

# Histone Deacetylase/Acetylase Activity in Total Synovial Tissue Derived From Rheumatoid Arthritis and Osteoarthritis Patients

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**Objective.** Rheumatoid arthritis (RA) is a chronic inflammatory disorder of unknown origin. Histone deacetylase (HDA) activity is considered to play a major role in the transcriptional regulation of proinflammatory genes. We undertook this study to investigate the balance of histone acetylase and HDA activity in synovial tissue from RA patients compared with that from patients with osteoarthritis (OA) and normal controls.

**Methods.** Activity of histone acetylases and HDAs was measured in nuclear extracts of total synovial tissue samples, which were obtained from RA and OA patients undergoing surgical joint replacement, and compared with the activity in synovial tissues from patients without arthritis. Tissue expression of HDAs 1 and 2 was quantified by Western blotting. In addition, immunohistochemistry was performed for HDA-2.

**Results.** Mean  $\pm$  SEM HDA activity in synovial tissue samples derived from patients with RA was measured as  $1.5 \pm 0.3$   $\mu\text{moles}/\mu\text{g}$ , whereas the activity levels in OA ( $3.2 \pm 0.7$   $\mu\text{moles}/\mu\text{g}$ ) and normal ( $7.1 \pm$

$4.2$   $\mu\text{moles}/\mu\text{g}$ ) synovial tissue samples were significantly higher. Histone acetylase activity reached similar levels in RA and OA tissues and in normal tissues. The ratio of HDA activity to histone acetylase activity in RA synovial tissue was significantly reduced ( $12 \pm 2\%$ ) compared with that in OA synovial tissue ( $26 \pm 3\%$ ). The activity ratio in normal control samples was arbitrarily set at  $100 \pm 40\%$ . In addition, the tissue expression of HDA-1 and HDA-2 proteins was clearly lower in RA samples than in OA samples.

**Conclusion.** The balance of histone acetylase/HDA activities is strongly shifted toward histone hyperacetylation in patients with RA. These results offer novel molecular insights into the pathogenesis of the disease that might be relevant to the development of future therapeutic approaches.

Rheumatoid arthritis (RA) is a chronic polyarticular disease that is characterized by inflammation and progressive destruction of the articular cartilage. Gene transcription of chemotactic and inflammatory mediators is regulated, at least in part, by the tight balance between histone acetylation and histone deacetylation (1–4). Histone acetylases and histone deacetylases (HDAs) induce posttranslational modifications of the N-terminal tails of the nuclear histone proteins that impact chromatin structure and gene transcription. The chromatin is compactly organized in nucleosomes as an octomeric core unit of 4 histones. Modifications are regulated by 2 groups of enzymes (i.e., histone acetylases and HDAs). In inactivated cells, the dense DNA–protein package prevents accessibility of transcription factors and nucleic acid polymerases (5,6). Acetylation of histones is thought to occur on actively transcribed chromatin only (7), thus allowing gene transcription during cell activation (8,9).

Histone acetylases can be separated into 2 categories (i.e., type A and type B) depending on their

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**Table 1.** Characteristics of the study subjects\*

	RA patients	OA patients	Normal subjects
Age, mean $\pm$ SEM (range) years	63 $\pm$ 6 (43–79)	73 $\pm$ 3 (62–80)	68 $\pm$ 10 (49–81)
No. of women/no. of men	6/1	5/1	2/3
Disease duration, mean $\pm$ SEM (range) years	26 $\pm$ 5 (10–38)	>10	NA
Origin of synovial tissue			
Shoulder	2	2	1
Elbow	1	0	0
Finger	2	0	0
Hip	1	1	0
Knee	1	3	1
Sternoclavicular joints	0	0	3
Medication			
Oral corticosteroid	2	0	1
Methotrexate	2	0	0
Anti-TNF $\alpha$	3	0	0

\* Except where indicated otherwise, values are the number of subjects. RA = rheumatoid arthritis; OA = osteoarthritis; NA = not applicable; anti-TNF $\alpha$  = anti-tumor necrosis factor  $\alpha$ .

subcellular localization. Type A histone acetylases are found in the nucleus. Since histone acetylases acetylate nucleosomal histones, they are closely linked to the transcriptional regulation of gene expression. On the other hand, type B histone acetylases acetylate newly synthesized histones that are free in the cytoplasm. Once acetylated, these histones are shuttled into the nucleus, where they may be deacetylated and incorporated into chromatin. Some histone acetylases also have coactivating roles for different transcription factors.

The reverse reaction (deacetylation of histone proteins) is catalyzed by HDAs, which can be divided into 3 different classes based on sequence homologies to yeast proteins. Class I HDAs (HDAs 1, 2, 3, 8, and 11) are closely related to the yeast transcriptional regulator reduced potassium dependency 3. Class II HDAs (HDAs 4, 5, 6, 7, 9, and 10) show homologies to the yeast deacetylase HDA-1. Class II HDAs are similar to the silent information regulator 2 family of NAD<sup>+</sup>-dependent HDAs. HDAs of class I are expressed in all cell types, whereas the expression of class II HDAs is more restricted and might show tissue-specific expression patterns (10).

The extent of gene transcription is regulated by the equilibrium of histone acetylation (increasing gene transcription) and histone deacetylation (blocking gene transcription). Hyperacetylation of histones is achieved through an increase in histone acetylase activity and, conversely, through a decrease in HDA activity. In either case, the local unwinding of nucleosomal DNA results in a loosened state of DNA, which allows transcription factors and RNA polymerase II to bind.

So far, decreased levels of active HDAs have mainly been reported in inflammatory lung diseases

(11). In this context, HDAs have been described as key enzymes in the repression of proinflammatory cytokines in alveolar macrophages, as seen for example in the clinical exacerbation of chronic obstructive pulmonary disease (COPD) (12). Increased acetylation of histones, moreover, has been associated with the activation of NF- $\kappa$ B and activator protein 1, two major transcription factors involved in the pathogenesis of RA (13–15).

In the present study, we addressed the activity levels of HDAs and histone acetylases in nuclear extracts of synovial tissue of RA and osteoarthritis (OA) patients. Novel insights into the imbalanced expression of epigenetic markers such as histone acetylase/HDA provide important information about the molecular features of the rheumatoid synovium.

## PATIENTS AND METHODS

**Reagents.** Recombinant human tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) was purchased from R&D Systems (Minneapolis, MN). Trichostatin A was from Sigma (St. Louis, MO). Rabbit anti-human HDA-1 and HDA-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human  $\alpha$ -tubulin was from Sigma. Polyclonal rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP) were from Dako (Zug, Switzerland), and goat anti-rabbit IgG conjugated to HRP were purchased from Jackson ImmunoResearch (Soham, UK). Mouse anti-human CD68 was from Dako.

**Patients.** Synovial tissue specimens were obtained from 7 RA patients and 6 OA patients undergoing surgical joint replacement at the Clinic of Orthopedic Surgery, Schulthess Hospital Zurich. All RA patients fulfilled the 1987 revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (16). An overview of the clinical characteristics of the subjects is provided in Table 1.

To establish HDA and histone acetylase activity as

epigenetic markers, as well as to determine their protein expression, normal synovial tissue was required for quality assurance. Synovial tissues (<5 mm<sup>3</sup> in size) from patients without arthritis undergoing surgical amputation of a limb (n = 2) and from sternoclavicular joints removed during autopsies (n = 3) were used as normal controls. All tissue analyses were performed according to the regulations of the Ethical Committee Zurich.

**Preparation of nuclear extracts.** Total synovial tissue specimens (~3–5 mm<sup>3</sup> in size) were used for preparation of nuclear extracts as described previously (12,17). Briefly, fresh tissue was transferred in hypotonic buffer consisting of 10 mM HEPES (pH 7.9), 1.5 mM magnesium chloride, 10 mM potassium chloride, 10 mM 2-mercaptoethanol, and a mixture of protease inhibitors (2 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A [Sigma]) as well as 1 mM phenylmethylsulfonyl fluoride (PMSF; Calbiochem, Dietikon, Switzerland) and homogenized in a tissue lyser (Qiagen, Hilden, Germany) for 2 minutes twice at 20 Hz. The samples were left on ice for 15 minutes before adding Nonidet P40 to a final concentration of 0.5%. The samples were centrifuged at 3,000 revolutions per minute for 1 minute to pellet the larger cellular debris. The resulting supernatants were centrifuged at 14,000 rpm for 30 seconds at 4°C to obtain the nuclear-rich fraction. The pellet was then resuspended in 100 µl nuclear buffer (20 mM HEPES [pH 7.9], 0.42 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, and 1 mM PMSF, which was added immediately before use) and incubated on ice for 15 minutes with additional vortexing every 5 minutes. Finally, the samples were centrifuged at 14,000 rpm for 15 minutes at 4°C, the supernatants (nuclear extracts) were transferred to ice-chilled tubes, and the protein concentration of each sample was analyzed (Bradford Bio-Rad Protein assay kit; Bio-Rad, Hercules, CA) with bovine serum albumin (Sigma) used as a standard.

**Measurement of histone acetylase activity and HDA activity.** Total histone acetylase activity and HDA activity were measured using colorimetric assay kits (BioVision, Mountain View, CA) according to the manufacturer's instructions. In this way, acetylation of peptides by active histone acetylases releases the histone acetylase cofactor acetyl-coenzyme A (acetyl-CoA). Free CoA serves as an essential coenzyme for producing NADH. New generated NADH can be measured spectrophotometrically upon reacting with a soluble tetrazolium dye. To measure the HDA activity, samples are incubated with a colorimetric substrate, which includes an acetylated lysine side chain. Deacetylation of the substrate sensitizes the substrate to react with a chromophore that can be measured spectroscopically.

Briefly, 25–100 µg of nuclear extracts was prepared in 40 µl water and added to a 96-well plate including blank samples and positive controls (for histone acetylase activity, CoA [Sigma]; for HDA activity, HeLa nuclear extract provided with the kit). After addition of assay buffer and enzyme mix, the plate was incubated at 37°C for 2 hours. Samples were then read at 500 nm (for histone acetylase activity) or incubated after the addition of a lysine developer (HDA activity kit) for an additional 30 minutes at 37°C before reading the plate at 405 nm. Data were analyzed by using Revel software (version G 3.2; Dynex Technologies, West Sussex, UK). Activity was analyzed as the relative optical density (OD) value per µg of protein sample and was recalculated using the deacetylated

standard (for HDA) or the CoA standard curve (for histone acetylase) as  $\Delta\text{OD}/\mu\text{M}$ .

**Immunohistochemistry.** Immunohistochemistry was performed using a standard indirect immunoperoxidase method (18). Sections from formalin-fixed, paraffin-embedded tissues were deparaffinized and pretreated at 80°C for 30 minutes in 10 mmoles/liter citrate buffer (pH 6.0) for antigen retrieval.

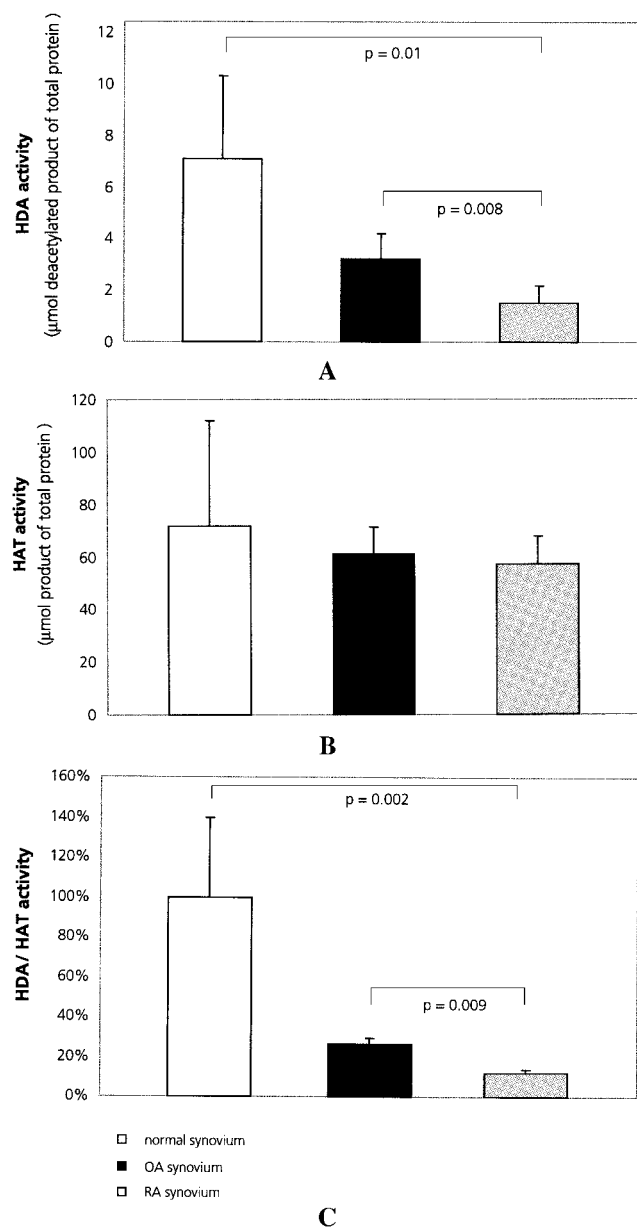
To determine the cell type expressing HDA-2 in synovial tissues, double labeling with immunohistochemistry was performed. Mouse anti-human CD68 (1:100) was used for double staining visualized by nitroblue tetrazolium/BCIP. In control experiments, matched mouse IgG isotypes (1:50,000; Dako) were used instead of the primary antibodies. To block nonspecific binding, slides were incubated for 1 hour in blocking solution consisting of 4% nonfat dry milk and 2% horse serum in Tris buffered saline (TBS) at pH 7.4. Slides were then incubated for 1 hour with polyclonal rabbit anti-human HDA-2 antibodies (200 µg/ml diluted 1:500 in phosphate buffered saline [PBS]). Bound primary antibodies were detected using biotinylated goat anti-rabbit IgG (1 mg/ml, diluted 1:1,000). Labeling was performed for 20 minutes with an HRP-conjugated streptavidin complex (BioGenex, San Ramon, CA). Antigens were visualized using aminoethylcarbazole chromogen and H<sub>2</sub>O<sub>2</sub> as substrate. All steps were performed at room temperature.

**Western blot analysis.** For Western blot analysis, whole cell lysates were prepared by lysing confluent cells ( $1 \times 10^6$ ) in 2× concentrated Laemmli buffer (100 mM Tris HCl [pH 6.8], 40% glycerol, 10% sodium dodecyl sulfate [SDS], 0.7M β-mercaptoethanol, and 0.0005% bromophenol blue). Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk with 0.05% Tween 20 in TBS (pH 7.4) and were probed overnight at 4°C with antibodies against HDA-1 or α-tubulin. After incubation for 30 minutes at room temperature with HRP-conjugated secondary antibodies (HRP-conjugated goat anti-rabbit or HRP-conjugated rabbit anti-mouse) in 5% nonfat dry milk with 0.05% Tween 20 in TBS (pH 7.4), bound antibodies were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK). Evaluation of the expression of specific proteins was performed by the Alpha Imager Software system (Alpha Innotech, San Leandro, CA) via pixel quantification of the electronic image.

**Statistical analysis.** All data are expressed as the mean ± SEM. Statistical analysis was performed using GraphPad Prism software, version 4.03 (GraphPad Software, San Diego, CA). For analysis between different groups, the Mann-Whitney U test was used. *P* values less than 0.05 were considered significant.

## RESULTS

**HDA activity and histone acetylase activity in total synovial tissue.** Decreased levels of HDA activity have been associated with inflammatory diseases, in particular with inflammatory lung diseases. In this context, we investigated the levels of active HDA in synovial tissues. The HDA activity in synovial tissues from pa-

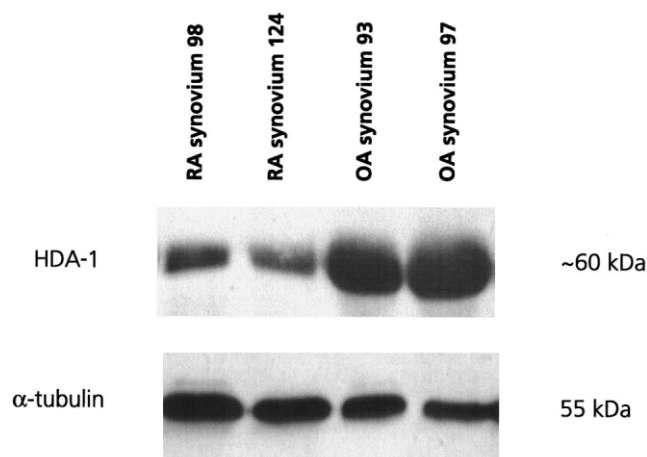


**Figure 1.** Histone deacetylase (HDA) activity versus histone acetylase (HAT) activity in synovial tissue. **A**, HDA activity in synovial tissue from normal controls compared with that in synovial tissue from patients with osteoarthritis (OA) or rheumatoid arthritis (RA). HDA activity was significantly reduced in RA patients ( $1.5 \pm 0.3 \mu\text{moles}/\mu\text{g}$ ) compared with OA patients ( $3.2 \pm 0.7 \mu\text{moles}/\mu\text{g}$ ) and normal controls ( $7.1 \pm 4.2 \mu\text{moles}/\mu\text{g}$ ). Data were calculated as  $\mu\text{moles}$  of deacetylated product from  $\mu\text{g}$  of total protein content. **B**, Histone acetylase activity measured in the same samples as in **A**. Data were calculated as  $\mu\text{moles}$  of product from  $\mu\text{g}$  of total protein content. No significant differences in histone acetylase activity could be detected between OA, RA, and normal samples. **C**, Ratio of HDA activity to histone acetylase activity. Activity is strongly shifted toward hyperacetylation in RA synovial tissue samples ( $12 \pm 2\%$ ) compared with OA synovial tissue samples ( $26 \pm 3\%$ ) and normal synovial tissue samples (arbitrarily set at  $100 \pm 40\%$ ). Values are the mean and SEM.

tients with RA was  $\sim 2$ -fold lower than that in synovial tissues from patients with OA or from normal controls. In particular, the mean  $\pm$  SEM HDA activity levels in RA were determined to be  $1.5 \pm 0.3 \mu\text{moles}/\mu\text{g}$ , expressed as  $\mu\text{moles}$  of deacetylated product from  $\mu\text{g}$  of total protein. On the other hand, the levels were  $3.2 \pm 0.7 \mu\text{moles}/\mu\text{g}$  in OA samples and  $7.1 \pm 4.2 \mu\text{moles}/\mu\text{g}$  in normal control samples. As shown in Figure 1A, the difference between the HDA activity in RA synovial tissue versus OA synovial tissue ( $P = 0.008$ ) as well as between HDA activity in RA synovial tissue versus normal control tissue ( $P = 0.01$ ) was statistically significant (Figure 1A), whereas no significant difference was observed between OA and normal control samples.

The extent of gene transcription is regulated by the tight balance between HDA and histone acetylase activities. Thus, we next tested whether the decreased activity levels of HDA in RA synovial tissue samples were compensated by an adequate decrease in histone acetylase activity. As shown in Figure 1B, we found similar activity levels of histone acetylase in synovial tissues from RA patients ( $57.0 \pm 25.1 \mu\text{moles}/\mu\text{g}$ ) and OA patients ( $61.6 \pm 26.0 \mu\text{moles}/\mu\text{g}$ ) and in those from normal controls ( $72 \pm 39.2 \mu\text{moles}/\mu\text{g}$ ).

To detect the resulting activity levels within RA and OA synovial tissues, the ratio of HDA activity to histone acetylase activity was calculated. The HDA activity:histone acetylase activity ratio in normal synovial tissue was arbitrarily set at 100% ( $100 \pm 40\%$ ). In OA samples, the ratio decreased to  $26 \pm 3\%$ . In RA samples, a further decrease in the HDA activity:histone



**Figure 2.** HDA-1 protein expression in synovial tissue. Representative Western blot showing down-regulation of HDA-1 protein in RA synovial tissue compared with OA synovial tissue. Whole cell lysates were analyzed and normalized to  $\alpha$ -tubulin protein. Numbers represent individual patients. See Figure 1 for definitions.

acetylase activity ratio to  $12 \pm 2\%$  could be observed. The alteration toward acetylation of histones in RA patients reached statistical significance between OA and RA synovial samples ( $P = 0.009$ ) (Figure 1C), but not between normal samples and OA synovial samples.

**Protein expression of HDA-1 and HDA-2 in synovial tissue.** To analyze whether the activity levels correlate with protein expression, we performed Western blotting for HDA-1 and HDA-2. After normalizing protein to  $\alpha$ -tubulin protein, Western blots were quantified by Alpha Imager Software as electronic images. Reduced levels of HDA-1 ( $51 \pm 26\%$ ) (Figure 2) and HDA-2 ( $70 \pm 18\%$ ) were found in RA synovial tissues ( $n = 8$ ) compared with expression in OA synovial tissues ( $n = 6$ ), which was arbitrarily set at 100%.

To determine the morphologic localization of HDA expression in the synovium, HDA-2 protein was analyzed by immunohistochemistry and evaluated by analyzing several tissue slides. In normal healthy synovium as well as in OA synovial tissue, most of the cells were strongly positive for nuclear HDA-2 expression (Figures 3a and b). However, in RA synovial tissue ( $n = 4$ ), the expression of nuclear HDA-2 was strongly reduced (Figure 3c). When the tissue slides were double stained against HDA-2 and CD68, no merging of CD68 and HDA-2 could be observed (Figures 3e and f).

Taken together, our results show that the total HDA activity as well as distinct isoforms of HDA proteins are down-regulated in RA synovial tissue compared with OA synovial tissue. These data suggest a pathophysiologic association between reduced HDA activity and chronic inflammatory processes of the joint.

## DISCUSSION

Enhanced histone acetylation or histone hyperacetylation leads to local unwinding of chromatin and is generally associated with induction of gene expression due to increased gene transcription rates. The acetylation status is regulated by the action of 2 distinct groups of enzymes (i.e., histone acetylases and HDAs). Persistent alterations in the tight equilibrium between histone acetylase and HDA activities have been associated with pathologic gene expression patterns and the development of chronic diseases, such as COPDs and other related inflammatory disorders of the lungs (for review, see refs. 11 and 19).

Our present data show that the levels of total HDA activity are strongly decreased in synovial tissue homogenates from patients with RA compared with the respective activity in those from OA patients and normal controls. In addition, no different activity levels of the

enzymatic counterpart histone acetylase have been found between all conditions investigated.

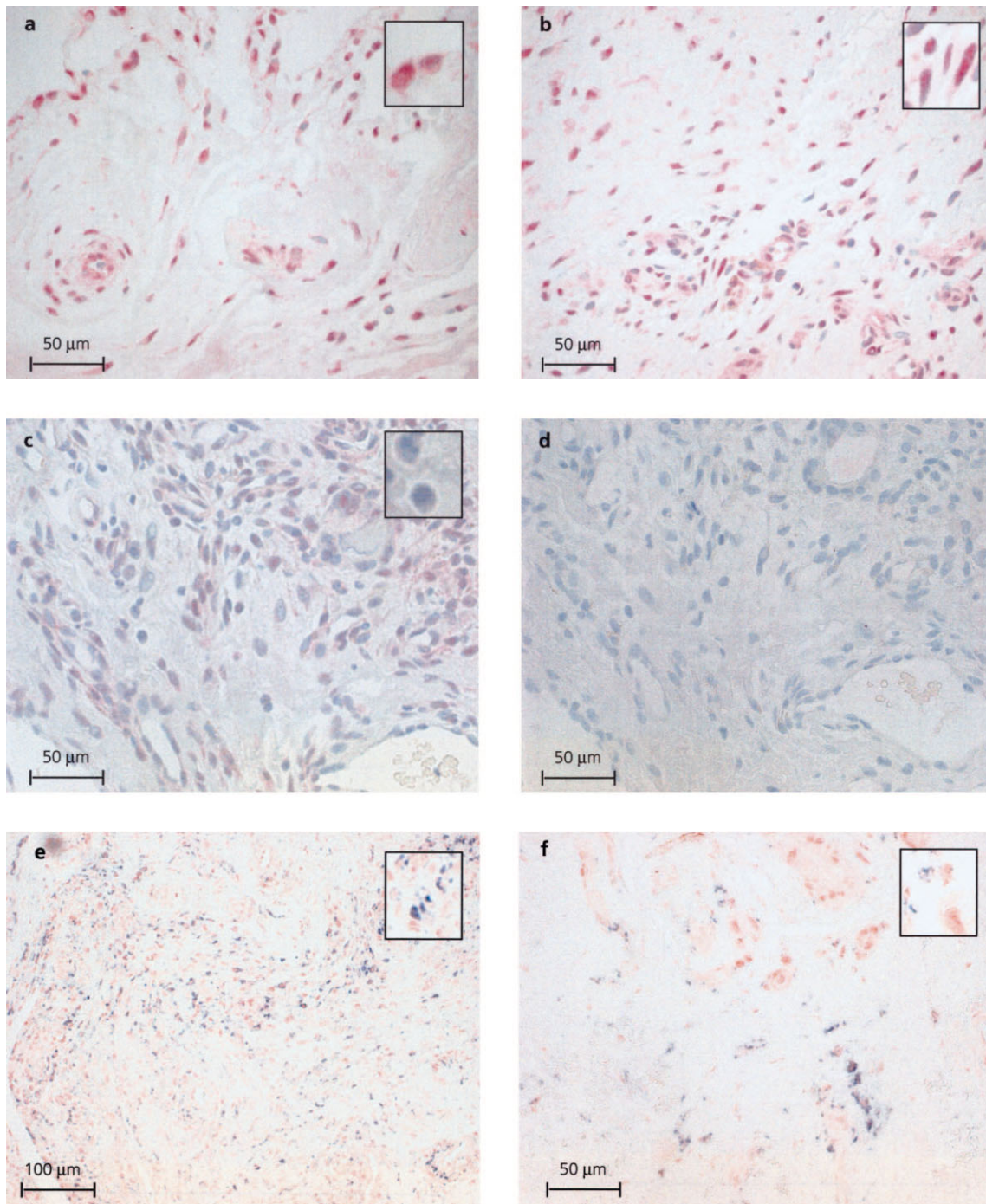
When the ratio of both involved molecular players was calculated, the overall histone status was shifted toward histone hyperacetylation in RA. These changes reached significance between RA and OA synovial tissue, indicating that chronic inflammatory processes are closely linked to reduced activity of HDAs. This tendency is further supported by our observation that the levels of HDA activity are even higher within the total synovium of healthy individuals.

Our data are consistent with other studies that showed histone hyperacetylation in chronic inflammatory lung diseases due to decreased HDA activity without alterations of histone acetylase activity (12). To date, however, this is the first study to analyze HDA activity within the synovial tissue. Of interest, several studies have suggested beneficial effects of HDA inhibitors in the treatment of RA and other inflammatory processes (20,21). By showing a clear reduction of HDA activity in the rheumatoid synovium, however, our data strongly challenge the idea of epigenetically modulating molecular targets by HDA inhibitors for therapeutic purposes in RA.

With respect to NF- $\kappa$ B, which is one of the best-characterized proinflammatory transcription factors, it is a matter of debate whether HDA inhibitors lead to induction (22,23) or inhibition (24) of NF- $\kappa$ B. Both scenarios are probably possible, depending on specificity, the proinflammatory mediator investigated, or the cell type (21).

Previous work by Ito et al (12) revealed that the reduction of total HDA activity in inflammatory diseases is mainly due to changes in the expression of class I HDAs (i.e., HDAs 1, 2, 3, 8, and 11), particularly HDA-2. We therefore focused on the tissue expression of HDA-1 and HDA-2 proteins, both of which we found to be clearly reduced in RA synovial tissue compared with OA or normal synovial tissue.

Still, it remains rather unclear whether the observed reduction in HDA activity is "the chicken or the egg" in the pathogenesis of RA. We cannot exclude the possibility that the reduced HDA activity reflects an epiphenomenon of ongoing inflammation. However, Ito et al (23) have shown that HDA-2 suppresses NF- $\kappa$ B-mediated gene expression. In RA synovial cells, NF- $\kappa$ B is highly activated, leading subsequently to the expression of several proinflammatory mediators including TNF $\alpha$ , interleukin-6 (IL-6), IL-8, and cyclooxygenase 2, as well as matrix-degrading enzymes (for review, see ref. 25). In concert with the findings of Ito and coworkers, we hypothesize that class I HDAs appear to act up-



**Figure 3.** HDA-2 protein expression in synovial tissue. **a–c**, Representative immunohistochemistry for HDA-2 protein in normal synovial tissue (**a**), OA synovial tissue (**b**), and RA synovial tissue (**c**). **d**, Isotype control. **e** and **f**, Double labeling for CD68 after immunohistochemistry for HDA-2 protein in synovial tissues from patients with RA shown at different magnifications. Double labeling indicates that synovial macrophages do not express HDA-2 protein. Color for CD68 was developed with nitroblue tetrazolium/BCIP. Boxed areas show higher magnification views of selected nuclei. (Original magnification  $\times 100$  in **a–d** and **f**;  $\times 50$  in **e**;  $\times 400$  in boxed areas in **a–c**, **e**, and **f**.) See Figure 1 for definitions.

stream of NF- $\kappa$ B and other related transcription factors in RA.

We report here for the first time an overall status of histone hyperacetylation within RA synovium due to

low activity levels of total HDA enzymes, which was further underpinned by reduced protein expression of HDAs 1 and 2. We conclude that the observed reduction in the activity levels of class I HDAs contributes to the pathogenesis of RA, probably by activation of proinflammatory transcription factors. These findings have potential implications for the development of novel, molecule-targeted therapies against inflammatory diseases of the joint.

#### AUTHOR CONTRIBUTIONS

Dr. Huber had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study design.** Huber, Brock, J. H. W. Distler, R. E. Gay, S. Gay, O. Distler, Jüngel.

**Acquisition of data.** Huber, Brock, Hemmatzad, Giger, Moritz, Trenkmann, Kolling.

**Analysis and interpretation of data.** Huber, S. Gay, O. Distler, Jüngel.

**Manuscript preparation.** Huber, J. H. W. Distler, R. E. Gay, Moch, Michel, S. Gay, O. Distler, Jüngel.

**Statistical analysis.** Huber.

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