Regulation of inflammation by histone deacetylases in rheumatoid arthritis: beyond epigenetics
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Citation for published version (APA):
Grabiec, A. M. (2012). Regulation of inflammation by histone deacetylases in rheumatoid arthritis: beyond epigenetics
CHAPTER 4

RELATIONSHIPS BETWEEN PROTEIN ACETYLATION IN RHEUMATOID ARTHRITIS SYNOVIAL TISSUE AND CLINICAL AND MOLECULAR PARAMETERS OF DISEASE ACTIVITY

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ABSTRACT

Background: Reversible acetylation of histones and non-histone proteins plays a pivotal role in regulating cellular inflammatory responses. Aberrant activity and expression of histone deacetylases (HDACs) contributes to pathology in inflammatory lung diseases, and recent reports have suggested that HDAC activity is deregulated in rheumatoid arthritis (RA). The purpose of this study was to analyze potential associations of synovial protein acetylation and HDAC expression, with disease activity and synovial expression of inflammatory mediators in RA.

Methods: Synovial protein acetylation and cellular composition of the synovial tissue was assessed by immunohistochemistry and quantified by digital image analysis. mRNA expression of HDAC family members, TNFα, IL-6 and MMP-1 in the synovial tissue, and changes in mRNA levels of HDACs expressed in RA fibroblast-like synoviocytes (FLS) were measured by quantitative PCR.

Results: Similar levels of acetylated lysine and acetylated histone 3 (H3) were detected in RA and osteoarthritis (OA) synovial tissue, while a modest reduction in H4 acetylation was observed in RA compared to OA. Total protein acetylation levels failed to correlate with clinical parameters and synovial T cell or FLS numbers. A strong positive association of acetylated lysine, acetylated H3, acetylated H4 and dimethylated H3 levels with synovial macrophage numbers was observed. Synovial expression of TNFα and MMP-1 positively correlated with mRNA levels of HDAC1 and other class I HDACs. A significant negative correlation was observed between HDACS expression and both disease activity and synovial IL-6 expression. mRNA expression of HDAC5 in RA FLS, but not other Class I or Class II HDACs, was rapidly suppressed following IL-1β stimulation.

Conclusions: This study provides several novel findings regarding the relationship between HDAC-mediated epigenetic regulatory mechanisms and inflammation in RA. First, our data suggests that differences in HDAC expression or activity that might be present in RA synovial tissue are not sufficient to result in global changes in total protein or histone acetylation associated with clinical parameters of disease activity. Second, cellular protein hyperacetylation represents a cell type-specific phenomenon characteristic for synovial macrophages in RA, not directly related to diagnosis and disease activity. Finally, our results confirm previous studies suggesting that high synovial expression of class I HDACs is associated with local expression of inflammatory mediators, but identify cytokine-mediated reduction of HDAC5 as a potential marker of systemic inflammatory processes.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by excessive production of cytokines, chemokines and other inflammatory mediators. Tumour necrosis factor α (TNFα), interleukin (IL)-1β, IL-6 and IL-8 secreted by activated immune cells infiltrating the synovial tissue stimulate hyperplastic growth and activation of stromal fibroblast-like synoviocytes (FLS), directly contributing to irreversible bone and cartilage damage. At the cellular level, expression of inflammatory cytokines is tightly regulated by a complex interplay between activation of conserved intracellular signalling pathways, recruitment of activated transcription factors to gene promoters, epigenetic regulatory mechanisms, as well as modulation of mRNA stability, protein biosynthesis and secretion. In RA and other immune-mediated inflammatory diseases (IMIDs) aberrant regulation of individual components of this network of biological events causes chronic activation of the immune system and pathology. However, while characterization of some of the processes underlying pathological inflammatory activation in RA has led to successful development of anti-cytokine therapies, and therapeutic targeting of intracellular signaling pathways has also demonstrated initial clinical efficacy, a substantial fraction of RA patients fails to respond to these novel therapeutic strategies. Therefore, there is a continuing need for improving our understanding of alternative mechanisms of gene regulation which might be involved in RA pathology and serve as novel therapeutic targets.

Multiple epigenetic regulatory mechanisms modulate expression of inflammatory mediators, among which reversible acetylation and methylation of histones plays a prominent role. The activation of transcriptional coactivators possessing intrinsic histone acetyl transferase (HAT) activity, leading to histone acetylation and opening of chromatin structure, is an essential prerequisite for gene transcription. Acetyl groups are subsequently removed from N-terminal histone tails by histone deacetylases (HDACs), four classes of which have been identified in mammals, allowing for termination of ongoing transcriptional processes. The family of zinc-dependent classical HDACs contains the ubiquitously expressed class I HDACs (HDACs 1-3 and 8), the tissue-enriched class II HDACs (HDACs 4-7, 9, 10), and HDAC11 as the sole member of class IV HDACs. Structurally distinct nicotinamide adenine dinucleotide-dependent sirtuins (Sirt1-7) comprise the class III mammalian HDACs. In contrast to histone acetylation which is generally associated with transcriptional activation, methylation of histones plays a more complex biological role. High levels of methylated H3K4, H3K36 and H3K79 denote areas of relaxed chromatin, while H3K9, H3K27 and H4K20 methylation events are characteristic for transcriptionally inactive genes.

An association between deregulated HAT and HDAC activity and chronic inflammation was first reported in inflammatory lung disease. Elevated HAT and reduced HDAC activity are found in asthma patient bronchial biopsies and alveolar macrophages. In chronic obstructive pulmonary disease (COPD), decreased HDAC activity and expression correlates with disease severity and IL-8 levels, and restoration of HDAC activity in COPD alveolar macrophages normalizes cytokine responses. In particular, reduced expression of HDAC2 is thought to play a central role in asthma and COPD patient resistance to glucocorticoid (GC) therapy. These findings have recently been translated into clinical applications as treatment of COPD patients with oral theophylline, which restores HDAC activity and expression in vitro and in vivo, in combination with GCs, results in improved lung function and decreased inflammatory cytokine levels.
Initial studies analyzing potential deregulation of HDAC activity and expression in RA synovial tissue have generated discordant observations. One study has suggested that HDAC activity, as well as HDAC1 and HDAC2 protein expression, is reduced in RA synovial tissue compared with disease controls and healthy individuals, possibly contributing to inflammatory cytokine transcription. However, others have observed increased HDAC activity and HDAC1 protein expression in RA synovium compared to osteoarthritis (OA) patients and healthy subjects, which positively correlated with TNFα levels. Other independent studies have also reported elevated total HDAC activity in RA patient peripheral blood mononuclear cells compared to healthy subjects, and increased HDAC1 expression in isolated RA FLS compared to FLS obtained from OA patients. While it is thus unclear if (patho)physiological changes in HDAC activity contribute to disease in RA, many lines of experimental evidence suggest that pharmacological manipulation of HDAC activity, through the use of HDAC inhibitors (HDACi), might have potent anti-inflammatory effects in RA. HDACi uniformly suppress inflammatory cytokine production by immune and stromal cells derived from RA patients, and are protective in animal arthritis models. These latter findings provide strong evidence supporting a central role for HDAC activity in the regulation of cellular inflammatory responses.

It still remains unknown if alterations in HDAC expression and activity in RA result in significant changes in acetylation of histones and/or non-histone HDAC targets, and whether such alterations are directly involved in initiating inflammatory mediator production, or are secondary to the ongoing inflammatory processes. In light of a recent clinical trial demonstrating initial clinical efficacy of the HDACi ITF2357 (givinostat) in the treatment of systemic onset juvenile idiopathic arthritis (SOJIA), answering these questions might allow for identification of RA patient subsets most likely to respond to novel therapeutic strategies targeting the epigenome. In this study we directly examined whether reported alterations in synovial HAT and HDAC activity might result in changes in cellular protein acetylation status in RA synovial tissue, and analyzed potential associations of synovial protein acetylation and HDAC expression, with RA patient disease activity and synovial expression of inflammatory mediators.

MATERIALS AND METHODS

Patients and synovial tissue samples. Synovial tissue specimens for immunohistochemical analyses were obtained from 12 RA patients and 12 OA patients undergoing surgical joint replacement at the Clinic of Orthopedic Surgery, Schulthess Hospital Zurich, fixed in paraformaldehyde and embedded in paraffin. Synovial biopsy samples for paired immunohistochemical and mRNA expression studies were obtained by arthroscopy from an actively inflamed knee or ankle joint of 20 patients with RA as previously described, embedded in TissueTek OCT (Miles Diagnostics, Elkhart, IN), snap-frozen by immersion in methylbutane (−80°C), and stored in liquid nitrogen until further processing. All RA patients fulfilled the 1987 revised criteria of the American College of Rheumatology. Clinical characteristics of the patients are summarized in Table 1. Written informed consent was provided by all patients prior to the start of this study. The studies were approved by the medical ethics committees of the Academic Medical Center, University of Amsterdam, The Netherlands and of the Schulthess Hospital Zurich, Switzerland.
Immunohistochemical analyses. Serial sections from six different TissueTek-embedded biopsy samples per patient were cut with a cryostat (5 μm), fixed with acetone, and endogenous peroxidase activity blocked with 0.3% hydrogen peroxide in 0.1% sodium azide/phosphate-buffered saline. Sections were stained overnight at 4°C with rabbit antibodies against acetylated lysine, acetylated H3, acetylated H4 (all from Cell Signaling Technology, Beverly, MA), dimethylated H3K4 (Epitomics, Burlingame, CA), CD3 (Becton Dickinson, San Jose, CA), CD55 (Bioconnect, Huissen, The Netherlands) and CD68 (Dako, Glostrup, Denmark). Equivalent concentrations of irrelevant control rabbit antibodies (anti-fluorescein isothiocyanate (FITC), Thermo Scientific, Waltham, MA) were applied for control sections. Sections were then washed and incubated with swine anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Dako), followed by incubation with biotinylated tyramide and streptavidin-HRP, and development with amino-ethylcarbazole (AEC, Vector Laboratories, Burlingame, CA). Sections were subsequently counterstained with Gill’s hematoxylin (Klinipath, Duiven, The Netherlands) and mounted in Kaiser’s glycerol gelatin (Merck, Darmstadt, Germany). Paraffin-embedded sections were deparaffinized, pretreated at 80°C for 30 minutes in 10 mM citrate buffer (pH 6.0) for antigen retrieval, and endogenous peroxidase activity was blocked with 1% hydrogen peroxide. Sections were then washed and incubated with swine anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Dako), followed by incubation with biotinylated tyramide and streptavidin-HRP, and development with amino-ethylcarbazole (AEC, Vector Laboratories, Burlingame, CA). Sections were subsequently counterstained with Gill’s hematoxylin (Klinipath, Duiven, The Netherlands) and mounted in Kaiser’s glycerol gelatin (Merck, Darmstadt, Germany). Paraffin-embedded sections were deparaffinized, pretreated at 80°C for 30 minutes in 10 mM citrate buffer (pH 6.0) for antigen retrieval, and endogenous peroxidase activity was blocked with 1% hydrogen peroxide. Sections were then blocked with in Tris buffered saline (TBS) pH 7.4 containing 5% horse serum solution and 1% bovine serum albumin, and incubated overnight at 4°C with primary antibodies. After extensive washing sections were incubated with biotinylated goat anti-rabbit antibody (Jackson, Suffolk, UK) followed by incubation with HRP-conjugated streptavidin complex (Vector Laboratories). Bound antibodies were then visualized using AEC and sections counterstained as described above.

Digital image analysis. For quantitative analysis of protein acetylation and methylation, stained sections were randomly coded by an independent observer, blinded to antibodies used and clinical diagnosis. Stained slides were analyzed by computer-assisted image analysis using the Qwin analysis system (Leica, Cambridge, UK) as previously described in detail. Values of integrated optical densities (IOD)/mm² were obtained and corrected for total number of nucleated cells/mm². For quantitative analysis of cell type-specific markers data were presented as number of positive cells/mm².

Table 1. Clinical features of RA and OA patients included in the study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Frozen sections</th>
<th>Paraffin sections</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>RA (n = 20)</td>
<td>RA (n = 12)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>63 (39-68)</td>
<td>63 (54-81)</td>
</tr>
<tr>
<td>Male:female (n)</td>
<td>7:13</td>
<td>10:2</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>79 (1-169)</td>
<td>n/a</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>18.5 (5-104)</td>
<td>n/a</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>7.85 (1-122.6)</td>
<td>46.1 (5-74)</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.7 (2.45-7.49)</td>
<td>n/a</td>
</tr>
</tbody>
</table>

*ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = disease activity score 28, parameters are presented as median (range); n/a = values not available
RNA extraction and quantitative PCR. For analysis of synovial tissue gene expression, total RNA was isolated from biopsies of 20 RA patients using RNA STAT-60™ (Invitrogen, Breda, the Netherlands) according to the manufacturer’s instructions. The total RNA fraction was then cleaned using RNeasy spin columns (Qiagen, Venlo, The Netherlands) including a DNAsase step to remove genomic DNA. Quantity and purity of the RNA was assessed using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). 500 ng of RNA was

| Table 2. Sequences of primers used for qPCR analysis. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| HDAC1 forw      | CCGCATGACTCATAAATTTGC | rev             | TGGAGCGCAAGAATTTAATG |               |
| HDAC2 forw      | AATTTGCGCATTTACCTCA | rev             | TATCCGCTTGCTAGCTC |               |
| HDAC3 forw      | GATGCCATTAGACCAGAAG | rev             | GATCACAGCCAGAGAGTA |               |
| HDAC4 forw      | CGTCCCGGTACTTGTAGTG | rev             | ATTTCCACGAAAACGATAC |               |
| HDAC5 forw      | TTCTTTGACCAGATCTCC | rev             | GTTGCGTGCAAGCTGTTC |               |
| HDAC6 forw      | CGGAGGTCTTTATCGTAGA | rev             | GTAGCGGTGAGAGAAGAT |               |
| HDAC7 forw      | TTCTCTCAGACTGCAGAG | rev             | TTGCAGAGCCAGGAATCT |               |
| HDAC8 forw      | AGCTGAGGACTGCTGCTAT | rev             | CCTGCAGTCACAAAATCCC |               |
| HDAC9 forw      | GTCATTCAACAGCAACACC | rev             | TTCCTCTGCTTCAAGGT |               |
| HDAC10 forw     | CCTGAGCGAGGACAGAGAAG | rev            | AGAGGTACAGGAGATC |               |
| TNFα forw       | CCCAGTTGGAGGAAAACCT | rev             | TGGAGTACAGGGGCTTCC |               |
| MMP1 forw       | AGTGACTGGGAACACAGATGCTGAT | rev   | GCTTTCGGAAATCGCCTGTA |               |
| IL-6 forw       | GACAGCCACTCACTCTTCCA | rev             | CTCCTTTGCTGTTGACAC |               |
| 18S forw        | CGGCTACCACATCCAAGGAA | rev             | GCTGGAATTACCGCGCT |               |
| GAPDH forw      | GCCAGCGCGCCACATC | rev             | TGACGCGCGCACAATAC |               |
reverse transcribed using a First-Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) and quantitative (q)PCR reaction was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA) using Power SybrGreen PCR Master Mix (Applied Biosystems). Sequences of the primers used are listed in Table 2. All qPCR reactions were performed in duplicate and specific amplification of PCR products was confirmed by dissociation curve analysis for each primer pair. Relative gene expression was calculated using StepOne Software v2.1 (Applied Biosystems) and expressed as the ratio between the gene of interest and average expression of 18S and GAPDH.

**FLS culture and stimulation.** FLS were isolated from synovial biopsies of RA patients fulfilling the ACR revised criteria for RA, cultured as previously described, and used for experiments between passages 4-9. 24 h prior to each experiment culture medium was replaced with medium containing 1% fetal bovine serum (Invitrogen). FLS were then stimulated with 1 ng/ml IL-1β (R&D Systems, Minneapolis, MN) for 1-8 hours. Total RNA was extracted using the RNeasy mini kit (Qiagen), reverse transcribed and changes in HDAC isoform mRNA expression analyzed by qPCR as described above.

**Statistical analyses.** The Mann-Whitney U test was used for the comparison of protein acetylation status between the diagnostic groups. Analyses of correlations between experimental groups were performed using the Spearman’s rank correlation coefficient. There was no correction for multiple comparisons due to the exploratory nature of the study.

**RESULTS**

**RA and OA synovial tissue contain comparable levels of acetylated proteins.** Previous studies of proteins and pathways regulating protein acetylation in RA and other rheumatic diseases have been limited to analyses of total HDAC activity in peripheral blood and synovial tissue protein extracts, or synovial HDAC mRNA and protein expression. However, it has yet to be tested whether aberrant HDAC expression and activity translate into altered acetylation of histones and non-histone proteins in the rheumatoid joint. To gain more insight into potential involvement of changes in protein acetylation status in RA, immunohistochemical staining was performed on synovial tissue of RA and OA patients using antibodies specific for acetylated H3, acetylated H4 and acetylated lysine. While no specific staining was observed with irrelevant control rabbit antibodies, robust nuclear staining was detected in the synovial tissue with anti-acetylated H3 and anti-acetylated H4 antibodies. Consistent with histones being major HAT and HDAC substrates, prominent nuclear staining was also observed in sections stained with antibodies against acetylated lysine, but faint cytoplasmatic staining representing acetylation of non-histone proteins was also detected. Protein acetylation was apparent both in the mononuclear cells infiltrating the synovial sublining and in the intimal lining layer (Fig. 1). Quantitative analysis of protein acetylation status revealed similar levels of acetylated proteins in RA and OA synovial tissue. We observed no significant differences in acetylated lysine and acetylated H3 levels between RA and OA (Fig. 2A and B). On the other hand, possibly consistent with studies reporting elevated HDAC activity in RA patients, we found a significant decrease in H4 acetylation levels in RA compared to OA tissue (p = 0.04, Fig. 2C).
Figure 1. Detection of acetylated protein and dimethylated histone 3 in RA synovial tissue. Representative photomicrographs (100x magnification) of tissue sections stained with irrelevant control rabbit antibody (control Ig), or with antibodies recognizing acetylated lysine, acetylated histone 3 (H3), acetylated H4, and dimethylated H3K4. Synovial tissue composition was visualised by staining with antibodies against CD3 (T cells), CD55 (fibroblast-like synoviocytes) and CD68 (macrophages). Stainings were developed using biotin tyramide enhancement followed by horseradish peroxidise and aminoethylcarbazole, and subsequently counterstained with Gill’s hematoxylin.
Protein acetylation in RA synovial tissue correlates with macrophage numbers but not with disease activity. To test the potential relationship of protein acetylation status in the synovial tissue of RA patients with disease activity and synovial tissue composition, we next performed immunohistochemical staining on synovial tissue from an independent cohort of 20 RA patients with antibodies recognizing acetylated H3, acetylated H4, acetylated lysine and dimethylated H3K4. No correlation was observed between serum C-reactive protein (CRP) levels and either acetylation markers or H3K4 dimethylation, a well-described marker of transcriptionally active chromatin (Fig. 3A). Synovial protein acetylation and H3K4 dimethylation status also failed to correlate with either erythrocyte sedimentation rate (ESR), or with DAS28 score (data not shown), indicating no direct link between the global open chromatin state in cells infiltrating RA synovium and disease activity.

To examine whether protein acetylation and histone modification status are associated with the cellular composition of RA synovium, synovial tissue sections from the same RA patients were stained with antibodies against markers specific for T cells (CD3), FLS (CD55) and macrophages (CD68) (see Fig. 1 for representative photomicrographs). We failed to find any relationship between protein acetylation and the numbers of T lymphocytes and FLS detected in the rheumatoid synovium (Fig. 3B and C). Synovial H3K4 dimethylation also did not correlate with T cell and FLS numbers (Fig. 3B and C, right panels). However, consistent with our previous report in which we noted prominent protein acetylation in RA synovial macrophages, we observed strong positive correlations between macrophage numbers and the acetylation status of both histones and total proteins (Fig. 3D; AcLys: $R = 0.61$, $P = 0.004$; AcH3: $R = 0.81$, $P < 0.0001$; AcH4: $R = 0.54$, $P = 0.014$). A positive correlation between numbers of macrophages detected in RA synovial tissue and dimethylated H3K4 was also noted (Fig. 3D, right panels; $R = 0.74$, $P < 0.0001$). Collectively, these results demonstrate that high levels of acetylated histones and dimethylated H3, indicative of open chromatin conformation, are not associated with global disease parameters but rather represent a cell type-specific phenomenon mostly restricted to macrophages infiltrating RA synovial tissue.
Figure 3. Protein acetylation and histone 3 dimethylation in RA synovial tissue are associated with high numbers of synovial macrophages but not with disease activity. Synovial biopsy sections from 20 RA patients were stained with antibodies against acetylated lysine (AcLys), acetylated histone 3 (H3) (AcH3), acetylated H4 (AcH4), dimethylated H3K4 (MeH3), CD3, CD55 and CD68. (A) Correlations of integrated optical density (IOD)/mm² values for AcLys, AcH3, AcH4 and MeH3 staining, calculated as in Figure 2, with the C-reactive protein (CRP) levels analyzed using Spearman’s correlation coefficient. Alternatively, IOD/mm² values for staining of the synovial tissue for AcLys, AcH3, AcH4 and MeH3 were correlated with numbers of positive cells expressing (B) CD3, (C) CD55 and (D) CD68. Circles indicate individual patient values, and Spearman R values and P values are indicated in each graph.

Histone 3 dimethylation rather than histone acetylation is associated with the expression of inflammatory mediators in RA synovial tissue. Since protein acetylation pivotally contributes to the expression of cytokines through both epigenetic and non-epigenetic mechanisms, we next examined whether protein acetylation status is associated with local mRNA expression of inflammatory mediators relevant to RA pathobiology. Total RNA was extracted from synovial biopsies of 20 RA patients previously included in immunohistochemical analyses and expression of TNFα, IL-6 and MMP-1 measured by qPCR. While no association between IL-6 mRNA expression and protein acetylation or H3K4 dimethylation was observed (data not shown), we found a strong positive correlation of dimethylated H3K4 levels with TNFα (Fig. 4A, bottom panel: R = 0.786, P = 0.0002) as well as MMP-1 (Fig. 4B, bottom panel: R = 0.743, P = 0.0006). Although synovial expression of TNFα and MMP-1 also positively correlated with acetylated H3 and acetylated lysine, but not acetylated H4 signal (Fig. 4), these trends were much less pronounced, indicating that H3K4
Figure 4. Synovial expression of TNFα and MMP-1 is associated with H3K4 dimethylation rather than histone or total protein acetylation. Total RNA was extracted from synovial tissue specimens from 20 RA patients, cDNA was synthesized and expression of TNFα and MMP-1 determined by quantitative PCR. Correlations of relative expression of (A) TNFα and (B) MMP-1 with integrated optical densities (IOD)/mm² for stainings with antibodies recognizing acetylated lysine (AcLys), acetylated H3 (AcH3), acetylated H4 (AcH4), and dimethylated H3K4 (MeH3) of respective synovial tissue sections (See figure 3 for details) were calculated using Spearman’s correlation coefficient. Circles indicate individual patient values, and Spearman R values and P values are indicated in each graph.

dimethylation rather than histone or protein hyperacetylation may serve as a better biomarker for predicting local production of mediators of inflammation and tissue destruction.

High levels of class I but not class II HDACs correlate with elevated expression of inflammatory mediators. A recent report has demonstrated that HDAC activity and HDAC1 mRNA expression in RA synovial tissue positively correlates with TNFα levels.23 Here, we examined if this association is restricted to HDAC1 or can be extended to other HDAC isoforms, and whether similar relationships can be found between HDAC expression and other inflammatory mediators contributing to RA pathobiology. We therefore analyzed relative mRNA levels of class I and class II HDACs in RA synovial tissue. TNFα expression demonstrated a strong positive correlation not
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Figure 5. Expression of TNFα and MMP-1 in RA synovial tissue correlates with class I HDAC mRNA levels, while IL-6 expression is negatively associated with the class II HDAC family member HDAC5.

mRNA expression of HDAC1-10, TNFα, MMP-1 and IL-6 was analyzed in synovial tissue of 20 RA patients by qPCR and calculated relative to mean 18S and GAPDH expression. Analyses of correlations between expression of HDAC family members and inflammatory mediator mRNA levels were performed using Spearman’s correlation coefficient. Spearman R values and P values are indicated in graphs representing correlations of selected HDAC isoforms with (A) TNFα, (B) MMP-1 and (C) IL-6. Circles indicate individual patient values.

only with expression of HDAC1 (Fig. 5A, top panel, R = 0.651, P = 0.003), but also to a lesser extent with other class I HDAC family members (Fig. 5A middle and bottom panel; HDAC2: R = 0.523, P = 0.022; HDAC3: R = 0.570, P = 0.011). Synovial MMP-1 expression was also positively associated with the expression of class I HDACs (Fig. 5B): significant correlations were observed for both HDAC1 (R = 0.501, P = 0.029) and HDAC2 (R = 0.512, P = 0.025), while a trend towards positive correlation between MMP-1 and HDAC3 was noted, but did not reach statistical significance. At the same time no correlation was observed between TNFα or MMP-1 mRNA expression and any of the class II HDACs, nor the class I member HDAC8 (data not shown). In contrast, while expression of IL-6 was not associated with any of the class I HDACs (Fig. SC top and middle panel and data not shown), we found a significant inverse relationship between expression of IL-6 and the class II member HDAC5 (Fig. SC bottom panel, R = -0.477, P = 0.039).

HDAC5 expression is inversely related with disease activity and is suppressed by inflammatory stimuli. To gain more insight into the potential involvement of changes in HDAC expression in the pathology of RA we analyzed the relationship between HDAC mRNA levels and clinical parameters of disease activity. Significant negative correlations were observed between HDAC5 expression and the levels of serum CRP (Fig. 6 left panel; R = -0.664, P = 0.007)
and ESR (Fig. 6 middle panel; $R = -0.556, P = 0.013$). We also found a negative association of CRP levels with expression of HDAC6 ($R = -0.632, P = 0.012$) and HDAC8 ($R = -0.561, P = 0.030$), as well as ESR levels with HDAC6 expression ($R = -0.499, P = 0.030$) (data not shown). HDAC5 mRNA levels were also negatively associated with the DAS28 score (Fig. 6 right panel; $R = -0.567, P = 0.011$). Notably, there were no correlations between patient DAS28 score and mRNA levels of any other HDAC family member (data not shown). To verify whether the negative relationship between disease activity and HDAC5 expression might be causative for inflammatory processes, or rather secondary to cellular inflammatory activation, we analyzed the effects of inflammatory stimulation on HDAC mRNA expression in RA FLS. While FLS exposure to IL-1β failed to modulate expression of class I HDACs (Fig. 7 left panel), it caused a time-dependent downregulation of most of the class II HDAC family members (Fig. 7 right panel). Among class II HDACs, HDAC5 was most susceptible to regulation by an inflammatory stimulus – 8 h treatment with IL-1β resulted in 70% reduction of HDAC5 mRNA levels. Collectively, these results indicate that while elevated class I HDAC expression is associated with the local production of mediators of inflammation and tissue damage in the rheumatoid joint, reduced expression of class II HDAC family members, particularly HDAC5, is secondary to ongoing synovial inflammation in RA.

Figure 6. HDAC5 expression negatively correlates with RA patient disease activity. Correlations of HDAC5 mRNA expression levels in RA synovial tissue determined by qPCR with the C-reactive protein (CRP) level (left panel), erythrocyte sedimentation rate (ESR) (middle panel), and disease activity score 28 (DAS28) (right panel) were calculated by Spearman’s correlation coefficient.

Figure 7. IL-1β downregulates expression of HDAC5 and other class II but not class I HDACs in RA FLS. FLS were left untreated or were stimulated with 1 ng/ml IL-1β for the indicated time (h), total RNA was extracted, reverse transcribed and temporal changes in mRNA accumulation of class I (left panel) and class II (right panel) HDACs analyzed by quantitative PCR. Data are presented as mean +/- SEM expression of each HDAC isoform relative to GAPDH of two independent experiments.
DISCUSSION

In asthma and COPD patients, depressed HDAC activity has been clearly shown to contribute to lung inflammation and GC resistance, and restoration of HDAC activity in these patients has demonstrated initial therapeutic potential.\textsuperscript{16,18,20} Translating these observations to understanding and treating pathology in RA, however, have initially failed to generate a consensus in regard to whether HDAC activity is altered in RA, and how HDACs regulate synovial cell activation. Early observations indicated analogies between inflammatory lung disease and RA, noting reduced HDAC expression in RA synovial tissue as compared with OA. This suggested that reduced HDAC activity in RA might stimulate the transcription of inflammatory mediators through epigenetic mechanisms.\textsuperscript{22} In line with this possibility, genetic silencing of HDAC1 and HDAC4 expression in RA FLS \textit{in vitro} potentiates MMP-1 production, pointing towards MMP-mediated acceleration of bone and cartilage destruction as a direct consequence of reduced HDAC levels in RA.\textsuperscript{25,42} Subsequent independent studies, however, reported increased expression of HDAC1 and HDAC activity in RA synovial tissue.\textsuperscript{23,25} Additionally, HDAC activity is increased in peripheral blood mononuclear cells of RA patients compared to healthy controls.\textsuperscript{24} It is as yet uncertain if the different conclusions reached in these studies derive from the relatively small patient cohorts examined in each report or treatment history, and it is also unknown if the reported changes in HDAC activity and expression are sufficient to result in alterations in the acetylation status of histones and non-histone proteins in the synovial tissue or modulate inflammatory activation.

Here, we show that H4 acetylation levels are slightly reduced in RA tissue compared to OA, while comparable levels of acetylated lysine and acetylated H3 are detected in both RA and OA synovium. However, the differences between median H4 acetylation levels in RA and OA patients were relatively minor, and we found no correlation between acetylated H4 and clinical parameters as well as local expression of TNF\textalpha{} and MMP-1. Together, these findings suggest minimal, if any, diagnostic or prognostic value for global histone or protein acetylation status in RA. Although histone acetylation levels are associated with disease activity in other chronic inflammatory disorders, such as systemic lupus erythematosus,\textsuperscript{43} we also find no evidence for correlation between cellular protein acetylation and clinical parameters. This observation, together with a weak relationship of H3 and total lysine acetylation with local expression of inflammatory mediators, argues against a model in which global hyperacetylation of histones can promote expression of cytokines by increasing the accessibility of their promoter regions for transcription.\textsuperscript{44} Our study does not rule out the idea that analysis of epigenetic modification of histones at specific gene promoters may be useful in understanding pathology in RA. In this regard, a recent study has shown enhanced H4 acetylation at the MMP1 promoter, associating with enhanced transcription at this locus.\textsuperscript{42} Future studies will likely need to consider multiple histone markers however, as exemplified by examination of histone modifications following cellular HDACi treatment. For instance, treatment of murine bone marrow-derived macrophages (BMDM) with HDACi induces rapid H4 hyperacetylation in the IL-6 promoter region even though IL-6 mRNA expression is downregulated.\textsuperscript{45} Furthermore, in cancer patients treated with HDACi, increases in global histone acetylation fail to correlate with tumour response to therapy,\textsuperscript{36-48} providing additional evidence for the lack of a straightforward relationship between either global or local changes in histone acetylation status and expression of genes relevant to disease pathology.

Curiously, we did observe an association between protein acetylation status and the cellular composition of RA synovial tissue. The levels of acetylated lysine, acetylated H3 and acetylated
H4 positively correlated with macrophage numbers, but not with FLS or T cells. This finding is consistent with our previous study demonstrating that protein acetylation is most readily detected in CD68-expressing synovial macrophages. A similar positive relationship was noted between dimethylated H3K4 levels and macrophages infiltrating synovial tissue, indicating that macrophages express relatively highest levels of histone modifications characteristic for actively transcribed chromatin regions comparing to other cell types in the inflamed synovium. While independent studies are needed to verify whether histone hyperacytlation observed in RA synovial macrophages is caused by reduced expression of specific HDACs, is secondary to chronic activation of HATs due to persistent exposure to inflammatory stimuli, or a general feature of macrophages relative to other cell types, this observation suggests that macrophages might represent the cell type most vulnerable to anti-inflammatory and/or pro-apoptotic activities of HDACi.

Consistent with the study of Kawabata and colleagues, we find a strong positive correlation of HDAC1 mRNA levels with TNFα expression in the synovial tissue. A positive relationship was also noted between TNFα mRNA levels and expression two other class I HDACs: HDAC2 and HDAC3, and similar trends were observed for MMP-1. A previous report demonstrating that TNFα transiently increases HDAC1 expression and activity in RA FLS raised the possibility that alterations in HAT/HDAC balance in RA synovium might be secondary to persistent synovial cell exposure to inflammatory cytokines and/or treatment history. While this has not been tested formally, a recent study has failed to find an effect of anti-TNFα therapy on cellular HDAC activity in RA peripheral blood mononuclear cells. The positive correlations we observe between class I HDAC expression, TNFα and MMP-1 suggest that class I HDAC activity might be important in promoting inflammation and tissue damage in RA, and partly explain the suppressive effects of HDACi on TNFα and MMP-1 production observed in human immune cells and chondrocytes, respectively. In line with this possibility, the class I-selective HDACi MS-275 is more effective in suppressing inflammation and bone destruction in collagen-induced arthritis in rats and mice than the pan-HDACi suberoylanilide hydroxamic acid (SAHA). However, HDAC1 knockdown can promote MMP-1 production by RA FLS. It remains to be determined if the different influences of class I HDAC inhibition and knockdown on MMP-1 production reflect off-target effects of the inhibitors, or point to important protein-protein interactions between HDACs and transcriptional co-factors which contribute to the effects of HDACi.

Surprisingly, we observed a significant negative association between synovial mRNA expression of class II HDAC5 and disease activity. In RA FLS, IL-1β stimulation caused rapid downregulation of HDAC5 and, to a lesser extent, other class II HDAC family members. Class II HDACs display markedly lower deacetylase activity compared to class I HDACs, which indicates that their non-enzymatic interactions with other proteins might be more important for class II HDAC cellular functions. Our results suggest that class II HDACs are not only regulated by posttranslational modifications affecting their enzymatic activity and nuclear localization, but also undergo regulation at the level of mRNA expression upon inflammatory stimulation. It remains to be explored whether this results in reduced HDAC5 protein expression. Decreased expression of HDAC5 has also been noted in peripheral lung tissue of COPD patients, suggesting that reduction of HDAC5 levels might serve as a sensitive biomarker of local inflammation regardless of triggers underlying the inflammatory process. Indeed, stimulation of murine BMDM with LPS also results in a transient reduction of HDAC5 mRNA levels. Although nothing is currently known about the role of HDAC5 in FLS activation, silencing HDAC5 expression in other cell types contributes to pro-inflammatory processes relevant to RA. HDAC5 is a
negative regulator of nuclear factor of activated T-cells c1 (NFATc1), a key transcription factor regulating osteoclast differentiation, and overexpression of HDAC5 reduces receptor activator of NF-κB ligand (RANKL)-induced NFATc1 activation. In endothelial cells, HDAC5 has an anti-angiogenic effect dependent on its enzymatic activity and nuclear localization, and genetic HDAC5 silencing stimulates endothelial cell migration, sprouting and tube formation. It is therefore possible that inflammation-mediated downregulation of HDAC5 contributes to RA pathobiology by stimulating osteoclast-dependent bone destruction, and by promoting angiogenesis which, as a consequence, facilitates recruitment of activated immune cells to the synovium. Further studies using overexpression and silencing strategies are needed to characterize the biological consequences of reduced HDAC5 expression in RA FLS, facilitating hypothesis-driven decisions as to the suitability of either targeting bulk HDAC activity or inhibiting specific HDAC family members in therapeutic strategies.

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