concentrations was used to assess the relationship of the observed concentrations with clinical and laboratory criteria, and Mann–Whitney tests were used to assess differences between two patient groups. Non-parametric analysis of variance was used for more than two groups (Kruskall–Wallis, with Dunn’s post-hoc test to correct for multiple comparisons). Where multiple samples were available from a patient, mean levels were used over the study time in order to maintain independence of all data points in the analyses.

**Results**

**Patients and laboratory inflammation**

The patients studied showed a range of ages and disease duration at entry into the study, allowing the comparison of MMP levels longitudinally through the young with recent diagnosis to adults with several decades of disease. The mean (range) age of onset was 7 (1–16) yr, and the mean (range) age and disease durations at the time of study entry were 14 (1–36) yr and 7 (1–33) yr, respectively. For analytical purposes, we divided the patients into groups based on disease duration: 1–10 yr (n = 24), 10–18 yr (n = 26) and 18–36 yr (n = 14) to represent early JIA, teenage and transitional JIA, and adults with active JIA.

Laboratory measures of inflammation were collected from patient case notes. AJC was available corresponding to 64 SF and 73 sera, ESR for 50 SF and 57 sera, CRP for 50 SF and 50 sera, and PLT, NEC and WCC for 35 SF and 35 sera. The mean AJC was 2. Mean (±s.d.) ESR and CRP values were 2.1 ± 1.7 mm/h and 17.4 ± 21.4 mg/l, respectively. For the cell counts, mean (±s.d.) total PLT, WCC and NEC (×10⁹ cells/l) were 384.5 ± 107.6, 7.3 ± 2.7 and 4.1 ± 1.7, respectively. None of these clinical or laboratory indicators of disease activity were significantly different when patients were grouped by subtype, age or disease duration.
The level of TIMP-1 was determined in SF for 64/65 (98%) patients, and sera for 70/73 (96%) patients. Samples with undetectable levels were assigned a value of 10 ng/ml for analysis, and mean (±s.d.) values were 2.66 ± 1.3 µg/ml in SF and 0.72 ± 0.39 µg/ml in serum, ranging up to 7.0 µg/ml and 1.9 µg/ml, respectively. No significant differences were observed for mean TIMP-1 levels between the JIA subtypes in either SF or serum (Table 1).

Finally, we collected data on medication regimen at the point of sampling to determine the effect of medications on our measurements. Of the 101 SF and 103 sera collected over the course of the study, 24 SF and 15 sera were taken during corticosteroid therapy, 58 SF and 53 sera during NSAID treatment, 37 SF and 35 sera during methotrexate treatment and four SF and five sera during etanercept treatment.

Collagenase levels in JIA patients

Detectable MMP-1 was measured in 59/65 (91%) of the patients donating SF. Serum MMP-1, however, was poorly detectable in JIA patients, with only 7/73 (10%) patients showing levels above the assay limit of detection, and samples below this limit were allocated the arbitrary value of 10 ng/ml for statistical analysis. The mean (±s.d.) patient SF MMP-1 level was 0.32 ± 0.3 µg/ml, and ranged up to 1.4 µg/ml in the JIA population studied. The mean (±s.d.) patient serum value was 0.04 ± 0.14 µg/ml with a range up to 0.9 µg/ml despite the low detection rate. Despite a tendency for lower levels in persistent oligoarticular patients, no significant differences were observed between JIA subtypes (Fig. 1A and Table 1). A significant positive correlation was observed between SF MMP-1 levels and PLT (Fig. 1B). We did not find any differences between samples compared by medication regimen in SF or sera. MMP-13 was not detectable in any SF or serum sample analysed.

Determination of TIMP-1 and MMP-3 in JIA samples

The level of TIMP-1 was determined in SF for 64/65 (98%) patients, and sera for 70/73 (96%) patients. Samples with undetectable levels were assigned a value of 10 ng/ml for analysis, and a significant positive correlation was observed between the different subtypes. Plots show median value (line), interquartile range and total range (whiskers). (B) Spearman’s rank correlation of SF MMP-1 and PLT in the whole JIA population. A significant correlation is observed.

Table 1. Concentrations of MMP-1, MMP-3 and TIMP-1 measured in JIA samples in the different subtypes studied. No significant differences were observed between subtypes for any of the MMP or TIMP levels in SF or serum.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Persistent oligo</th>
<th>Extended oligo</th>
<th>Polyarticular</th>
<th>Systemic</th>
<th>Psoriatic</th>
<th>Enthesitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1 SF</td>
<td>0.27 ± 0.15 (n = 33)</td>
<td>0.32 ± 0.15 (n = 11)</td>
<td>0.30 ± 0.22 (n = 12)</td>
<td>0.80 ± 0.52 (n = 3)</td>
<td>0.34 ± 0.13 (n = 2)</td>
<td>0.70 ± 0.63 (n = 3)</td>
</tr>
<tr>
<td>MMP-1 Serum</td>
<td>0.05 ± 0.17 (n = 33)</td>
<td>0.01 ± 0.00 (n = 11)</td>
<td>0.04 ± 0.01 (n = 12)</td>
<td>0.01 ± 0.06 (n = 3)</td>
<td>0.01 ± 0.63 (n = 3)</td>
<td>0.01 ± 0.00 (n = 3)</td>
</tr>
<tr>
<td>MMP-3 SF</td>
<td>92.21 ± 68.59 (n = 29)</td>
<td>114.92 ± 85.15 (n = 10)</td>
<td>78.09 ± 40.07 (n = 11)</td>
<td>153.11 ± 59.35 (n = 3)</td>
<td>57.97 ± 33.69 (n = 2)</td>
<td>115.31 ± 93.57 (n = 2)</td>
</tr>
<tr>
<td>MMP-3 Serum</td>
<td>0.21 ± 0.35 (n = 11)</td>
<td>0.16 ± 0.19 (n = 10)</td>
<td>0.55 ± 0.94 (n = 11)</td>
<td>1.16 ± 0.60 (n = 2)</td>
<td>0.09 ± nd (n = 1)</td>
<td>0.01 ± nd (n = 1)</td>
</tr>
<tr>
<td>TIMP-1 SF</td>
<td>2.29 ± 1.03 (n = 33)</td>
<td>2.79 ± 1.33 (n = 11)</td>
<td>3.46 ± 1.51 (n = 12)</td>
<td>2.98 ± 0.35 (n = 3)</td>
<td>3.10 ± 2.70 (n = 2)</td>
<td>0.86 ± 0.64 (n = 3)</td>
</tr>
<tr>
<td>TIMP-1 Serum</td>
<td>0.68 ± 0.33 (n = 33)</td>
<td>0.65 ± 0.26 (n = 11)</td>
<td>0.87 ± 0.42 (n = 12)</td>
<td>0.93 ± 0.24 (n = 3)</td>
<td>0.51 ± 0.05 (n = 2)</td>
<td>0.46 ± 0.04 (n = 3)</td>
</tr>
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</table>

Age and disease duration

No correlation was found between SF MMP-3 or MMP-1 and age at study entry, or with disease duration. Neither could any differences be seen between patients grouped by age (2–10 yr, 11–15 yr, 16–20 yr, 21–25 yr, >25 yr) or gender.
10–18 yr, 18–36 yr; Fig. 5). No relationship was found either between serum levels of MMP-3 and TIMP-1 and the age variables tested; thus MMP and TIMP-1 are present from early in the disease course and persist throughout the duration of active disease.

Paired samples and the MMP:TIMP ratio

The availability of paired SF and serum samples allowed the comparison of local and systemic levels of MMPs and TIMP-1 by calculating the SF:serum ratio. MMP-3 and TIMP-1 SF and serum values were significantly correlated ($r = 0.39$ and 0.36, respectively, both $P < 0.0001, n = 89$). The mean (± S.D.) SF:serum ratio of MMP-3 was $2758 ± 5853$, and a considerable gradient from SF to serum was found for all sample pairs with available data. The mean (± S.D.) SF:serum ratio for TIMP-1 was $4.57 ± 4.1$, and therefore a gradient from SF to serum was present in all patients, but not as great as that for MMP-3.

The assays used measure both free MMP and TIMP-1, as well as MMP–TIMP complex. This allowed calculation of the ratio MMP:TIMP, which is a useful indicator of the relative production of MMP and TIMP-1 during active disease. The MMP:TIMP ratio was calculated using the molecular weights of 59 kDa corresponding to pro-MMP-3, 52 kDa to pro-MMP-1, and 28 kDa to TIMP-1. It was observed that the ratio in SF favoured MMP over TIMP-1 (Fig. 6A). This is in contrast to paired sera, where the majority of samples showed a ratio <1, i.e. favouring TIMP over MMP. The MMP:TIMP ratio correlated with AJC (Fig. 6B), but no differences were seen between JIA subtypes. Due to the high MMP-3 concentrations detected, MMP-3 is responsible for the vast majority of enzyme in this calculation, and the concentration of MMP-3 compared with MMP-1 meant that the ratio of the collagenase activator MMP-3:MMP-1 always favoured the activator; with a mean (± S.D.) of $1099 ± 3995$ in SF.

**Discussion**

This is the first report of collagenase levels in JIA patients. Collagenases are considered to be crucial to the progression of destructive arthritis due to their ability to cleave native type II collagen.
collagen, a pivotal step in joint breakdown since it is essentially irreversible [13, 35]. Furthermore, destruction of the type II collagen network in JIA, may contribute to damage of the growth plate, a common feature of JIA, which has been linked to production of pro-inflammatory cytokines [36]. MMP-1 was readily detectable in most JIA SF, and closely correlated with PLT, indicating an association with general inflammatory activity. The levels of MMP-1 detected were within the range previously reported for RA patients [37–39]. However, MMP-1 was not readily detected in JIA serum. MMP-1 has been detected in RA sera in several studies [33, 40] at levels approaching the limit of detection of the assay employed here—generally between 10 and 70 ng/ml and not markedly elevated above control or OA patient levels [24, 33, 41]. Thus, whilst MMP-1 in SF appears to be closely linked to inflammation, and is of significance in the degradation of type II collagen, serum measurements were not suitable as markers of disease activity.

MMP-3 has previously been proposed as a good marker of disease activity in RA [10, 42], and it may also be predictive of clinical progression [24, 25]. It has been suggested that MMP-3 is an acute-phase reactant [10, 42], and the correlation reported herein of serum MMP-3 with the standard acute-phase inflammatory measurements of ESR and PLT, as well as AJC, suggest that this is also the case in JIA [27]. The high level in SF and lack of correlation with overall inflammation is consistent with an early up-regulation of MMP-3 reaching a plateau. MMP-3 has been proposed to play a role in breakdown of proteoglycan [43], but perhaps more importantly is capable of activating many pro-MMPs including MMP-1 [22, 23]. Because MMP-3 is present at such high levels, some 1000-fold higher than MMP-1, it is also of significance during disease since MMP-3 levels contribute most of the total MMP that would appear to overwhelm the levels of TIMP-1. The ELISA assays employed detect free pro-MMP or active MMP, and TIMP-bound active MMP. Thus, whilst not
necessarily reflecting collagenolysis, collagenase activation or MMP inhibition, the comparative measurement of levels of MMP-1, MMP-3 and TIMP-1 and the calculation of the MMP:TIMP ratio are an indirect measure of the relative production of these molecules within the diseased tissue, where activation presumably occurs in localized areas where joint destruction is occurring.

The levels of MMP-3 reported here are higher than those previously reported by Gattorno et al. [26] in JIA SF (4–5 μg/ml). We report mean patient SF levels of MMP-3 ranging up to 115 μg/ml, with levels in some samples reaching over 300 μg/ml. In fact, these levels are similar to those reported in RA patients [39–44]. A unique feature of the JIA population utilized in this study is the range of age and disease duration of the patients recruited. We are able to (i) confidently assign subtypes in most patients by the ILAR criteria, as patients that progress to an extended oligoarticular course generally do so within the first 5 yr of disease [45] and (ii) compare levels of degradative enzymes in relatively recent diagnoses with those of patients with long-standing disease. This latter point is important given that many patients (up to 50%) have active disease well into adulthood with resulting poor outcome [3, 4], and joint damage, previously considered a late event in JIA, may occur early in disease with clear implications for developing joints [21]. We therefore tested the hypothesis that the high MMP levels found may be related to age, with higher levels in the older patients included in this study—adults with many decades of active disease. However, this was not the case, MMP and TIMP-1 levels were independent of age and disease duration, and are thus present from early in disease and persist with active disease into adulthood. Thus the potential for irreversible joint breakdown is present from early disease and throughout the disease course. It is notable that whilst the serum MMP-3 levels reported here are also higher than those reported by Gattorno et al. [26] at 0.02–0.5 μg/ml, they are comparable with a previous study by Matsuyama et al. [27], who reported 0.01–7.6 μg/ml.

TIMP-1 has been found previously to be concordantly up-regulated with MMP production [9], and the correlation with MMP-3 shown here supports this. A correlation between serum MMP-3 and TIMP-1 was also identified in a previous study on JIA patients, and notably existed only in patients that progressed to show joint erosions [27]. Although other cellular sources cannot be excluded, higher levels in SF compared with paired serum imply that both MMP-3 and TIMP-1 are produced in a concordant manner by cells within the joint such as synovial fibroblasts and chondrocytes. The correlation of TIMP-1 in SF with blood NEC/WCC would also support a link between the production of MMP-3 and TIMP-1 and the overall inflammatory response during disease.

The determination of high SF levels and lower serum levels of MMP-1, MMP-3 and TIMP-1 are as expected if they are produced by cells within the joint during inflammatory disease, with subsequent diffusion into the serum. We tested SF and serum samples taken at the same time point to test whether a gradient favouring such diffusion was present for all samples examined by calculation of the SF:serum ratio, and indeed SF levels of MMP-3 and TIMP-1 were always higher than in paired sera; some 2500-fold for MMP-3 and 5-fold for TIMP-1, which may be related to the smaller size of the TIMP-1 protein allowing more rapid diffusion.

The failure to detect MMP-13 is interesting, since MMP-13 is capable of degrading type II collagen with a greater efficiency than other collagenases [20], and MMP-13 has been immunolocalized in synovial tissue from JIA patients in a similar pattern to MMP-1 [6]. MMP-13 mRNA is up-regulated in response to pro-inflammatory cytokines [46], although the protein can be internalized by several cell types, which may explain the lack of detectable MMP-13 in SF [47]. Since it is an efficient collagenase with respect to type II collagen, it may therefore contribute towards joint damage despite being undetectable in SF. It is thought to be closely involved in the destruction of cartilage collagen in OA [20]; however, its role in JIA is not clear.

In conclusion, the data presented here show the presence of MMP-1 and MMP-3 in SF linked to inflammatory activity in JIA, and suggest that enzymes capable of degrading cartilage, including collagenases and collagenase activators, are present from an early point in disease. MMP-1 and MMP-3 were detected at similar concentrations to those found in previous work on RA patients, and MMP-3 levels were high. The high SF level and lack of correlation with inflammatory variables, coupled with the gradient into serum where levels did correlate with inflammation, suggest that local MMP-3 production may be an early event in disease and may also be important in saturation of TIMP-1. The data further support the use of serum MMP-3 and TIMP-1 as specific markers of disease activity in JIA.

### Acknowledgements

We thank the Faculty of Medical Sciences, University of Newcastle-upon-Tyne, for the award of a research studentship to Nick Peake, the Arthritis Research Campaign for the award of a senior clinical lectureship to Helen Foster, and the Dunhill Medical Trust for essential support for this work. We would also like to thank the Daiichi Fine Chemical Co. for supplying the MMP-3 ELISA kits, and the monoclonal antibodies to MMP-13. Finally, we thank Dr Simon Kometika of the statistics support service, University of Newcastle, for his helpful advice on statistical methods.

The authors have declared no conflicts of interest.

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