Apoptosis-induced histone H3 methylation is targeted by autoantibodies in systemic lupus erythematosus

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ABSTRACT

Objectives In systemic lupus erythematosus (SLE) apoptotic chromatin is present extracellularly, which is most likely the result of disturbed apoptosis and/or insufficient removal. Released chromatin, modified during apoptosis, activates the immune system resulting in the formation of autoantibodies. A study was undertaken to identify apoptosis-induced histone modifications that play a role in SLE.

Methods The lupus-derived monoclonal antibody BT164, recently established by selection using apoptotic nucleosomes, was analysed by ELISA, western blot analysis and immunofluorescence staining using chromatin, cells, plasma and renal sections. Random peptide phage display and peptide inhibition ELISA were used to identify precisely the epitope of BT164. The reactivity of plasma samples from lupus mice and patients with SLE with the epitope of BT164 was investigated by peptide ELISA.

Results The epitope of BT164 was mapped in the N-terminal tail of histone H3 (27-KSAPAT-32) and included the apoptosis-induced trimethylation of K27. siRNA-mediated silencing of histone demethylases in cultured cells resulted in hypermethylation of H3K27 and increased nuclear reactivity of BT164. This apoptosis-induced H3K27me3 is a target for autoantibodies in patients and mice with SLE and is present in plasma and in glomerular deposits.

Conclusion In addition to previously identified acetylation of histone H4, H2A and H2B, this study shows that trimethylation of histone H3 on lysine 27 is induced by apoptosis and associated with autoimmunity in SLE. This finding is important for understanding the autoimmune response in SLE and for the development of translational strategies.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease that is characterised by the formation of autoantibodies against nuclear antigens for which the nucleosome is considered a major autoantigen.¹ The nucleosome is comprised of an octamer of two copies of histones H2A, H2B, H3 and H4 with 146 bp of DNA wrapped around it. In SLE these nucleosomes are released from apoptotic cells that are not removed owing to insufficient clearance.^{2–3} This leads to the presence of chromatin (DNA, histones, nucleosomes) in the circulation and tissues.⁴ During apoptosis chromatin can be modified, and several apoptosis-induced modifications have been described including the phosphorylation of H2B on serine 14, considered a hallmark for apoptosis.⁵ In addition to the phosphorylation of serine 14 on histone H2B, the

phosphorylation of serine 28 and threonine 45 and methylation of lysine 27 on histone H3 have been related to apoptosis.^{5–7} Several apoptosis-associated histone modifications have been identified that are targeted by lupus autoantibodies, such as specific acetylation of histone H4, H2A and H2B which we have recently shown.^{8–9}

Apoptosis-induced modifications may enhance the immunogenic potential of chromatin, most likely initiated by ligation of specific receptors on antigen presenting cells. Thereafter, the modified chromatin will be ingested, digested and presented in an immunogenic way by antigen presenting cells.^{8–10–12} We have recently shown that dendritic cells (DC) can be activated, in particular by apoptotic blebs containing histones with apoptosis-associated hyperacetylation or by isolated hyperacetylated nucleosomes.^{8–11–12} Importantly, we identified a mixed Th1/Th17 response induced by apoptotic bleb-matured DC.¹¹

We hypothesised that, after activation by mature DC, autoreactive T helper cells specific for these apoptosis-associated chromatin modifications will stimulate B cells to produce autoantibodies. The autoantibodies will form immune complexes with circulating chromatin and deposit in basement membranes such as the glomerular basement membrane in the kidney, thereby inciting severe glomerulonephritis.¹³

We have recently established a panel of new monoclonal antibodies derived from lupus mice by selection on apoptotic chromatin. One of these monoclonal autoantibodies, BT164, preferentially binds to apoptotic chromatin and, in preliminary experiments, it has been identified as an anti-histone H3 antibody (van Bavel *et al*, unpublished).

In this study we aim to map the exact epitope of BT164 and to identify the presence of apoptosis-associated modifications. Furthermore, we evaluate whether this modified epitope is targeted by autoantibodies from patients with SLE and lupus mice.

METHODS

Plasma

Plasma from 24 patients with SLE with biopsy-proven proliferative lupus nephritis (International Society of Nephrology/Renal Pathology Society class III or IV), 16 patients with SLE without lupus nephritis, 12 patients with rheumatoid arthritis (RA) and 12 patients with systemic sclerosis (SSc) was collected at the Radboud University Nijmegen Medical Centre and Erlangen University. All patients with SLE had ≥ 4 American College of Rheumatology (ACR) criteria.

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Plasma samples were collected from 18 prediseased (6–10 weeks) and 28 diseased (14–22 weeks) MRL/*lpr* mice as approved by the local animal ethics committee.

Cell culture and apoptosis induction

HeLa, Jurkat and 32Dcl3¹⁴ cells were routinely cultured in complete DMEM or RPMI-1640 DM medium (Invitrogen, Breda, The Netherlands). Apoptosis was induced by treatment with 4 µg/ml camptothecin (Sigma-Aldrich, Zwijndrecht, The Netherlands) for indicated periods. The progress of apoptosis was determined by staining with Annexin V (AnV)-fluorescein isothiocyanate and propidium iodide (PI; ITK Diagnostics, Uithoorn, The Netherlands) and analysed by flow cytometry.

Antibodies

BT164 is a lupus-derived monoclonal antibody that was recently established by selection on apoptotic chromatin (van Bavel *et al*, unpublished data). The lupus-derived monoclonal antibody #34 specifically recognises unmodified H3.1/2.¹⁵ Antibodies against methylated H3K27 were provided by Dr A Peters and described previously.¹⁶ Other antibodies used included anti-H3S28p, anti-H3K18ac, anti-H3K23ac (Invitrogen), anti-H3K27ac (Abcam, Cambridge, UK) and anti-EHS laminin (laboratory collection) reacting with laminin-1.

Preparation of cell extracts, histones and immunoprecipitation

Preparation of cell extracts, extraction of histones and protein determination has been described previously.^{8,9}

For immunoprecipitation, cell extracts of apoptotic cells were incubated with BT164. After 2 h, immune complexes were caught by anti-mouse IgG agarose beads (Sigma-Aldrich). After five washing steps, immunoprecipitated proteins were denatured by heating and resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (18%) as described elsewhere.¹⁷

Isolation of chromatin from plasma

Purified BT164 (1 mg) was covalently coupled to 1 ml (bed volume) of cyanogen bromide-activated Sepharose 4B. The column was loaded by circulating 6 ml plasma from patients with SLE or controls for 16 h. Chromatin was eluted with 50 mM citrate buffer pH 2 with 1 M NaCl and immediately neutralised.

Western blot analysis

Proteins resolved by SDS-PAGE were blotted as previously described.¹⁸ Immunoblots were incubated with the indicated primary antibodies and the appropriate IRDye800-conjugated antibodies (Westburg, Leusden, The Netherlands) for detection and analysed with the Odyssey infrared scanner (LI-COR Biosciences, Lincoln, Nebraska, USA).

Random peptide phage display

Random peptide phage display with the 12-mer Ph.D.-12 Phage Display Peptide Library kit (New England Biolabs, Ipswich, UK) was used to determine the epitope of BT164 according to the manufacturers' protocol. After three panning and amplification rounds with decreasing antibody concentrations, individual clones were selected for isolation of ssDNA with the Qiagen Spin M13 kit (Qiagen, Hilden,

Figure 1 Higher reactivity of lupus-derived clonal antibody BT164 with apoptotic chromatin. (A) BT164 shows a higher reactivity with apoptotic nucleosomes (dashed line) in ELISA compared with control nucleosomes (continuous line). (B) BT164 shows an increased immunofluorescent staining with apoptotic cells compared with control cells (blue, diamidino-2-phenylindole; green, BT164; red, anti-H2BS14p). The epitope of BT164 appears to be translocated to the surface of the apoptotic blebs as most staining in the apoptotic cell is observed in the blebs. Apoptosis was induced by camptothecin. (C) Enhanced reactivity of BT164 with apoptotic histone H3 on western blot analysis is observed 4 h after induction of apoptosis. As a control, #34 recognising non-modified histone H3 is used as a probe. With #34 no difference was observed between control and apoptotic histones and there is equal loading. Apoptosis-associated triacetylation of H4 as probed by KM-2 also increases in apoptotic extracts as reported previously.⁸ The rate of apoptosis is measured by staining with Annexin V (AnV) and propidium iodide (PI). AnV+/PI- cells represent early apoptotic cells; AnV+/PI+ cells represent late apoptotic cells.

Germany) according to the manufacturer's protocol. Phage ssDNA was sequenced on the 3730 Sequence Analyzer (Applied Biosystems, Foster City, California, USA) according to the Sanger method and aligned using ClustalW2.

Peptide (inhibition) ELISA

The peptide (inhibition) ELISA was performed as described previously.⁹ BT164 (150 ng/ml) was preincubated with the indicated competitor peptides and subsequently transferred to a Maxisorp 96-well plate (Nunc, Uden, The Netherlands) coated with 0.1 µm unmodified H3 (23–34) peptide. Peptides H3 (18–37), H3.3 (18–37), H3S28p (18–37), H3 (23–34), H3K27ac (23–34), H3K27me1 (23–34), H3K27me2 (23–34) and H3K27me3 (23–34) were synthesised in our laboratories in Strasbourg or obtained commercially.

Immunofluorescence staining

Cells or 2 µm frozen sections of mouse kidneys were fixed with 2% (w/v) paraformaldehyde and permeabilised with 0.05% (v/v) Triton X-100, blocked with 2% (w/v) BSA and subsequently incubated with the primary antibody. Appropriate Alexa 488- or Alexa 594-labelled secondary antibodies (Invitrogen) were used for detection. All cells and sections were embedded in VectaShield mounting medium H-1200 containing the DNA stain 4',6-diamidino-2-phenylindole (Vector Laboratories, Peterborough, UK).

siRNA transfection

HeLa cells were transfected with siRNA specific for the histone demethylases JMJD3 and UTX, or control siRNA (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) with Oligofectamine (Invitrogen) according to the manufacturer's protocol. Briefly, 54 pmol siRNA or 1.08 µl oligofectamine were each diluted in 135 µl serum-free Opti-MEM (Invitrogen).

After 5 min of incubation, siRNA was gently mixed with oligofectamine for 20 min. The culture medium from the wells was replaced with the 270 µl transfection mix. Cells were analysed 48 h after transfection.

Statistical analysis

Statistical analysis was performed by Mann–Whitney U test and the Student t test using Graphpad Prism V.4.0 (GraphPad Software, San Diego, California, USA). A p value <0.05 was considered significant.

RESULTS

The lupus-derived monoclonal antibody BT164 preferentially recognises apoptotic chromatin

BT164 has been selected for its higher reactivity with apoptotic nucleosomes in ELISA than control nucleosomes (figure 1A). This enhanced reactivity was also observed with apoptotic cells and blebs (figure 1B). It appeared that BT164 in particular stained apoptotic cells and blebs that also stained positively with anti-H2BS14p, the latter antibody defining an established apoptosis-induced histone modification.⁵ Western blot analysis revealed an increased staining by BT164 of histone H3 isolated from apoptotic cells (46% AnV+/PI–; 17% AnV+/PI+) compared with H3 isolated from normal cells (8% AnV+/PI–; 5% AnV+/PI+) (figure 1C). Inhibition of apoptosis by Z-VAD-FMK prevented the induction of the epitope of BT164 (data not shown). This finding with BT164 preferentially reacting with apoptotic H3 was similar to the previously identified apoptosis-induced hyperacetylation of H4 as defined by the lupus-derived monoclonal

Figure 2 (A) Fine mapping of the epitope of BT164. The epitope of BT164 as mapped with random peptide phage display. The alignment of several clones with the amino acid sequence of histone H3 (27-KSAPAT-32) is shown. The consensus of all sequences is IAAPAS. The lysine on position 27, which can be modified, is never found in any sequence. (B) BT164 recognises methylated H3K27 with a preference for trimethylated H3K27 as shown by inhibition ELISA. The amount of peptide necessary to inhibit the binding of BT164 to coated H3 peptide (23–34) shows that trimethylation of H3K27 gives the strongest inhibition. H3 peptides acetylated at K27, phosphorylation at S28 and the H3.3 variant peptide do not inhibit the binding of BT164. *p < 0.05 vs non-modified peptide. (C) BT164 has a higher nuclear reactivity with cells containing hypermethylated chromatin. Immunofluorescence staining of cells transfected with siRNA of H3K27-specific histone demethylases UTX and JMJD3, which results in hypermethylation of H3K27, shows increased staining of BT164 (left panel) and anti-H3K27me3 (right panel). Because UTX and JMJD3 are H3K27me2- and H3K27me3-specific demethylases, blocking these enzymes leads to a decreased amount of H3K27me1, as revealed by staining with anti-H3K27me1 (middle panel).

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antibody KM-2, whereas the reactivity of antibody #34 specific for unmodified H3.1/3.2 was not affected by apoptosis (figure 1C).⁸ Apparently, BT164 recognises an epitope within histone H3 that is modified during apoptosis.

Fine mapping the epitope of BT164

To map the exact epitope of BT164 we performed random peptide phage display. After three panning rounds a consensus was obtained with sequence IAAPAS/T corresponding to amino acids 27-KSAPAT-32 from histone H3 (figure 2A). However, the lysine on position 27 was never identified in the selected random peptide expressing phage clones. The identified amino acid on position 27 in the isolated clones was in general neutrally charged, indicating that this lysine residue is probably neutralised by post-translational histone modifications, and the most likely candidates are acetylation or methylation (figure 2A). Note that these post-translational modifications are not synthesised in the bacterial random peptide expressing system, thus explaining the presence of neutrally charged residues mimicking methylated K27 in the consensus motif.

Peptide inhibition ELISAs were conducted in which the binding of BT164 to coated non-modified H3 peptide (23–34) was tested after preincubation with defined modified and non-modified H3 peptides. We observed significantly decreased binding of BT164 with the coated unmodified H3 peptide only when inhibited with peptides containing three methyl groups on lysine 27 (figure 2B). H3 peptide acetylated on lysine 27 or phosphorylated on serine 28 or the non-modified H3.3 variant (alanine at position 31 is replaced by a serine) did not inhibit binding to unmodified H3 peptide. These findings indicate preferential binding of BT164 to, in particular, the H3 peptide trimethylated at lysine K27.

BT164 preferentially reacts with trimethylated H3K27 in situ

Histone H3K27 methylation is carried out by histone methyltransferase EZH2 as part of the polycomb complex PCR2. The site-specific demethylases for H3K27, UTX and JMJD3, which catalyse demethylation of the tri- and dimethylated forms to the monomethylated H3K27 have recently been discovered.^{19–22} To induce a possible hypermethylation of histones in situ, we applied siRNA-mediated gene silencing of the histone H3K27me2- and H3K27me3-specific demethylases UTX and JMJD3 in HeLa cells. We then stained these manipulated cells with BT164 and antibodies specific for H3K27me1 and H3K27me3 (figure 2C). Nuclear staining by BT164 was strongly increased in cells transfected with siRNA specific for the demethylases UTX and JMJD3 compared with cells transfected with control siRNA (figure 2C, left panels). As expected, nuclear staining by an anti-H3K27me3 antibody was also increased in cells transfected with UTX/JMJD3 siRNA (figure 2C, right panels), while nuclear staining by anti-H3K27me1 was decreased (figure 2C, middle panels).

In addition, we found that immunofluorescence double staining of apoptotic cells with BT164 and antibodies against methylated H3K27 (mono- di- and trimethylation), acetylated H3K27, phosphorylated H3S28 and #34 (anti-H3.1/H3.2) only revealed co-localisation with antibodies against methylated histone H3K27 (figure 3A). We also performed immunoprecipitation experiments with BT164 using apoptotic chromatin as input. Probing of blots of resolved immunoprecipitates with BT164, anti-H3K27me3 and anti-H3K27ac only revealed precipitated bands with BT164, as expected, and with anti-H3K27me3 but not with anti-H3K27ac (figure 3B). Apparently, methylated histone H3 in apoptotic chromatin is predominantly

Figure 3 BT164 specifically stains and precipitates H3K27me3. (A) BT164 (green) immunofluorescence staining co-localises (yellow) only with antibodies against methylated H3K27 (red). No co-localisation is observed with antibodies (red) directed against other possible modifications present in the epitope of BT164. Antibody (Ab) #34, which recognises unmodified H3, does not show co-localisation with BT164. (B) BT164 immunoprecipitates apoptotic trimethylated histone H3K27. Apoptotic chromatin immunoprecipitated with BT164 is recognised by BT164, as expected, and by the anti-H3K27me3 antibody, while precipitated chromatin does not contain acetylated H3K27 since no staining is observed with anti-H3K27ac.

precipitated by BT164. Taken together, all these in situ data are in agreement with the peptide inhibition ELISA data, indicating that histone H3 trimethylated at K27 is the preferred epitope of BT164.

Methylation of histone H3 in SLE

The immunogenicity of apoptotic chromatin containing trimethylated H3 at K27 is in part dictated by its release and presence in the circulation and other tissues. When we

analysed kidney sections of control mice (MRL^{+/+}) and lupus mice (MRL/lpr) by staining with BT164, nuclear staining was observed in both mice as expected. However, glomerular staining of BT164 outside nuclei was observed only in lupus mice, as visualised by co-staining with anti-laminin (figure 4A). Using an affinity purification column with BT164 we were also able to specifically isolate chromatin containing trimethylated H3 at K27 from plasma of patients with SLE as probed with BT164 (figure 4B). Plasma from healthy controls did not contain any chromatin. The epitope of BT164 (methylated histone H3) therefore seems to be present in extracellular chromatin deposited in the glomerular basement membrane in diseased lupus mice and in the circulation of patients with SLE.

We further investigated the relevance of apoptosis-associated methylation in SLE by analysing the reactivity of plasma with the epitope of BT164 in peptide ELISA. We used plasma from prediseased and diseased lupus mice, from patients with SLE with and without lupus nephritis and from control patients with RA and SSc. The reactivity of the majority of plasma samples from (pre)diseased lupus mice and patients with SLE with or without lupus nephritis was significantly higher with the trimethylated H3 peptide compared with the control peptide, whereas almost no reactivity was observed with plasma from control disease patients (RA and SSc) (figure 4C). In addition, there was a significantly higher reactivity with either H3 peptide of plasma from diseased mice compared with plasma from

Figure 4 H3K27me3 is present in glomerular deposits and in plasma and is recognised by lupus autoantibodies. (A) Extracellular glomerular staining of kidney sections of MRL/lpr lupus mice by BT164. In MRL^{+/+} mice only nuclear staining of the glomerular cells is observed. In MRL/lpr mice extracellular staining is observed which co-localises with anti-laminin-1 staining expressed in the glomerular basement membrane. In the merge, co-localisation of BT164 (green) with the nuclei (diamidino-2-phenylindole) and laminin-1 (red) is seen. (B) Trimethylated H3 (15 kDa) is present in the plasma of patients with systemic lupus erythematosus (SLE) but not controls (C). Plasma from a patient with SLE or control was affinity-purified with a BT164 column and the eluate was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, blotted and probed with BT164 (molecular weight is shown in kDa). (C) Higher reactivity with the H3 peptide trimethylated on K27 compared with non-modified H3 peptide of 13/18 plasma samples from pre-diseased lupus mice, 24/28 plasma samples from diseased lupus mice, 17/24 plasma samples from patients with systemic lupus erythematosus (SLE) with lupus nephritis and 13/16 plasma samples from patients with SLE without lupus nephritis. Plasma from control patients with rheumatoid arthritis or systemic sclerosis do not show (enhanced) reactivity with the trimethylated peptide H3 peptide. Plasma from healthy control humans and mice do not show any reactivity with either peptide (data not shown). tri-me, trimethylated; unm, non-methylated. *p<0.05; **p<0.01; ***p<0.001.

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prediseased mice. Plasma samples from healthy control humans and mice did not show any reactivity with either H3 peptide (data not shown). These findings indicate that autoantibodies derived from lupus plasma samples preferentially recognise the apoptosis-associated trimethylation of H3 at K27 compared with the non-methylated H3K27.

DISCUSSION

We recently established the lupus-derived monoclonal antibody BT164 that was specifically selected on apoptotic chromatin. In the current study, using different approaches, we identified the exact epitope of BT164 in histone H3 (amino acids 27–32). Importantly, recognition of apoptosis-induced trimethylation on lysine 27 (H3K27me3) is an important characteristic of this lupus-derived autoantibody.

The association of trimethylation of H3K27 with apoptosis has also been described recently by others.⁷ The mechanisms and consequences of apoptosis-specific histone H3K27 methylation are not known yet. Trimethylation of lysine residues neutralises the positive charge which may induce changes in the chromatin structure required for facilitating the process of apoptosis. In particular, methylation of histone H3K27 is commonly associated with repression of genes that may be linked to condensation of chromatin.^{16 23 24} Kim *et al*²⁵ showed that methylation of H3K27 by the histone methyltransferase WHISTLE is important for the induction of caspase 3-mediated apoptotic cell death. Epigenetically, enhanced histone H3K27 methylation is found in CD4 T cells of lupus mice,²⁶ which coincides with the decreased acetylation of histone H3 in lupus-prone mice and patients with SLE.^{26 27} Finally, methylation of H4K20 has also been related to apoptosis.²⁸

Serum samples from patients with SLE have previously been found to react with the N-terminal tail of histone H3,^{29 30} and recently the epitope of another lupus-derived monoclonal antibody (#34) has also been mapped in the N-terminal tail of histone H3 from amino acids 28–33.¹⁵ In contrast to these B cell epitopes, histone H3-specific T cell epitopes have so far been identified mainly outside the N-terminal tail of H3.^{31–33}

This study shows that methylated histone H3 is a target for lupus autoantibodies. Apoptosis-associated trimethylation of H3K27 seems to be associated with the increased immunogenic potential of apoptotic chromatin, similar to the specific acetylation patterns we have recently described for H2A, H2B and H4.^{8 9} It is conceivable that a pattern of combined chromatin modifications associated with apoptosis specifically ligates to receptors on antigen presenting cells such as DC, thereby activating these cells and resulting in an immunogenic presentation. The predominant staining of BT164 of apoptotic blebs indicates that the trimethylated histone H3K27 is translocated during apoptosis from the nucleus to apoptotic blebs which is consistent with reports on the presence of autoantigens in apoptotic blebs.^{34 35} It is noteworthy that we have recently shown that apoptotic blebs containing apoptosis-associated histone modification induce maturation of DC and subsequently activation of Th1 and Th17 T cells.^{11 12}

The enhanced reactivity of autoantibodies present in the plasma from lupus mice and patients with the trimethylated H3K27 peptide compared with the non-modified peptide was specific for SLE as there was hardly any reactivity in plasma samples from patients with RA or SSc and healthy controls. Furthermore, released apoptosis-induced methylated histone H3 was present in glomerular deposits in lupus mice and not in non-lupus mice. Importantly, we also showed the presence

of apoptosis-induced methylated histone H3 in the plasma of patients with SLE but not in controls. Apoptosis-associated H3K27 methylation appears to be a target for the immune system leading to the production of autoantibodies which deposit with apoptotic chromatin in the glomerular capillary filter. These chromatin deposits are an important feature of lupus nephritis.³⁶ This shows that, in SLE, modified apoptotic chromatin is released which triggers the immune system.

Peptides or combinations of peptides containing apoptosis-induced modifications associated with SLE such as specific acetylation of H2A, H2B, H4^{8 9} and methylation of H3 (this study) could lead to the development of novel diagnostic and prognostic approaches. Furthermore, application of such peptides with apoptosis-induced modifications may even lead to tolerance against these immunogenic key autoantigens in SLE.^{37 38}

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Competing interests None.

Ethics approval This study was conducted with the approval of the local ethics committee of the Radboud University Nijmegen Medical Centre.

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