REDUCTION OF SERUM MATRIX METALLOPROTEINASE 1 AND MATRIX METALLOPROTEINASE 3 IN RHEUMATOID ARTHRITIS PATIENTS FOLLOWING ANTI-TUMOUR NECROSIS FACTOR-\(\alpha\) (cA1) THERAPY

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SUMMARY
Matrix metalloproteinase (MMP)-1 and MMP-3 levels were measured in serum samples from rheumatoid arthritis (RA) patients undergoing a double-blinded placebo-controlled trial with the chimaeric anti-tumour necrosis factor (TNF)-\(\alpha\) antibody cA2. Both MMP-1 \((P < 0.015)\), but to a larger extent MMP-3 \((P < 0.001)\), levels were elevated in all RA patients prior to the commencement of the trial compared with normal control sera. Following cA2 therapy, MMP-1 and MMP-3 levels were assessed in the placebo, and 1 and 10 mg/kg cA2-treated groups at 7, 14, 21 and 28 days. In both the 1 and the 10 mg/kg cA2-treated groups, a significant decrease in serum MMP-3 levels at all time points was observed, reducing maximally to 41% of pre-infusion values at day 7. MMP-1 levels were also reduced, but less dramatically than MMP-3, to 85% of pre-infusion values after 14 days in the 10 mg/kg cA2 treated group. In a separate non-placebo-controlled study, we also evaluated the tissue inhibitor of metalloproteinase (TIMP)-1 levels in plasma following cA2 infusion. Pre-infusion TIMP-1 levels were above the normal control range, but were significantly reduced \((P < 0.035)\) 14 days after infusion to 72% of pre-infusion values. This study confirms previous reports that MMP-3 levels are elevated and correlate with measures of inflammation in RA, and furthermore demonstrate that serum MMP-3 and MMP-1 levels are downregulated following anti-TNF-\(\alpha\) antibody therapy. Whilst serum MMP-3 levels correlated with C-reactive protein (CRP) both prior to and following anti-TNF-\(\alpha\) antibody therapy, it remains to be demonstrated that serum MMP-3 and/or MMP-1 levels reflect the cartilage and bone resorptive processes which are evident in this disease.

KEY WORDS: Rheumatoid arthritis, Tumour necrosis factor, Anti-TNF-\(\alpha\) antibody, Metalloproteinase, Tissue inhibitor of metalloproteinase.

RHEUMATOID arthritis (RA) is an autoimmune disease characterized by inflammation of the synovial joints, infiltration by blood-derived cells, and the eventual destruction of cartilage and bone. It is now generally accepted that the activity of the cells within the synovium and, in particular, the cytokine and enzyme products which they generate, are involved in the destruction of the underlying matrix components (reviewed in [1]). Of the cytokines released in RA synovial tissue, tumour necrosis factor (TNF)-\(\alpha\) and interleukin-1 (IL-1) are considered to be of major importance in inflammatory tissue destruction as they induce prostaglandin E\(_2\) (PGE\(_2\)) production, cartilage destruction and bone resorption [2–6]. Furthermore, in RA synovial cell cultures, TNF-\(\alpha\) is important in driving the cytokine cascade since its neutralization reduces IL-1 [7], granulocyte-macrophage colony-stimulating factor (GM-CSF) [8], IL-6 and IL-8 levels [9] (reviewed in [1]). The destruction of cartilage and other components of connective tissue is thought to involve the actions of the matrix metalloproteinase (MMP) enzymes (reviewed in [10]) released by a wide range of cells in the synovial tissue, including synovial fibroblasts, chondrocytes and monocytes in response to pro-inflammatory cytokines such as IL-1 and TNF-\(\alpha\), and growth factors including epidermal growth factor and platelet derived growth factor [11].

The MMPs are a growing family of molecules, currently \(\sim 15\) members, which are synthesized and secreted as latent pro-enzymes, contain a zinc binding active site and require Ca\(^{2+}\) for activation. They include the classical MMP enzymes which are secreted from the cell in a latent form and are grouped into three main classes including: (1) interstitial (MMP-1) and neutrophil (MMP-8) collagenase; (2) 72 kDa gelatinase A (MMP-2) and 92 kDa gelatinase B (MMP-9); and (3) stromelysin-1 (MMP-3), stromelysin-2 (MMP-10) and PUMP-1 (MMP-7) (reviewed in [12]). The MMP family also includes enzymes recently cloned and characterized that are not secreted, but anchored in the membrane. These include the membrane-type MMP (MT MMP-1) [13] and a related enzyme, MT MMP-2 [14], which both induce processing of pro-gelatinase A. In addition, the TNF-\(\alpha\) convertase enzyme (TACE), which was identified as a metalloproteinase enzyme, has recently been cloned and characterized, and is also a membrane-anchored enzyme (R. Black, unpublished personal communication).

Of the conventional MMP enzymes, MMP-1 (interstitial collagenase) and MMP-3 (stromelysin-1) are thought most likely to be involved in the pathogenesis of RA as MMP-1 degrades collagen type II, and MMP-3 degrades a range of matrix proteins including proteoglycans, laminin, fibronectin and gelatin found in connective tissue in the synovial joint (reviewed in [12]). This concept is supported by the

Please note: The text above is a summary of the research article and is not the full article.
observation that MMP-1 [15, 16] and, in particular, MMP-3 levels [16, 17] are raised in RA synovial fluid, with MMP-3 levels also correlating with markers of disease activity such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). Serum levels of MMP-3, but not of MMP-1 [17–20], are also increased compared with normal control levels, and show a highly significant correlation with matched synovial fluids [19, 20], indicating that serum pro-MMP-3 is derived chiefly from that synthesized in the synovium.

As the MMP enzymes are capable of matrix degradation, they are potentially pathogenic and as such their function must be tightly controlled. This is achieved not only by the transcriptional and translational control of MMP production, but also released from cells they can be neutralized by the binding of inhibitors. These include non-specific inhibitors such as &alpha;2-macroglobulin (&alpha;2M), a 750 kDa protein produced in the liver which binds many classes of enzymes including the MMPs [10]. &alpha;2M is found at high levels in normal and RA serum as well as RA synovial fluid, although its large size probably largely excludes it from the extracellular compartment in the synovial membrane, rendering it ineffective in this inflammatory environment. The second class of MMP inhibitors are the specific tissue inhibitors of metalloproteinases (TIMPs) which only bind the active form of the MMP [10]. Three human TIMPs have been identified which specifically inhibit MMP activity by forming a 1:1 non-covalent high-affinity complex with the active MMP molecule. In RA synovium, TIMP-1 production has been immunolocalized to synovial lining cells, endothelial cells and chondrocytes [21], and the production of TIMP-1 can be upregulated by a different spectrum of cytokines and growth factors to those which induce MMP transcription. These include transforming growth factor &beta; (TGF-&beta;) [22, 23], IL-6 [24–26] and IL-11 [27]. The pro-inflammatory cytokines including IL-1 and TNF-z have variable effects on TIMP. For example, in one study TIMP expression was not modulated by either IL-1 or TNF-z in RA fibroblast cultures although MMP-3 expression was strongly unregulated [28], whereas in a separate study TIMP expression was upregulated by IL-1 in synovial cells and chondrocytes, but downregulated in endothelial cells [26].

Although RA patients show elevated levels of TIMP-1 in synovial fluid and in serum [17, 20], it is considered that an imbalance of the enzyme:inhibitor ratio is apparent and matrix destruction is ongoing. There is a wide range in the published serum levels of TIMP-1 which is likely to be due to its release from activated platelets during blood clotting [29]. Thus, in order to obtain accurate measurements of TIMP-1 in plasma, it is necessary to extract the plasma carefully to avoid platelet aggregation.

In recent clinical trials on RA conducted by our group, TNF-z has been the target of immunotherapy using an anti-human TNF-z chimaeric monoclonal antibody (cA2). In the initial open-phase trial, RA patients with active disease received 20 mg/kg cA2 [30] and in a second, placebo-controlled trial, RA patients received either placebo, 1 mg/kg cA2 or 10 mg/kg cA2 [31]. Patients in both trials showed a marked improvement in both clinical and laboratory parameters, with a corresponding improvement in disease status. In this study, we investigated whether anti-TNF-z therapy also resulted in a reduction in the levels of circulating MMP-1, MMP-3 and TIMP-1. Immunoassays were employed which would detect both free MMP or TIMP, or that complexed together MMP-TIMP. Using serum derived from the double-blinded placebo-controlled trial, our results confirm that serum MMP-3 and also serum MMP-1 levels are raised in RA patients, and that the levels of these proteins are significantly diminished following anti-TNF-z therapy. Secondly, in a preliminary investigation using EDTA-plasma obtained from the non-placebo-controlled trial, we observed that the levels of TIMP-1 in plasma were also elevated in the RA patients, but that following anti-TNF-z treatment a small, but significant decrease in TIMP-1 levels was observed. These results extend our previous observation regarding the pivotal role of TNF-z in RA (reviewed in [1]) and indicate that the pro-inflammatory cytokine TNF-z is also important in regulating those events which lead to increased levels of connective tissue degradative enzymes such as MMP-1 and MMP-3. Hence, TNF-z blockade may prove to be of importance in slowing down those processes which lead to cartilage destruction in the synovial joints.

METHODS

Patients and samples

Serum samples for the MMP-1 and MMP-3 assay were obtained from RA patients (n = 73) participating in the second trial with the chimaeric anti-TNF-z monoclonal, cA2, which was placebo controlled and double blinded as described previously [30]. Patients received either placebo, or 1 or 10 mg/kg of cA2 in a single infusion. Owing to incomplete sampling, serum samples from only 57 patients (placebo = 21, 1 mg/kg cA2 = 19, 10 mg/kg cA2 = 17) were examined. Serum samples were taken between 08:00 and 12:00 h prior to infusion, and at weekly intervals up to 4 weeks after infusion, and stored at −80°C until assayed.

EDTA-plasma samples for the TIMP-1 assay were obtained from RA patients participating in the first open-label (non-placebo-controlled) trial with cA2 as described previously [30]. Patients included in the open trial received a total of 20 mg/kg of cA2 administered in two or four i.v. infusions over a 14 day period. However, due to an incomplete set of samples available after other tests were performed, plasma samples from only seven of these patients were available for TIMP-1 assessment. Bloods taken subsequent to day 1 were taken in the morning into tubes containing EDTA and the plasma extracted. Samples were taken at 24 h and weekly during the trial up to 12 weeks after the initial infusion.
Clinical assessments
All patients in both trials fulfilled the revised American College of Rheumatology criteria for the diagnosis of RA, and had a history of failed therapy with at least one disease-modifying anti-rheumatic drug (DMARD) at trial entry. All DMARDs were withdrawn 4 weeks prior to trial entry, although low-dose corticosteroids or non-steroidal anti-inflammatory drugs were allowed [30, 31]. Patients were assessed for overall disease activity using the Paulus index [32] as previously described [31]. This index incorporates six different clinical and laboratory measures of disease activity: tender and swollen joint scores, duration of morning stiffness, ESR, and the patient’s and observer’s assessment of disease activity. The Paulus 20% response is defined as improvement in four of the six parameters with at least 20% improvement in the continuous variables and at least two grades improvement in disease severity.

Immunoassays
MMP-1 was assayed in a blinded fashion by ELISA (Amersham Life Science, UK) according to the manufacturer’s instructions. The assay detects pro-MMP-1 and MMP-1 complexed with TIMP, but not MMP-1 complexed with α2M. The range of the assay was from 0.625 to 50 ng/ml with a sensitivity of 1.7 ng/ml. Serum samples were undiluted and individual patients were assayed in duplicate on the same plate. MMP-1 detection was not altered by the addition of diluent containing up to 100 μg/ml cA2 (data not shown). MMP-3 was assayed in a blinded fashion by ELISA using reagents provided by Dr Jaspar, Biosource Europe S.A, Belgium. The assay recognizes pro-MMP-3, activated MMP-3, MMP-3 in complex with TIMP-1, but not MMP-3 complexed with α2M (data not shown). The range of the assay was 1.25–20 ng/ml. Briefly, blocked 96-well plates were received coated with a monoclonal antibody specific to human MMP-3. Serum samples were diluted from 1:10 to 1:200 and 50 μl added to the plates in duplicate; individual patients were assayed on the same plate. An anti-MMP-3 antibody conjugated to horseradish peroxidase (HRP) was diluted and 50 μl added to each well. The plates were incubated at room temperature for 2 h on a horizontal shaker set at 700 ± 100 r.p.m. The liquid from each well was aspirated and the plates washed four times with 0.4 ml of Medgenix wash solution in each well. Plates were developed using 100 μl of freshly prepared TMB solution for 15 min at room temperature on a horizontal shaker set at 700 ± 100 r.p.m. The reaction was stopped by the addition of 200 μl of 0.3 N H2SO4, and the absorbance was read at 450 nm within 30 min. MMP-3 concentrations were calculated by reference to a standard curve. Up to 100 μg/ml cA2 were added to the standard curve and did not significantly affect the results (data not shown).

TIMP-1 was also assayed by ELISA (Amersham Life Sciences, UK) according to the manufacturer’s instructions. The range of the assay was from 3.13 to 50 ng/ml with a sensitivity of 1.25 ng/ml. The assay recognizes free TIMP-1 and that complexed with MMPs, and the TIMP-1 concentration was calculated by reference to a standard curve. Plasma-EDTA samples were diluted 1:10 (RA patients) or 1:5 (normal serum and plasma) and individual patients were assayed on the same plate.

Statistical analysis
Statistical analyses were performed using the Minitab 8.2 software for the Macintosh computer. The comparison between treatment groups using the raw data and percentage changes relative to pre-infusion values was analysed using the non-parametric Mann–Whitney U-test for non-matched samples, whereas for comparison of matched samples with their pre-infusion value the non-parametric Wilcoxon signed rank test was used. The significance values were further adjusted for comparisons between multiple groups using the Bonferroni correction. Thus, for the Mann–Whitney U-test the P value was multiplied by two (placebo compared with treatment group) and for the Wilcoxon rank test the P value was multiplied by four (pre-infusion value compared with day 7, 14, 21 or 28).

Routine laboratory parameters, CRP and ESR and the clinical assessment of swollen joint count were compared with MMP-1 and MMP-3 measurements by calculation of the correlation coefficient. Significance was achieved if P ≤ 0.05. The Paulus 20% response at week 4 was used to divide patients into responder versus non-responder groups. The groups were then analysed by the non-parametric Mann–Whitney U-test for non-matched samples.

RESULTS

Table I summarizes the pre- and post-infusion (days 7, 14, 21 and 28) MMP-1 levels (mean, median and range) in the RA sera (n = 57) analysed from the multicentre, placebo-controlled cA2 trial. The pre-infusion median MMP-1 concentrations in the RA sera (placebo group: 5.9 ng/ml; 1 mg/kg cA2 group: 7.1 ng/ml; 10 mg/kg cA2 group: 7.2 ng/ml) were not statistically different from each other but all showed a small but significantly elevated MMP-1 level (placebo: P = 0.016, 1 mg/kg cA2: P = 0.0105, 10 mg/kg cA2 P = 0.0025) compared with median levels (4.7 ng/ml) in normal control serum (n = 9).

For statistical analysis, the data within groups were analysed by the Wilcoxon rank test to compare median values at each time point with the pre-infusion value (Table I) and by the Mann–Whitney U-test to compare the placebo group at each time point with the cA2 (1 or the 10 mg/kg) treated group using the median data expressed as a percentage relative to pre-infusion values (Fig. 1). The results were adjusted according to the Bonferroni correction in which the P values were multiplied by the number of comparisons made, as a more stringent test of significance.

The median MMP-1 levels in the placebo-treated patients were relatively unchanged over the course of
the 4 weeks for which samples were assayed (Table I, Fig. 1). However, in the 10 mg/kg cA2-treated group, and to a lesser extent in the 1 mg/kg cA2-treated group, a reduction in median MMP-1 levels was observed, although not until 14 days after infusion of antibody. Thus, at day 14 in the 10 mg/kg cA2-treated group, a small but significant decrease ($P < 0.004$) from the pre-infusion median level of 7.2 ng/ml to 6.35 ng/ml was observed, and was further reduced to 6.1 ng/ml ($P < 0.001$) at day 21. The reduction at day 14 in the 10 mg/kg cA2-treated group was 85% of the pre-infusion values and was significantly different from the placebo-treated group at this time point ($P < 0.001$) (Fig. 1), and was also observed at day 21 ($P = 0.0012$) and at day 28 ($P = 0.015$) (Fig. 1). The reduction of serum MMP-1 in the 1 mg/kg cA2 group was very small (~94% of pre-infusion values) and was not significantly reduced compared with pre-infusion values at any time (Table I), although at day 14 post-infusion a small but significant ($P = 0.0376$) reduction compared with the placebo group was observed (Fig. 1).

**Serum levels of MMP-3 in patients treated with cA2**

Table II summarizes the pre- and post-infusion (day 7, 14, 21, 28) MMP-3 levels (mean, median and range) in the RA sera ($n = 57$) analysed from the multicentre, placebo-controlled cA2 trial. The median MMP-3 concentration in the pre-infusion RA serum samples was 79.2 ng/ml (placebo group), 118.6 ng/ml (1 mg/kg cA2 group) and 88.6 ng/ml (10 mg/kg cA2 group), not significantly different from each other, but all were significantly higher ($P < 0.001$) than the median MMP-3 serum levels (14.4 ng/ml) in the normal control group ($n = 44$).

As with the MMP-1 data, the MMP-3 data within groups were analysed by the Wilcoxon rank test to compare median values at each time point with the pre-infusion value (Table II) and by the Mann–Whitney U-test to compare the placebo group at each time point with the cA2 (1 or the 10 mg/kg) treated group using the median data expressed as a percentage relative to pre-infusion values (Fig. 2), and adjusted with the Bonferroni correction.

The median MMP-3 levels in the placebo-treated patients were relatively unchanged over the course of the 4 weeks for which samples were assayed (Table II, Fig. 3). However, in both the 10 mg/kg cA2-treated group and the 1 mg/kg cA2-treated group, a significant reduction in median MMP-3 levels was observed by

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**Table I**

<table>
<thead>
<tr>
<th>Days</th>
<th>Mean (range)</th>
<th>Median (range)</th>
<th>S.D.</th>
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**Table II**

<table>
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<td>6.60 (2.6-34.7)</td>
<td>10.69</td>
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Normal controls ($n = 9$), mean = 4.3 ± 1.2 ng/ml, median = 4.7 ng/ml, range 2.2–5.8 ng/ml. The MMP-1 serum levels in RA patients treated with either placebo, or 1 or 10 mg/kg of the anti-TNF monoclonal, cA2, were determined by double-antibody sandwich ELISA as described in Methods. The MMP levels were tested in samples taken before infusion and at weekly intervals after infusion. A summary of these results is shown in the above table with all measurements in ng/ml. Statistical differences between the median value and pre-infusion value were determined by the Wilcoxon rank test, and adjusted by the Bonferroni correction.

**$**P < 0.01; $***P < 0.001; ns, not significant.
day 7 and at all other subsequent time points. Thus, at day 7 in the 10 mg/kg cA2-treated group, a significant decrease \((P < 0.012)\) from the pre-infusion median level of 87 ng/ml to 34 ng/ml was observed, which was maintained at day 14 (41 ng/ml), day 21 (46 ng/ml) and day 28 (55 ng/ml) \((P < 0.004)\) (Table II). The MMP-3 level at day 7 was 41% of pre-infusion values (Fig. 2), and was significantly different \((P < 0.002)\) from the placebo group analysed by the Mann–Whitney U-test, and throughout the 28 day trial period: day 14 (to 43%, \(P < 0.001)\), day 21 (to 56%, \(P < 0.002)\), day 28 (to 62%, \(P < 0.001)\), respectively.

In the patients receiving 1 mg/kg cA2, the median pre-infusion level of 119 ng/ml reduced to 79 ng/ml \((P < 0.024)\) at day 7, 60 ng/ml \((P < 0.012)\) at day 14, 77 ng/ml \((P < 0.044)\) at day 21, and 81 ng/ml \((P < 0.032)\) at day 28 (Table II). These changes in MMP-3 levels were also significantly different from the placebo-treated group at each time point except day 28; thus at day 7 the MMP-3 level was 67% of pre-infusion values \((P < 0.017)\), 53% at day 14 \((P < 0.0038)\), and 75% at day 21 \((P < 0.02)\).

**Correlations of MMP levels with clinical and biochemical parameters of disease activity**

The MMP-1 and MMP-3 levels in sera were compared to each other, and also with clinical (swollen joint count) and laboratory parameters (CRP) routinely used to assess disease status (Table III). Pre-infusion MMP-3 and MMP-1 values correlated significantly with each other \((P < 0.05)\) and, in addition, MMP-3 (but not MMP-1) correlated with pre-infusion levels of CRP \((P < 0.05)\) as previously reported [17, 20]. Neither MMP-1 nor MMP-3 pre-infusion values correlated with swollen joint score count. The correlation between CRP and MMP-3 levels was maintained throughout the post-infusion period at day 7 \((r = 0.409, P < 0.001)\), day 14 \((r = 0.343, P < 0.02)\), day 21 \((r = 0.519, P < 0.001)\) and day 28 \((r = 0.494, P < 0.001)\). Interestingly, although the swollen joint score count did not correlate with MMP-3 levels before infusion, this parameter did correlate at all times post-infusion in the cA2-treated groups \((P < 0.02 day 7; P < 0.02 day 14; P < 0.05 day 21; P < 0.05 day 28)\). MMP-1 levels did not correlate with CRP levels in the pre-infusion samples or in the post-infusion sera, with the exception of day 28 \((r = 0.302, P < 0.05)\). However no correlation between MMP-1 and swollen joint count score was noted at any time before or after cA2 infusion.
To analyse further the association between serum MMP levels and the clinical response, the data from the patients treated with 1 or 10 mg/kg cA2 were pooled and then subdivided according to their 20% Paillus response 4 weeks after infusion of cA2. The median MMP-1 level at week 4 (expressed as a percentage of the pre-infusion value) in the 20% Paillus responder group was 92.6%, and was no different from the non-responder group (93.51%). For MMP-3, despite a difference in median values in the responder (63%) vs the non-responder group (71.3%), this difference was not significant (data not shown).

Levels of TIMP-1 in RA patients treated with cA2

As the endogenous inhibitor of MMPs has been reported to be upregulated in inflammation, it was of interest to determine whether this was also reflected in circulating TIMP-1 levels. Thus, in a preliminary investigation (data not shown), the TIMP-1 concentration of plasma obtained from RA patients in the open-label trial was determined. The pre-infusion plasma from the patients (n = 7) had TIMP-1 levels (248–493 ng/ml) above the normal range (142–198 ng/ml). Using the Wilcoxon rank U-test for non-parametric samples, we compared the median pre-infusion values with each sample after cA2 infusion for 12 weeks. The median pre-infusion level in plasma was 303 ng/ml which fell maximally to 212 ng/ml (72% of pre-infusion values) 2 weeks after cA2 infusion (P < 0.035). This reduction was maintained at week 3 (P = 0.036), but levels increased thereafter and were not significantly different from the pre-infusion values (data not shown).

DISCUSSION

The MMP enzymes are generally considered to play a role in the pathogenesis of RA, based on their local expression in the synovial joint and their capacity to destroy connective tissue components. As the pro-inflammatory cytokine TNF-α is a potent transcriptional inducer of MMPs [4, 33], it was of interest to determine whether serum levels of these enzymes, which are elevated in RA, were reduced following treatment with the chimaeric neutralizing anti-TNF-α antibody cA2. We focused on MMP-1 (collagenase) and MMP-3 (stromelysin) as these two MMPs, in particular, have been linked to the connective tissue changes which occur in this disease, and their serum levels correlate with disease activity. In our study we confirmed the previous observation [17–20] that compared with normal sera, MMP-3 (>5-fold), but also MMP-1 (1.5- to 2-fold) levels were elevated in RA sera. Following cA2 therapy, MMP-3 levels fell rapidly and in the first sample collected 7 days post-infusion were 40% of the pre-infusion values in the 10 mg/kg cA2 treatment group. The decrease in MMP-3 levels was significant throughout the study period (up to 4 weeks) and was apparent in both the high (10 mg/kg) and low (1 mg/kg) cA2 group. In this respect, the rapid modulation of serum MMP-3 levels was similar to that observed with many other parameters of disease activity, including IL-6 and CRP levels [30]. We do not know whether decreases in serum MMP-3 levels would have been apparent earlier, as samples prior to the 7 day time point were not available.

In common with MMP-3, we also observed a decrease in MMP-1 levels in the cA2-treated group, although this was only really apparent in the 10 mg/kg cA2 treatment group and was not evident until 14 days after infusion where a small, but significant reduction to 85% of pre-infusion MMP-1 levels was observed compared with the placebo group. At this stage, it is not clear why there is a difference between MMP-3 and MMP-1 with respect to the kinetics of reduction following cA2 treatment, but it may suggest that TNF-α is more directly involved with the regulation of MMP-3 than with MMP-1. In support of this hypothesis was the observation that in the cA2-treated patients the levels of MMP-3, but not of MMP-1, correlated with CRP and swollen joint count, two parameters which are significantly downregulated.

### TABLE III

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</tbody>
</table>

Total MMP-1 and MMP-3 levels at day 0, 7, 14, 21 and 28 were correlated with C-reactive protein (CRP) values and swollen joint count scores (SJC) as described in Methods. Correlation values which are statistically significant are denoted *P < 0.05, **P < 0.01 and ***P < 0.001, respectively.
following cA2 treatment [31]. The decrease in MMP-1 levels, which did not occur until 2 weeks after cA2 infusion, may be indirect, mediated through reduced cell trafficking to the joint which has recently been observed in the patients receiving cA2 treatment [34, 35].

We also included in this study a preliminary investigation of plasma TIMP levels following cA2 treatment. TIMPs are thought to control the activity of MMPs in tissue remodelling, but in destructive joint diseases such as RA the destruction of the extracellular matrix may be due to an imbalance between the metalloproteinase αs TIMP levels. In the literature, there are a number of conflicting reports on the effect of pro-inflammatory cytokines such as TNF-α and IL-1 on TIMP-1 production, and although some of these differences could be accounted for by different cell types, in other studies where differences regarding IL-1 levels on TIMP-1 production have been observed in the same cell types (e.g. RA explants) this clearly is not the case. We speculated that if TIMP levels were elevated in RA, these levels would be reduced following cA2 treatment, in common with our previous unpublished observation regarding the downmodulation of other endogenous regulators elevated in RA, such as the IL-1αa and soluble TNF receptors.

In order to measure circulating TIMP-1 levels accurately, it was necessary to use plasma preparations as platelets are a very rich source of TIMP-1, and differences observed between serum and plasma samples have previously been shown to be due to release of TIMP-1 by activated platelets during clotting. For this reason, we could not use the serum samples from the second, placebo-controlled trial to measure TIMP-1 levels as a large proportion of the TIMP-1 detected would be due to release from activated platelets. EDTA-plasma samples taken from the earlier phase I/II trial were used to measure TIMP-1 to determine whether anti-TNF-α treatment had any modulatory effect on TIMP-1. This trial was also chosen for testing as the RA patients received a high dose of cA2 and displayed a very good clinical improvement [30].

The TIMP-1 levels in the pre-infusion plasma samples of the RA patients were ~2-fold higher than those in normals. Following anti-TNF-α therapy, a decrease in these levels resulted, which reached significance 2 weeks after infusion, remained decreased at week 3, and thereafter gradually returned to the pre-infusion values. In this preliminary investigation, the results indicate that TIMP-1 levels are indeed modulated by anti-TNF-α therapy, indicating that TNF-α is in part responsible for elevated TIMP-1 levels. However, regulation by other cytokines, including those with anti-inflammatory or immunoregulatory potential such as IL-10 which can upregulate TIMP-1 expression [36], cannot be ruled out. Secondly, it was apparent from the data (not shown) that the TIMP-1 levels range widely between individual patients. This may reflect real differences between the patients, or it may indicate than even in EDTA-plasma there is some degree of platelet lysis. In support of this observation, we have compared TIMP-1 levels in normal individuals extracted with EDTA or citrate and have found that extraction of plasma into citrate media containing aspirin minimizes platelet lysis, with the result that in normal plasma the TIMP-1 levels fall within a tight range (data not shown). Currently, in a separate investigation, we are determining the modulation of circulating TIMP-1 following cA2 therapy using citrate/aspirin-extracted plasma.

In conclusion, we have demonstrated in this study that short-term treatment with cA2 leads to a significant and maintained reduction in serum MMP-3 and, to a lesser extent, MMP-1 levels. Although the percentage change in MMP-3 levels following cA2 therapy correlated with biochemical measures of inflammation such as CRP, it is clearly of importance to determine whether these reductions also mirror changes in the levels of active MMPs in the joint and in tissue damage and joint destruction. Previously, it had been demonstrated that serum MMP-3 levels in RA correlated strongly with CRP and ESR, whereas no correlation was found with radiological or functional joint scores [17]. Thus, it is not known whether serum MMP-3 levels merely reflect inflammatory episodes in RA, or whether MMP-3 and/or MMP-1 levels also reflect tissue destruction within the synovial joints. These questions could not be answered in the short-term study described in this paper, but it is intended to investigate this question in a longer term trial which has recently been completed. Such a study will enable us to compare the degree to which MMP-3 and MMP-1 levels change in individual patients following cA2 therapy over a 6 month period, and to compare this with X-ray changes and the number of erosions in the joints.

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