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Inhibition of ADAMTS4 (aggrecanase-1) by tissue inhibitors of metalloproteinases (TIMP-1, 2, 3 and 4)

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Abstract ADAMTS4 (aggrecanase-1) is considered to play a key role in the degradation of aggrecan in arthritides. The inhibitory activity of tissue inhibitors of metalloproteinases (TIMPs) to ADAMTS4 was examined in an assay using aggrecan substrate. Among the four TIMPs, TIMP-3 inhibited the activity most efficiently with an IC_{50} value of 7.9 nM, which was at least 44-fold lower than that of TIMP-1 (350 nM) and TIMP-2 (420 nM) and >250-fold less than that of TIMP-4 (2 μM for 35% inhibition). These results suggest that TIMP-3 is a potent inhibitor against the aggrecanase activity of ADAMTS4 in vivo.

Key words: Tissue inhibitor of metalloproteinases; A disintegrin and metalloproteinase; A disintegrin and metalloproteinase with thrombospondin motifs 4; Aggrecanase; Aggrecan degradation

1. Introduction

ADAM (a disintegrin and metalloproteinase) is a recently discovered gene family, and involved in various biological events including fertilization [1,2], myogenesis [3], cytokine shedding [4,5] and extracellular matrix (ECM) degradation [6–8] under pathophysiological conditions. The members can be classified into two groups according to their domain structures: membrane type ADAM and ADAM with thrombospondin motifs (ADAMTS) [9]. Among 10 members of the ADAMTS subgroup, ADAMTS4, also called aggrecanase-1, is notable in that it cleaves aggrecan, a major proteoglycan in cartilage, at the five specific sites of the core protein including the Glu^{373}–Ala^{374} bond in the G1–G2 interglobular domain [8]. Because of the expression and aggrecanase activity of the proteinase, ADAMTS4 is believed to play a key role in the destruction of articular cartilage through aggrecan degradation in human arthritides such as rheumatoid arthritis and osteoarthritis [10–12]. However, no information is available for the regulators of the aggrecanase activity of ADAMTS4 in vivo. Tissue inhibitors of metalloproteinases composed of four different molecules (TIMP-1, 2, 3 and 4) were originally cloned as inhibitors of matrix metalloproteinases (MMPs) [13], but recent studies have shown that TIMP-1 weakly inhibits the activity of ADAMTS4 [6,7]. However, little is known about the inhibitory activity of other TIMPs, i.e. TIMP-2, 3 and 4, to the proteinase.

In the present study, we examined the inhibitory activity of TIMP-1, 2, 3 and 4 against the purified recombinant human ADAMTS4. The data demonstrate that TIMP-3 most efficiently inhibits the aggrecanase activity of ADAMTS4 among the four TIMPs.

2. Materials and methods

2.1. Materials

Recombinant human TIMP-1 and TIMP-2 were purified as described previously [14]. Human TIMP-3 was also purified from placentas with ion-exchange and immunoaffinity column chromatography by modification of the method described by Apte et al. [15]. Mouse TIMP-4, which is 93% identical to human TIMP-4, was isolated from *Escherichia coli* [16] and provided by Dr. Vera Knäuper and Dr. Gillian Murphy (University of East Anglia, Norwich, UK). A polyclonal antibody (I19C) specific to the neo-epitope ($NITEGE^{373}$) of the ~80 kDa aggrecan fragment generated by the cleavage at the Glu^{373}–Ala^{374} bond with ADAMTS4 was prepared as described previously [17].

2.2. Purification of recombinant ADAMTS4

Recombinant human ADAMTS4 was purified according to our method [18]. Briefly, ADAMTS4 expression vector with the FLAG epitope tag added to the COOH-terminus was transfected into COS-7 cells. The culture media were harvested 3 days after the transfection, concentrated by ultrafiltration and subjected to anti-FLAG M2 affinity column chromatography (Sigma-Aldrich Corp., St. Louis, MO, USA). Recombinant ADAMTS4 was eluted with 6 M urea, dialyzed against 50 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 10 mM $CaCl_2$, 0.05% Brij-35 (TNCB buffer) and stored at 4°C before use. The purified ADAMTS4 showed a single band of 69 kDa on the silver-stained gel after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Concentration of the proteinase was determined using BCA protein assay reagents (Pierce Chemical Co., Rockford, IL, USA).

2.3. Aggrecan preparation

Bovine nasal cartilage (~10 g) was sliced and homogenized in 140 ml of 4 M guanidine–HCl in 50 mM sodium acetate (pH 5.8) containing proteinase inhibitors cocktail (Complete, Roche Diagnostics, Mannheim, Germany). Aggrecan was extracted from the cartilage for

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Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; CaPPS, calcium pentosan polysulfate; CBB, Coomassie brilliant blue R-250; MMP, matrix metalloproteinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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36 h at 20°C, and isolated by cesium chloride (1.56 mg/ml) density-gradient centrifugation at 100 000×*g* for 48 h at 20°C. Purified aggrecan was dialyzed against water, lyophilized and stored at −20°C.

2.4. Detection of aggrecanase activity and its inhibition by TIMPs

Aggrecan (100 μg) was first incubated with ADAMTS4 (4, 8, 16 or 32 nM at a final concentration) in TNCB buffer for 24 h at 37°C. The digestion products were treated with 0.025 units of chondroitinase ABC and 0.01 unit of keratanase (Seikagaku Corp., Tokyo, Japan) and subjected to SDS–PAGE (8 or 10% total acrylamide) under reducing condition. The gels were stained with 0.1% Coomassie brilliant blue R-250 (CBB) or silver nitrate. The proteins in the gels were also transferred onto nitrocellulose filters, and the digestion fragment by ADAMTS4 was detected by the I19C antibody according to the avidin-biotin-peroxidase complex method [17]. Time course digestion of aggrecan (100 μg) was performed by incubation with ADAMTS4 (8 nM at a final concentration) for 0–24 h, and the appropriate incubation time was determined.

For the inhibitor study with TIMPs, ADAMTS4 (8 nM) was incubated with TIMP-1, 2, 3 or 4 (1, 5, 10, 50, 100, 250, 500 or 1000 nM at a final concentration) for 2 h at 37°C before the assay. Aggrecan was then digested with the mixtures of ADAMTS4 and each TIMP for 8 or 12 h at 37°C, and the reaction was stopped with 20 mM EDTA. The samples were subjected to SDS–PAGE (10% total acrylamide) under reducing condition after deglycosylation, and the proteins transferred onto nitrocellulose filters. The digestion fragment of ∼80 kDa was detected by the neo-epitope specific antibody (I19C) as described above. They were also applied to SDS–PAGE (5% total acrylamide) to observe the processing of the intact aggrecan core protein into the ∼250-kDa fragment, and the gels stained with 0.1% CBB. Densities of immunoreactive bands with I19C antibody and CBB-stained intact aggrecan core protein were measured by scanning densitometry using NIH Image 1.62.

3. Results

When aggrecan (100 μg) was fully digested with a high concentration of ADAMTS4 (32 nM) for 24 h, aggrecan core protein was processed into the two major fragments of ∼250 kDa and ∼120 kDa (Fig. 1, lane 2). A minor fragment of ∼80 kDa was recognized after silver staining (data not shown), and detected by the I19C antibody (Fig. 2A), which

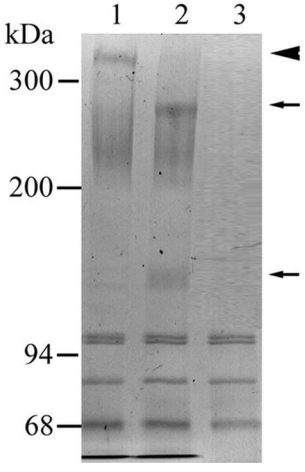

Fig. 1. Aggrecan degradation by ADAMTS4. Aggrecan (100 μg) was incubated in the presence and absence of ADAMTS4 (32 nM) for 24 h, and the digestion products were analyzed by SDS–PAGE (8% total acrylamide) after the deglycosylation with chondroitinase ABC and keratanase. Lane 1, aggrecan incubated with buffer alone and deglycosylated; lane 2, aggrecan digested with ADAMTS4 and deglycosylated; lane 3, chondroitinase ABC and keratanase alone. Arrowhead and arrows indicate intact aggrecan core protein and fragments of ∼250 kDa and ∼120 kDa, respectively.

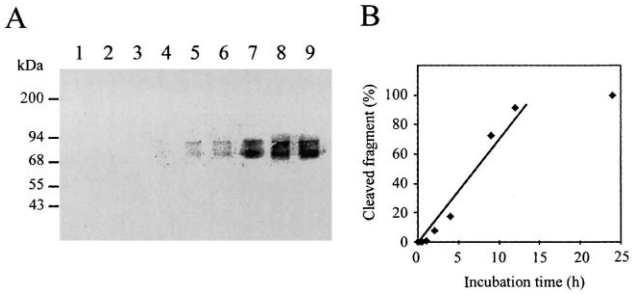

Fig. 2. Time course digestion of aggrecan by ADAMTS4. Aliquots of aggrecan (100 μg) were incubated with ADAMTS4 (8 nM) for 0 (lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), 8 h (lane 7), 12 h (lane 8) or 24 h (lane 9), and the digestion products were analyzed by immunoblotting using a neo-epitope specific antibody as described in Section 2. A: Immunoblotting of the digestion products. B: Densitometric analysis of immunoreactive fragments.

reacts with the neo-epitope (NITEGE^{373}) of the fragment generated by cleavage at the Glu^{373}–Ala^{374} bond [17]. To determine the appropriate incubation time for the inhibition study with TIMPs, aggrecan (100 μg) was incubated with ADAMTS4 (8 nM) for various times ranging from 0 to 24 h, and the specific band of ∼80 kDa immunoreactive with the I19C antibody was monitored by densitometry. As shown in Fig. 2A, the band appeared after a 2-h incubation. Densitometric analysis indicated that the intensity of the band increases linearly with time up to 12 h and then reached a plateau after a 24-h incubation (Fig. 2B).

Inhibition activity of TIMP-1, 2, 3 and 4 against the aggrecan-degrading activity of ADAMTS4 was measured by the densitometric analysis of the immunoreactive band of ∼80 kDa after the digestion of aggrecan (100 μg) with ADAMTS4 (8 nM) in the presence of TIMPs (0, 1, 5, 10, 50, 100, 250, 500 or 1000 nM). As shown in Fig. 3A, all the TIMPs except for TIMP-4 completely inhibited the generation of the band at different concentrations: 500, 1000 and 100 nM for TIMP-1, 2 and 3, respectively. In contrast, the complete inhibition was not obtained with 1000 nM TIMP-4 (Fig. 3A) or even at a

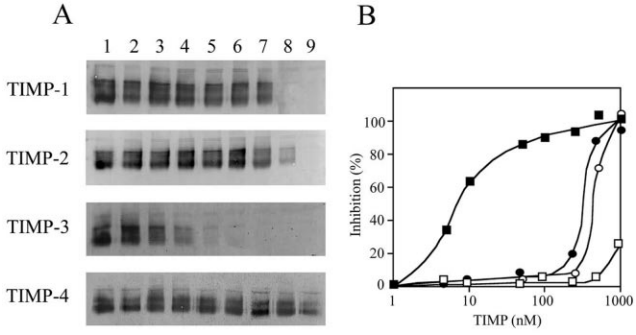

Fig. 3. Analysis of the inhibitory activity of TIMPs by immunoblotting. Aliquots of aggrecan (100 μg) were incubated with mixtures of ADAMTS4 (8 nM) and various concentrations of TIMP-1, 2, 3 or 4 for 12 h, and the digestion products were analyzed by immunoblotting with an antibody as described in Section 2. A: Immunoblotting of the digestion products. Lanes 1–9, aggrecan digested with the mixtures of ADAMTS4 and each TIMP (0, 1, 5, 10, 50, 100, 250, 500 and 1000 nM, respectively). B: Densitometric analysis of the immunoreactive bands. The percent inhibition (mean of the duplicate experiments) of ADAMTS4 activity with TIMP-1 (●), TIMP-2 (○), TIMP-3 (■) or TIMP-4 (□) is plotted.

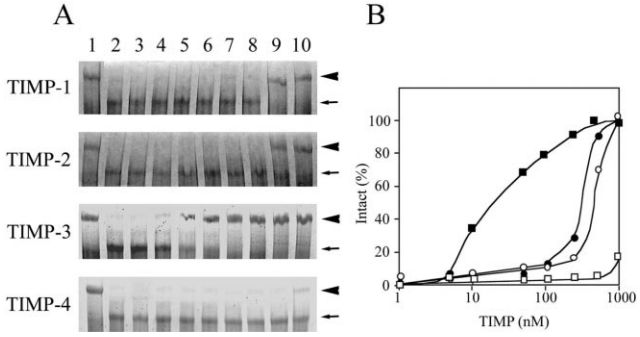

Fig. 4. Analysis of the inhibitory activity of TIMPs to the processing of intact aggrecan core protein. Aliquots of aggrecan (100 μg) were incubated with the mixtures of ADAMTS4 (8 nM) and various concentrations of TIMP-1, 2, 3 or 4 for 12 h, and the processing of the intact aggrecan core protein (arrowhead) into the ~250-kDa fragment (arrow) was monitored by densitometric analysis as described in Section 2. A: CBB-stained gels. Lane 1, aggrecan alone; lanes 2–10, aggrecan digested with mixtures of ADAMTS4 and each TIMP (0, 1, 5, 10, 50, 100, 250, 500 and 1000 nM, respectively). B: Densitometric analysis of the remained intact core protein. The percent inhibition (mean of the duplicate experiments) of ADAMTS4 with TIMP-1 (●), TIMP-2 (○), TIMP-3 (■) or TIMP-4 (□) is plotted.

concentration of 2000 nM (data not shown). When the data measured by the densitometric analysis were plotted, S-shaped inhibition curves were obtained for TIMP-1, 2 and 3 (Fig. 3B). Based on the curves, IC_{50} values (concentration at 50% inhibition) were determined to be 350, 420 and 7.9 nM for TIMP-1, 2 and 3, respectively. On the other hand, IC_{50} for TIMP-4 could not be obtained, since 1000 and 2000 nM TIMP-4 inhibited only 20 and 35% of the activity, respectively (Fig. 3A for 1000 nM TIMP-4 and data not shown for 2000 nM TIMP-4). When inhibition of the processing of the intact aggrecan core protein by TIMPs was monitored, very similar inhibitor profile was obtained. Almost complete inhibition was seen at the concentrations of 500, 500 and 100 nM of TIMP-1, 2 and 3, respectively, but only weak inhibition was detected with TIMP-4 (Fig. 4A). IC_{50} values were roughly calculated as 300, 400 and 18 nM for TIMP-1, 2 and 3, respectively (Fig. 4B). However, they appeared not to be accurate compared with those obtained by the analysis of the ~80-kDa immunoreactive band, since the difference with the densities of the intact core protein was subtle.

4. Discussion

We have demonstrated that the aggrecanase activity of ADAMTS4 is inhibited by TIMPs. Previous studies have shown that TIMP-1 weakly inhibits aggrecanase activity of ADAMTS4 with an IC_{50} value of 210 nM [6,7]. Since this value is close to that obtained in the present study (350 nM for TIMP-1), our study confirms the data. However, the present study further showed that TIMP-2 is also capable to inhibiting ADAMTS4 with a similar IC_{50} value (420 nM) to that of TIMP-1. This is different from the previous data which suggested that ADAMTS4 was insensitive to TIMP-2 at concentrations up to 1 μM [6]. Although the reason for the discrepancy is not clear, this may be because in the previous study culture media containing the proteinase were used for the experiment [6]. On the other hand, the present study has demonstrated for the first time that TIMP-3 most efficiently inhibits the aggrecanase activity of ADAMTS4. Judging from its IC_{50} value (7.9 nM), the inhibitory activity of TIMP-3 is about 50-fold higher than TIMP-1 and TIMP-2, and >250-fold stronger than TIMP-4. This suggests that TIMP-3 is a potent inhibitor of ADAMTS4.

The molecular mechanism of the preferable inhibition of ADAMTS4 by TIMP-3 is not apparent at present. However, recent biochemical studies have indicated that TIMP-3 can inhibit the activities of membrane-type ADAMs including ADAM-10, ADAM-12 and ADAM-17 (TNF α converting enzyme (TACE)) [16,19,20]. Thus, it is reasonable to think that TIMP-3 has some structural features that are critical to the inhibition of the activities of ADAM members, although the key structural element remains unknown. On the other hand, one of the most interesting characters of TIMP-3 is that the N-terminal domain has binding affinity to sulfated glycosaminoglycans [21]. This character may be suitable for TIMP-3 to be well-positioned to the cell surface through the interaction with cell membrane heparan sulfate proteoglycans such as syndecans [22] and to inhibit the membrane-type ADAMs responsible for the shedding of the membrane proteins. Since aggrecan is endowed with a large amount of chondroitin sulfate glycosaminoglycans, it is conceivable that the sulfated chains may also be involved in the efficient inhibitory activity of TIMP-3 to ADAMTS4 through the interference with the access of the proteinase to aggrecan.

The previous studies have demonstrated that the aggrecan fragments generated by the action of ADAMTS4 are commonly detected in the joint fluids in rheumatoid arthritis and osteoarthritis [23] and ADAMTS4 is expressed by the chondrocytes in the diseased cartilages [24], suggesting that this metalloproteinase plays an important role in the cartilage destruction in such joint diseases [13]. Thus, endogenous inhibitor(s) of ADAMTS4 would be one of the key regulators to prevent the cartilage destruction. Since the present data suggest that of the four TIMPs, TIMP-3 is a potent inhibitor in vivo, the up-regulation of TIMP-3 production may be beneficial to the retardation of joint destruction in arthritides. We have recently reported that TIMP-3 is expressed by rheumatoid synovial lining cells and its production is enhanced at the post-transcriptional level by the treatment with calcium pentosan polysulfate (CaPPS), an anti-arthritic agent [25]. CaPPS is also known to inhibit loss of glycosaminoglycans from articular cartilage in the explant cultures [26], and it increases TIMP-3 production in osteoarthritic chondrocytes (Takizawa et al., unpublished data). Thus, these data suggest the possibility of the introduction of a new chondroprotective therapy in human joint diseases by increasing the TIMP-3 levels in the local joint tissues such as articular cartilage.

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References

[1] Cho, C., Bunch, D.O., Faure, J.E., Goulding, E.H., Eddy, E.M., Primakoff, P. and Myles, D.G. (1998) Science 281, 1857–1859.
[2] Chen, M.S., Tung, K.S., Coonrod, S.A., Takahashi, Y., Bigler, D., Chang, A., Yamashita, Y., Kincade, P.W., Herr, J.C. and White, J.M. (1999) Proc. Natl. Acad. Sci. USA 96, 11830–11835.
[3] Yagami-Hiromasa, T., Sato, T., Kurisaki, T., Kamijo, K., Nabeshima, Y. and Fujisawa-Sehara, A. (1995) Nature 377, 652–656.
[4] Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P.,

Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J. and Cerretti, D.P. (1997) Nature 385, 729–733.
[5] Peschon, J.J., Slack, J.L., Reddy, P., Stocking, K.L., Sunnarborg, S.W., Lee, D.C., Russell, W.E., Castner, B.J., Johnson, R.S., Fitzner, J.N., Boyce, R.W., Nelson, N., Kozlosky, C.J., Wolfson, M.F., Rauch, C.T., Cerretti, D.P., Paxton, R.J., March, C.J. and Black, R.A. (1998) Science 282, 1281–1284.
[6] Arner, E.C., Pratta, M.A., Trzaskos, J.M., Decicco, C.P. and Tortorella, M.D. (1999) J. Biol. Chem. 274, 6594–6601.
[7] Tortorella, M.D., Burn, T.C., Pratta, M.A., Abbaszade, I., Hollis, J.M., Liu, R., Rosenfeld, S.A., Copeland, R.A., Decicco, C.P., Wynn, R., Rockwell, A., Yang, F., Duke, J.L., Solomon, K., George, H., Bruckner, R., Nagase, H., Itoh, Y., Ellis, D.M., Ross, H., Wiswall, B.H., Murphy, K., Hillman Jr., M.C., Hollis, G.F. and Arner, E.C. (1999) Science 284, 1664–1666.
[8] Tortorella, M.D., Pratta, M., Liu, R.Q., Austin, J., Ross, O.H., Abbaszade, I., Burn, T. and Arner, E.C. (2000) J. Biol. Chem. 275, 18566–18573.
[9] Kaushal, G.P. and Shah, S.V. (2000) J. Clin. Invest. 105, 1335–1337.
[10] Sandy, J.D., Flannery, C.R., Neame, P.J. and Lohmander, L.S. (1992) J. Clin. Invest. 89, 1512–1516.
[11] Lohmander, L.S., Neame, P.J. and Sandy, J.D. (1993) Arthritis Rheum. 36, 1214–1222.
[12] Fosang, A.J., Last, K., Stanton, H., Weeks, D.B., Campbell, I.K., Hardingham, T.E. and Hembry, R.M. (2000) J. Biol. Chem. 275, 33027–33037.
[13] Okada, Y. (2001) Kelly's Textbook of Rheumatology, 6th edn. (Ruddy, S., Harris, E.D., Jr., Sledge, C.B., Eds.), pp. 55–72, W.B. Saunders, Philadelphia, PA.
[14] Hayakawa, T., Yamashita, K., Ohuchi, E. and Shinagawa, A. (1994) J. Cell Sci. 107, 2373–2379.
[15] Apte, S.S., Olsen, B.R. and Murphy, G. (1995) J. Biol. Chem. 270, 14313–14318.
[16] Amour, A., Knight, C.G., Webster, A., Slocombe, P.M., Stephens, P.E., Knäuper, V., Docherty, A.J. and Murphy, G. (2000) FEBS Lett. 473, 275–279.
[17] Sugimoto, K., Takahashi, M., Yamamoto, Y., Shimada, K. and Tanzawa, K. (1999) J. Biochem. Tokyo 126, 449–455.
[18] Nakamura, H., Fujii, Y., Inoki, I., Sugimoto, K., Tanzawa, K., Matsuki, H., Miura, R., Yamaguchi, Y. and Okada, Y. (2000) J. Biol. Chem. 275, 38885–38890.
[19] Loechel, F., Fox, J.W., Murphy, G., Albrechtsen, R. and Wewer, U.M. (2000) Biochem. Biophys. Res. Commun. 278, 511–515.
[20] Amour, A., Slocombe, P.M., Webster, A., Butler, M., Knight, C.G., Smith, B.J., Stephens, P.E., Shelley, C., Hutton, M., Knäuper, V., Docherty, A.J. and Murphy, G. (1998) FEBS Lett. 435, 39–44.
[21] Yu, W.H., Yu, S.C., Meng, Q., Brew, K. and Woessner Jr., J.F. (2000) J. Biol. Chem. 275, 31226–31232.
[22] Fitzgerald, M.L., Wang, Z., Park, P.W., Murphy, G. and Bernfield, M. (2000) J. Cell Biol. 148, 811–824.
[23] Fosang, A.J., Last, K. and Maciewicz, R.A. (1996) J. Clin. Invest. 98, 2292–2299.
[24] Flannery, C.R., Little, C.B., Hughes, C.E. and Caterson, B. (1999) Biochem. Biophys. Res. Commun. 260, 318–322.
[25] Takizawa, M., Ohuchi, E., Yamanaka, H., Nakamura, H., Ikeda, E., Ghosh, P. and Okada, Y. (2000) Arthritis Rheum. 43, 812–820.
[26] Munteanu, S.E., Ilic, M.Z. and Handley, C.J. (2000) Arthritis Rheum. 43, 2211–2218.