Virchows Arch (1998) 433:153–160 

ORIGINAL ARTICLE

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# Immunohistochemical localization of metallothionein in synovial tissue of patients with chronic inflammatory and degenerative joint disease

Received: 25 July 1997 / Accepted: 23 March 1998

**Abstract** Metallothioneins (MTs) are low-molecular-weight cytosolic proteins, which are thought to participate in metal homeostasis and protection against metal toxicity and oxidative stress. MT synthesis can be induced by a variety of inflammatory mediators and antirheumatic drugs, and high levels of MT have been implicated in resistance of cells to some antirheumatic drugs. We studied the expression and localization of MT in synovial tissue samples from patients with rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis or osteoarthritis (OA) by quantitative immunohistochemistry. Immunostaining for MT was detected in a large number of intimal lining cells in most of the investigated synovial tissue samples (75%). In a smaller proportion of samples (42%), some of the fibroblast-like cells of the subsynovial layer were also MT positive. Immunostaining and double-staining experiments with antibodies against monocyte-, macrophage- and leucocyte-associated antigens suggested that most of the MT-positive cells were intimal fibroblast-like cells and subsynovial fibroblasts. However, there were no statistically significant differences in the intensity of staining for MT between the rheumatic diseases and OA at the single-cell level. Thus, MT is expressed in synovial tissue and may participate in homeostatic and protective functions. The inter-individual variability in the expression of MT in synovial tissue may be related to the therapeutic efficacy of the gold compounds and chemotherapeutic antirheumatic drugs sequestered by MT.

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**Key words** Metallothionein · Immunohistochemistry · Synovial tissue · Rheumatic diseases

## Introduction

Metallothionein (MT) is a low-molecular-weight protein with a high content of cysteine and metals, including cadmium, zinc (Zn) and copper (Cu) [13]. It is expressed in most vertebrate tissues and seems to be localized in both the cytoplasm and the nuclei of the cells [4, 9], but the physiological significance of MT is not understood. However, its ability to sequester and dispense metal ions is thought to have an important role in regulation of cellular homeostasis and functions of the essential trace elements Zn and Cu and in protection against toxicity of heavy metals, particularly cadmium [6]. In addition, MT seems to promote the resistance of cells to some alkylating agents and chemotherapeutic metal compounds, such as cisplatin [7, 14, 32].

Interestingly, MT has also been implicated in protection against the cytotoxic effects of oxidative stress, because it is a potent hydroxyl radical scavenger [41, 55]. In vitro, MT protects cells against the cytotoxic and DNA-damaging effects of nitric oxide and the DNA-damaging effects of $H_2O_2$ [1, 9, 51], and also against radical-induced lipid peroxidation [37]. In keeping with the protective roles of MT, its biosynthesis can be induced by a wide variety of factors, including divalent heavy metals, several cytokines, growth factors, tumour promoters, glucocorticoid hormones and xenobiotics [12, 13, 46, 54].

MT may be important in rheumatic diseases because of its protective effects against oxidative damage; it has been suggested that oxidative stress plays a role in the pathophysiology of synovitis in rheumatic diseases [28, 36, 40]. The cytokines interleukin(IL)-1, IL-6 and IL-11, which are present in increased amounts in inflamed joints [2], can induce MT expression in cultured cells [11, 12, 50, 59], so that the expression of MT in synovial tissue might be elevated in inflammatory joint disease,

an idea supported by studies with cultured rheumatoid and normal synovial fibroblasts from two patients [27]. It has also been shown that MT synthesis can be induced by some drugs that are used to treat patients with rheumatic diseases [5, 27, 38, 39, 42, 50, 54], and induced synthesis of MT and subsequent protection against reactive oxygen species might in part explain the therapeutic effects of these drugs. However, it is known that gold and some antirheumatic chemotherapeutic agents bind to MT [15, 16, 49], and in this context the presence of MT in synovial tissue could mediate the resistance of synovial cells to the effects of these drugs [14–16, 25, 26, 32, 42, 43, 57].

MT genes are expressed at the mRNA level in human chondrocytes [58] and synoviocytes [39, 53], and MT protein has been detected in cultured human synovial fibroblasts [27]. However, there are no detailed studies on the expression and localization of MT in synovial tissue. Because of the possible roles of MT in rheumatic diseases, we were interested in the expression of MT in synovial tissue and in the possible difference in MT levels between patients with inflammatory joint disease and patients with osteoarthritis (OA). We have studied, by means of quantitative immunohistochemistry, the in vivo expression and localization of MT in synovial tissue samples from 81 patients with OA, rheumatoid arthritis (RA), ankylosing spondylitis (AS or Bechterew's disease) or psoriatic arthritis (PsA).

## Materials and methods

The material studied consisted of synovial tissue samples from 81 patients with OA ($n$=18), RA ($n$=37), PsA ($n$=16) or AS ($n$=10), retrieved from the files of the Department of Pathology of Robert-Bosch-Krankenhaus (Table 1). The patients were operated on during the years 1980–1990 in different departments of orthopaedic rheumatology. All RA cases fulfilled the criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) for definitive or classic RA [48]. AS, OA and PsA were diagnosed by clinical and radiographic criteria. The patient characteristics in the different disease groups corresponded well with the clinical diagnoses (Table 1). Following operation, samples were fixed in nonbuffered or buffered 4–10% formalin within 24–72 h and embedded in paraffin. From the paraffin wax blocks, 3-µm-thick sections were prepared and mounted on slides.

**Table 1** Clinical features (mean±SD) of the patients with osteoarthritis (*OA* rheumatoid arthritis, *RA* ankylosing spondylitis, *AS* or psoriatic arthritis, *PsA*; *ESR* erythrocyte sedimentation rate)

| Feature | Diagnostic group | | | |
|---|---|---|---|---|
| | OA | RA | AS | PsA |
| Female:male ($n$) | 14:4 | 32:5 | 3:7 | 4:12 |
| Age (years) | 66±11 | 56±14 | 49±12 | 42±11 |
| (Range) | (48–83) | (12–74) | (32–67) | (23–65) |
| Disease duration (years) | 7.0±6 | 9.7±7.1 | 9.0±6.5 | 8.5±7.0 |
| Operated joint ($n$) | | | | |
| Knee | 12 | 33 | 3 | 6 |
| Hip | 5 | | 6 | 5 |
| Ankle | | | 1 | 3 |
| Hand or finger | 1 | 4 | | 2 |
| ESR (mm/h) | 15±15 | 21±17 | 16±10 | 8±4 |

A monoclonal mouse IgG antibody raised against horse MT-1 and MT-2 isoforms (Dako-MT, E9, Dako, Carpinteria, Calif.) was used for immunostaining of the tissue. According to the manufacturer, this antibody is specifically and equally well inhibited by human, horse, sheep or rat MT-1 and MT-2. It is well suited to use on formalin-fixed paraffin-embedded tissue sections. The staining procedure was performed with a modification of the avidin–biotin–peroxidase complex (ABC) technique [20, 30]. For all samples the procedure was performed at the same time to avoid possible interday variation in the intensity of staining.

When the sections had been deparaffinized an enzyme pretreatment was done with 0.1% trypsin (Boehringer Mannheim, Germany) in 0.1% calcium chloride for 30 min at 37°C. The slides were incubated with the primary antibody against MT (1:50 dilution) for 18 h and with the secondary biotinylated horse antibody against mouse-IgG (Vector Laboratories, Burlingame, Calif.), diluted 1:200, for 30 min at room temperature in a humidified chamber. Thereafter, Vectastain ABC-Elite (Vector Laboratories) was used to localize the secondary antibody with a chromogen of oxidized diaminobenzidine (DAB). Positive immunoreactions appeared as dark brown staining on a blue background (counterstaining with haematoxylin).

To control for the specificity of immunostaining, the following experiments were carried out: omitting the primary antibody (1); omitting the secondary antibody (2); omitting the ABC complex (3); omitting (1) and (2); omitting (1) and (3); omitting (2) and (3); omitting (1), (2) and (3); a nonsense control using a primary antibody against *Candida albicans*; and a positive control using a primary antibody against human IgM.

To label marrow-derived cells in the synovial tissue, adjacent synovial tissue sections from the study patients were stained with antibodies against CD68 diluted 1:100, CLA (common leucocyte antigen or CD45) diluted 1:10, and lysozyme diluted 1:500 (Dako) [3]. CD68 is a monocyte- and macrophage-associated antigen [44], and CLA is present on the cell membrane of most cells derived from the haematopoietic stem cell and some cells of the mononuclear phagocyte system [35]. Lysozyme has been shown to be localized in polymorphonuclear leucocytes, monocytes, giant cells and some lining cells, probably type A synoviocytes, of synovial tissue [18]. In addition, a double-staining experiment for CD68 and MT was performed with a sequential method. First, the sections were stained with the monoclonal antibody against CD68 by using the ABC technique. Colour development was performed by DAB/$H_2O_2$ with addition of 6.8 mg imidazole, 2 ml 1% nickel acetate and 2.5 ml 1% $CoCl_2$ (to each 100 ml of incubation solution), which produced a black colour instead of brown. Thereafter, the sections were stained for MT using an APAAP method with one repetition. The APAAP complex was developed with New Fuchsin and Naphthol-AS-BI (red colour) as described previously [10, 19].

All the slides were evaluated by a blinded observer. First, the localization of staining was evaluated visually. Thereafter, to quantify metallothionein expression, the intensity of staining in the synovial lining cell layer, sublining layer and blood vessel endothelium was estimated as follows: 0, none; 1, slight; 2, moderate; 3, strong. The estimated percentages of positive cells in each layer were classified as follows: 0, none; 1, ≤30%; 2, 30–60% ; 3, ≥60%. The product of the scores for staining intensity and percentage of positive cells was defined as the immunoreactivity score (IRS), which could thus have values 0–4, 6 or 9.

To measure staining intensity in the lining cell layer of synovial tissue more objectively, staining was also quantified videodensitometrically using an image analysis workstation (Histoanalyzer) as described previously [52]. The equipment consisted of a Leitz Aristoplan microscope (Leitz, Germany) connected to a Sun Workstation with a Sony videocamera. The slides were measured through an objective (Fluotar, Leica) with 40-fold magnification and a numerical aperture of 0.7 and a condenser with a numerical aperture of 0.4. Shading and gamma correction were employed for

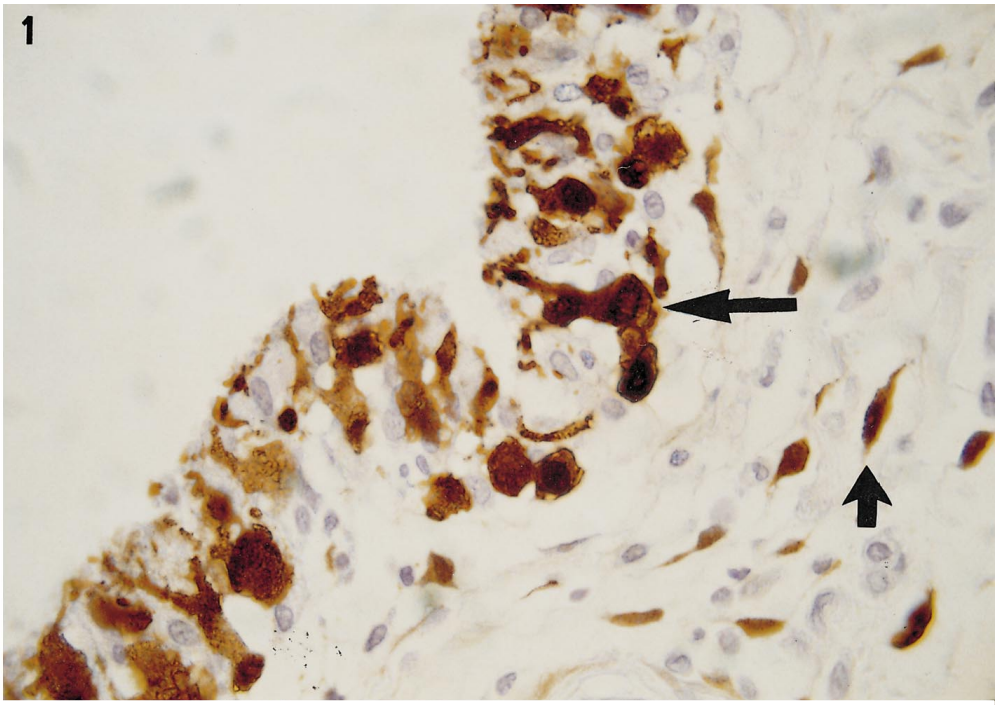


**Fig. 1** Immunohistochemical staining for metallothionein (MT) in a representative synovial tissue section (3 µm) from a patient with rheumatoid arthritis. A large number of MT-positive elongated cells (*long arrow*) with cytoplasmic processes in the synovial lining and some MT-positive fibroblast-like cells (*short arrow*) in the subsynovial layer. ×600

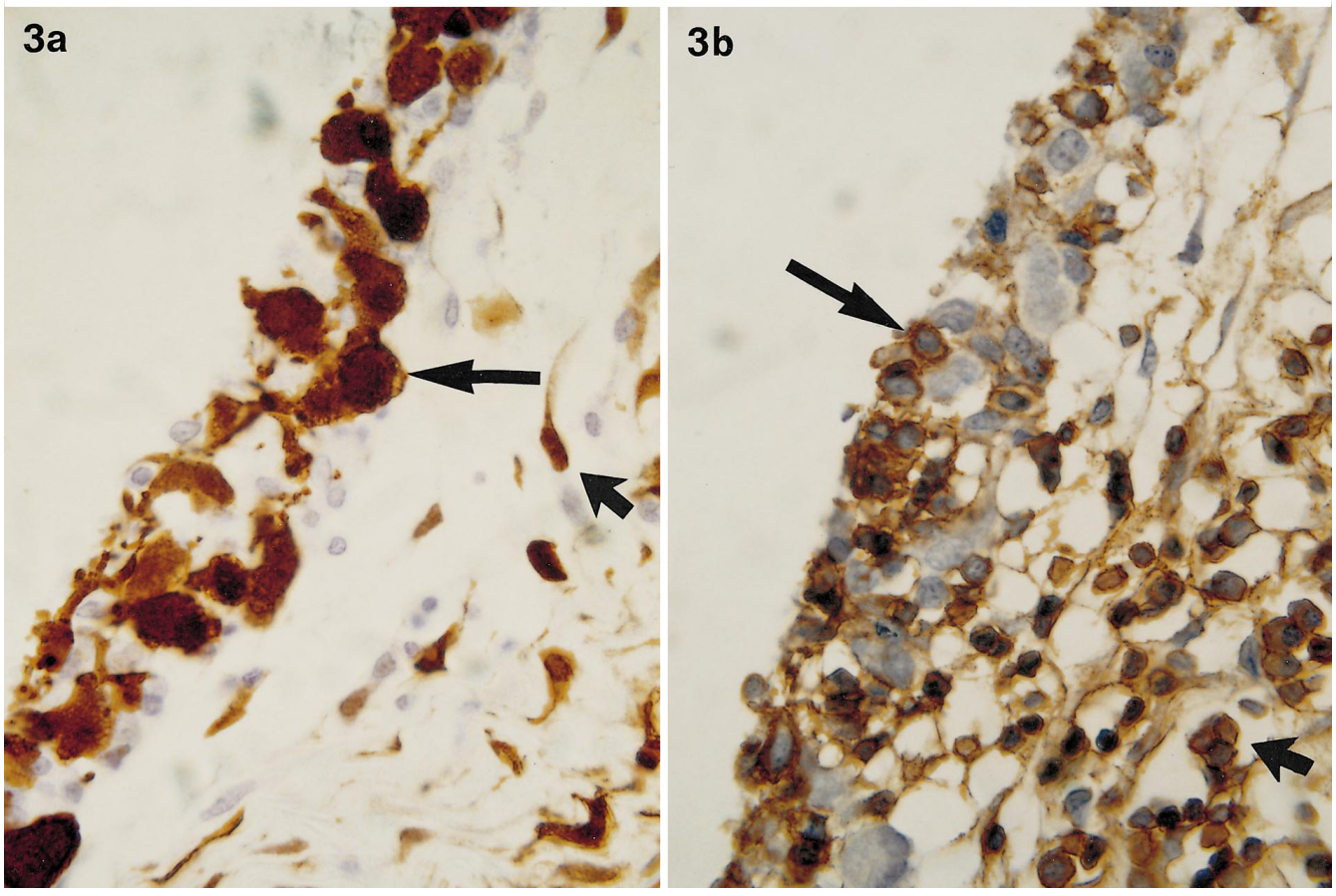


**Fig. 3** Immunohistochemical staining for **a** MT and **b** common leucocyte antigen (CLA) in a synovial tissue section from a patient with rheumatoid arthritis. **a** MT is localized in mainly basally located lining cells (*long arrow*) and some fibroblast-like subsynovial cells (*short arow*). **b** CLA is detected in rounded cells in the superficial parts of the synovial lining (*long arrow*) and a lymphoid infiltrate of the subsynovial layer (*short arrow*). The large MT-positive lining cells seem to be CLA-negative. ×600

stray light and camera correction. The area of interest on each slide was labelled with a cursor–mouse system under visual control. The staining intensity of the labelled area was given as the mean optical density per square micrometer (MOD) in the blue channel of the red/green/blue (RGB) camera signal. The mean of seven arbitrarily selected areas of the lining cell layer was used as the result after subtraction of the MOD of a nonimmunostained background area (not covered by tissue).

The sections were classified according to previously established histological classification criteria into four distinct morphological types of synovitis: (1) sero-fibrous, (2) lympho-plasmacytic, (3) ulcerous and (4) fibroblastic transformed synovitis [23]. In addition, the acute (amount of neutrophiles and fibrin) and chronic (lymphocytes and plasma cells) inflammatory reaction in the synovial tissue was evaluated, the result being noted as absent (0), slight (1), moderate (2) or strong (3) [24].

To test differences in the IRS values of staining for MT between the groups, the Kruskal–Wallis test was applied, followed by the Mann–Whitney rank sum test for paired comparisons. The statistical testing of the differences in MOD values between the inflammatory joint disease groups and the OA group was done by use of ANOVA followed by a posteriori testing with the Dunnett's test. Spearman's rank correlation coefficients were calculated to test the relationships between the amount of inflammatory reaction in synovium, the IRS values and the MOD of staining for MT. A two-tailed *P*-value was used. A result was considered to be statistically significant if $P<0.05$. Statistical analysis was performed with SPSS for Windows 7.0 (SPSS, Chicago, Ill.).

## Results

No immunostaining was detected in rheumatoid synovial tissue when any of the crucial steps of the immunostaining procedure were omitted or when an antibody against *Candida albicans* was used as the primary antibody. In contrast, when the antibody against IgM was used as the primary antibody, immunostaining was seen throughout the synovial tissue in IgM-synthesizing plasma cells and extravascular IgM deposits, as described previously [21, 22]. In liver tissue, a characteristic focal staining pattern with a random distribution of immunopositive hepatocytes was obtained with the anti-MT antibody (data not shown).

Positive immunostaining for MT (IRS>1) was observed in 80% (65/81) of the samples, with a similar distribution of staining in all investigated diagnostic groups (Fig. 1). In most MT-positive samples, intensive immunostaining was detected in synovial lining, especially in basally located large cells, which often had an elongated shape and narrow cytoplasmic processes. In a smaller proportion of samples, moderate staining was also seen in some of the fibroblast-like cells of the sublining layer. In some cases, large subsynovial MT-positive cells with cellular processes were observed close to the synovial lining. The blood vessels of the subsynovial layer were not stained.

There were no significant differences in the MOD of staining for MT in the synovial lining between the OA and RA, AS and PsA groups, although the mean MOD values appeared to be lower in the AS and PsA groups than in the OA group (Fig. 2). In good agreement with the MOD values, the IRS values in the AS and PsA groups also seemed to be lower than in the OA group, but no statistically significant differences were observed between the groups (Table 2). Moreover, there was a strong correlation between the IRS and MOD values in synovial lining ($r=0.799$, $P<0.001$). In addition, a significant correlation was observed between the IRS of staining for MT in the subsynovial layer and the IRS

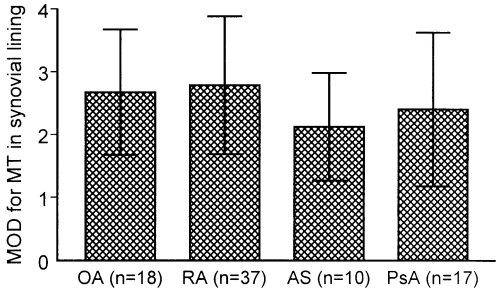


**Fig. 2** The results of the videodensitometric evaluation (MOD, mean optical density) of immunostaining for metallothionein (mean±SD) in synovial lining cells of patients with osteoarthritis (*OA*), rheumatoid arthritis (*RA*), ankylosing spondylitis (*AS*) or psoriatic arthritis (*PsA*). No statistically significant differences ($P<0.05$) were observed between the OA group and the other groups; ANOVA followed by a posteriori testing with the Dunnett's test

**Table 2** Results of the visual evaluation of immunostaining (*IRS*, immunoreactivity score) in the synovial lining and sublining layers, the histological classification of synovitis and the evaluation of synovial inflammation [median (range)] in sections from patients with osteoarthritis (*OA*), rheumatoid arthritis (*RA*), ankylosing spondylitis (*AS*) or psoriatic arthritis (*PsA*). No statistically significant differences ($P<0.05$) were observed between the groups; Kruskal-Wallis test followed by a posteriori testing with the Mann-Whitney rank sum test.

| Synovial area/ variable | Diagnostic group | | | |
|---|---|---|---|---|
| | OA (*n*=18) | RA (*n*=37) | AS (*n*=10) | PsA (*n*=16) |
| Lining layer | | | | |
| IRS | 4 (1–9) | 4 (0–9) | 2 (0–6) | 3 (0–9) |
| Sublining layer | | | | |
| IRS | 1 (0–6) | 1 (0–6) | 1 (0–4) | 1 (0–4) |
| Histological class (*n*) | | | | |
| Sero-fibrous | 14 | 15 | 5 | 5 |
| Lympho-plasmacytic | 3 | 6 | 4 | 4 |
| Ulcerous | 1 | 10 | 1 | 5 |
| Fibroblastic transformed | – | 6 | – | 2 |
| Inflammatory reaction | | | | |
| Acute | 0 (0–2) | 1 (0–2) | 1 (0–2) | 0.5 (0–2) |
| Chronic | 1 (1–3) | 2 (1–3) | 2 (1–3) | 2 (1–3) |
| Acute + chronic | 1 (1–5) | 3 (1–4) | 3 (1–5) | 2.5 (1–5) |

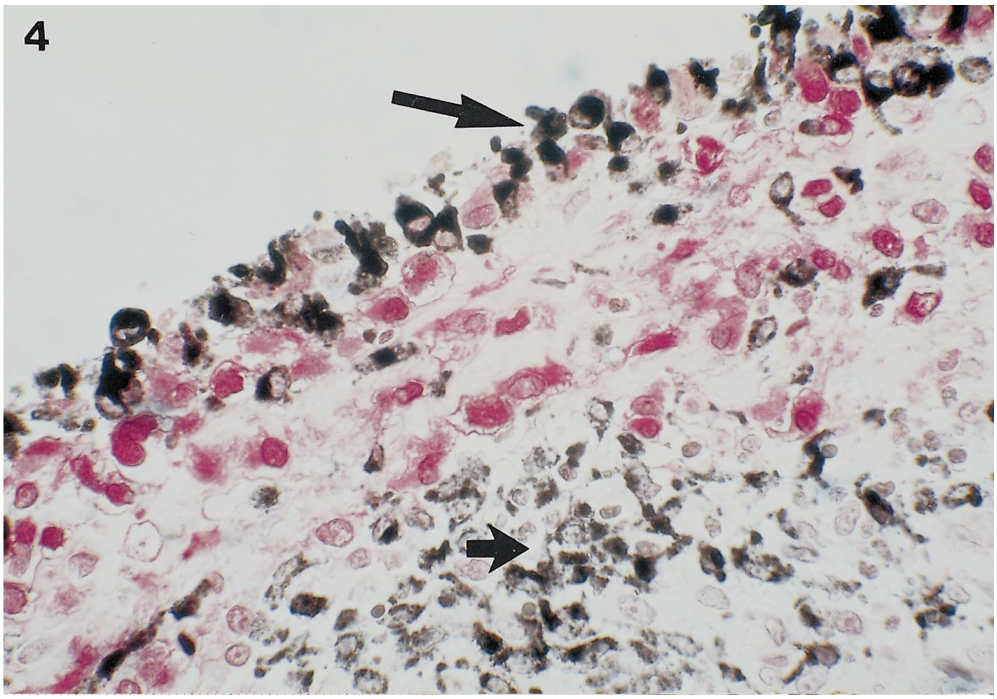


**Fig. 4** Immunohistochemical double staining for MT (*red*) and CD68 (dark brown) in a synovial tissue section from a patient with rheumatoid arthritis. CD68 is detected in some MT-negative lining cells (*long arrow*) and subsynovial reticular-like cells (*short arrow*). Localization of MT in some of the CD68-positive lining cells cannot be clearly excluded. ×240

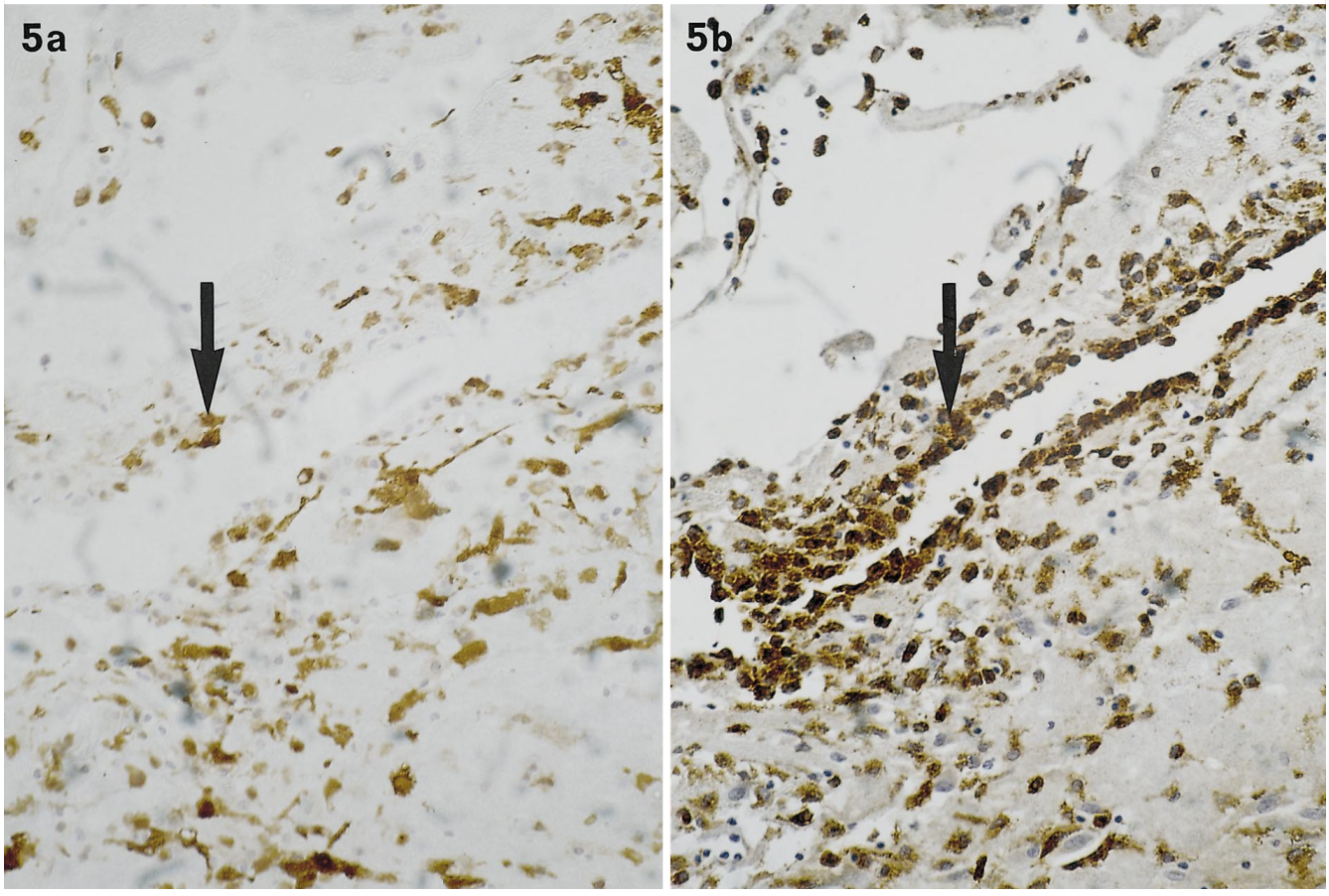


**Fig. 5** Immunohistochemical staining for **a** MT and **b** lysozyme in a synovial tissue section from a patient with rheumatoid arthritis. In the selected area, only some of the lining cells are MT-positive (**a**, *long arrow*) while most of them are lysozyme-positive (**b**, *long arrow*). In some cells of the subsynovial layer, MT and lysozyme appear to be colocalized. Note that the counterstaining in **b** is very light and the photomicrographs were taken without a condenser. ×240

($r$=0.281, $P$=0.018) and MOD ($r$=0.464, $P$<0.001) of staining for MT in synovial lining layer, indicating a direct relationship between the synovial sublining and lining layers in MT expression.

The localization of staining with the antibodies against CLA, CD68 and lysozyme was different from that with the antibody against MT (Figs. 3–5). CLA-positive cells were mainly localized in the lymphoid follicles of subsynovial layer and some of the most superficial cells of synovial lining (Fig. 3b). There did not seem to be any overlap in staining for CLA and MT. Staining for CD68 was observed in some rounded cells (monocytes/macrophages) throughout the synovial tissue and in the reticular-like cells of the subsynovial lymphoid follicles. The large cells with cellular processes located close below the lining layer were CD68 negative, as described previously [34]. In the double-staining experiment, MT was detected in most of the CD-68-negative synovial lining cells, but the localization of MT in some of the CD-68-positive lining cells could not be clearly excluded (Fig. 4). Lysozyme was detected in a large number of synovial lining and subsynovial cells which were MT negative (Fig. 5b). However, in some cells of the synovial lining, both MT and lysozyme may have been expressed.

The histological class of synovitis was most often sero-fibrous or lympho-plasmacytic in all diagnostic groups (Table 2). However, fibroblastic transformed synovitis was only seen in samples from patients with RA or PsA, reflecting the previously observed increased probability of a diagnosis of RA in this histological subtype. Although there was a tendency for increased acute and chronic subsynovial inflammatory reactions in the inflammatory arthritis groups, none of the groups differed statistically significantly from the OA group. However, there was a weak correlation between the acute inflammatory reaction and the IRS of staining for MT in the subsynovial layer when all the 81 cases were included ($r$=0.242, $P$=0.043).

## Discussion

In this immunohistochemical study we demonstrated the presence of MT in synovial tissue from patients with OA, RA, AS or PsA. Most of the synovial samples from all the diagnostic groups showed expression of metallothionein in a large number of synovial lining cells and in some of the fibroblast-like cells of the subsynovial layer. However, no difference in the intensity of staining for MT was observed between the inflammatory arthritides (RA, AS and PsA) and OA. In general, MT was not detected in the blood vessels and inflammatory cells of synovial tissue.

In our study, the most intensive immunostaining for MT was observed in basally located synovial intimal cells, which were mainly CD68 negative and often oval or oblong in shape. It is likely that a major proportion of these were type B synoviocytes (fibroblast-like synoviocytes), since CLA and lysozyme were also predominantly found in cells with a different localization and shape, and it seems that type B synovial lining cells are localized in the basal parts of the lining layer [56]. Previously, MT protein has also been detected in cultured human synovial fibroblasts [27], which are believed to be predominantly type B cells, because type A lining cells (macrophage-like synoviocytes) die during cell culture [17]. However, as it is possible that CD68 is not expressed in all the type A lining cells, we cannot, strictly speaking, exclude the expression of MT in some of the type A lining cells. Moreover, we observed some MT-positive cells with a fibroblast-like morphology in the subsynovial layer. They were most probably fibroblasts, since CLA, CD68 and lysozyme did not seem to be localized in these cells.

In vivo, the protective function of MT against oxidative damage is thought to require the upregulation of MT expression through cytokine induction in response to the stress situation [51]. Oxidative stress contributes to the pathology of rheumatic diseases [26, 36, 40], and elevated amounts of cytokines able to induce MT synthesis are present in inflamed synovium [2, 12]. However, in our study, equal amounts of MT were detected in synovial tissue from patients with RA, AS or PsA and OA, although it is likely that more inflammation and cytokines are present in rheumatic diseases than in OA. Nevertheless, as osteoarthritic joints are also often inflamed and we had no healthy control tissue, our results do not exclude the possibility that MT is induced in synovial cells in response to joint inflammation.

However, the lack of differences between the separate conditions might be due to low dietary intake and tissue concentrations of Zn in patients with rheumatic diseases [29, 33]. Because (at least in vitro) Zn is a strong inducer of MT and has a permissive effect on induction of MT synthesis by other mediators, low synovial Zn levels might have suppressed the induction of MT in the patients with the rheumatic diseases. In any case, MT is present and could thus contribute to protection against oxidative stress in synovial tissue, especially as the MT-rich synovial lining is thickened in inflammatory joint disease [45]. In addition, MT could, by its zinc-binding properties, modulate the function of the zinc-dependent matrix metalloproteinases, such as membrane-type-1 matrix metalloproteinase, which is expressed for example in osteoarthritic joints [31]. MT expression might thus influence the degradation of extracellular matrix components mediated by these enzymes in diseased joints.

Because of the possible protective functions of MT against oxidative stress, induction of MT synthesis has been thought to contribute to the therapeutic effects of some drugs [5, 38, 39, 54]. For example, induction of MT expression by gold compounds and glucocorticoids has been demonstrated in cultured human synovial fibroblasts [27, 39]. Moreover, the in vivo MT expression in hepatic tissue is induced by NSAIDs, chloroquine and penicillamine in mice or rats [38, 54]. In contrast to these antirheumatic drugs, cyclosporin has been shown to suppress MT induction in rat adjuvant arthritis [47].

Although it is likely that, compared with patients with OA, patients with the inflammatory arthritides are more often treated with drugs that can induce MT synthesis, the levels of MT in synovial tissue were not higher in the inflammatory arthritides than in OA in our study. Thus, the antirheumatic drugs may not be able to induce MT in synovial tissue in humans in vivo. However, we could not test this possibility, because information on drug use could not always be retrieved from the hospital files. An appropriate assessment of possible drug-mediated MT induction would require experimental studies in patients.

The fibroblast-like synoviocytes, and synovial lining cells in general, are thought to have a role in the pathogenesis of RA [17]. The observed presence and variability in the levels of MT in synovial lining cells and subsynovial fibroblast-like cells may have some implications for the treatment of rheumatic diseases, since high expression of MT has been shown to confer resistance of cells to anticancer drugs [14], some of which are also used in rheumatic diseases [8]. This reduction in the effects of chemotherapeutic agents is thought to be mainly caused by binding of the agents [15, 16] and scavenging of reactive oxygen species by MT [14]. MT has been shown to be involved in resistance of cells to cyclophosphamide and chlorambucil [14–16, 32, 43], which are both used as second-line agents against RA. Moreover, pre-existent or gold-induced MT can probably mediate the resistance of cells to the effects of gold chloride and auranofin, as demonstrated in experiments using cultured mammalian cells [25, 26, 42, 57]. Accordingly, patients with little or no MT in synovial tissue might have a better response to some antirheumatic drugs than patients with high levels of MT in synovial tissue.

In conclusion, MT was expressed in the synovial lining cells of most of the OA, RA, AS or PsA synovial tissue samples investigated. In a small proportion of samples, MT was also detected in some of the fibroblast-like cells of the subsynovial layer. As there were no significant differences in the intensity of staining for MT between the rheumatic diseases and OA, our data do not support the idea that MT can be induced in response to synovial inflammation in vivo. However, based on our knowledge about the roles of MT, it could contribute to metal homeostasis and protection against oxidative stress in synovial tissue. Moreover, since MT can bind gold compounds and some chemotherapeutic antirheumatic drugs, individuals with a high expression of MT in synovial tissue might be resistant to the therapeutic effects of these drugs.

**Acknowledgements** The study was supported by the Robert Bosch Foundation, Germany. J.T. Backman was supported by the Alexander von Humboldt Foundation (a research fellowship), Germany, and the Academy of Finland, Finland.

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