Participation of histones and ubiquitin in lupus nephritis

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To evaluate the role of histones and ubiquitin in lupus nephritis, we searched for glomerular deposits of histones and ubiquitin in renal biopsy specimens from 53 patients with systemic lupus erythematosus (SLE) and 30 with non-lupus glomerulonephritis. Glomerular immunofluorescence staining revealed positive for histone H2A, H1 + H3, H4 and ubiquitin in 49.1% (26/53), 45.3% (24/53), 32.1% (17/53) and 22.6% (12/53) of the SLE patients, respectively. Non-SLE renal biopsies revealed absence of positive staining with histone H2A, H1 + H3, H4 and ubiquitin. The positive incidence of histone H1 + H3 and ubiquitin in diffuse proliferative lupus nephritis was significantly different (p<0.01) from that in minor glomerular abnormality. Levels of CH50 in patients with glomerular deposition of histone H1 + H3 (p < 0.001) and ubiquitin (p < 0.01) were significantly lower than in patients without deposition. Levels of anti-DNA antibody in patients with glomerular deposition of histone H1 + H3 were significantly higher than in patients without deposition (p < 0.05). Only the positive incidence of glomerular deposition of ubiquitin was correlated with the histological activity index (p <0.05). These results suggest that histones and ubiquitin may play an important role in the induction of lupus nephritis.


Key words: lupus nephritis, histones, ubiquitin

Introduction

The development of glomerulonephritis is a frequent and prognostically unfavorable disease manifestation in patients with systemic lupus erythematosus (SLE). The pathogenesis of this form of glomerulonephritis, known as lupus nephritis, is still poorly understood in spite of extensive studies using human and animal models. However, recent reports [1, 2] have implicated histones and ubiquitin in the induction of glomerulonephritis in human SLE and lupus mice.

Histones are a set of highly cationic proteins essentially involved in the binding and compaction of DNA in the cell nucleus chromatin [3]. Experimentally cationized protein antigens are excellent nephritogens [4]. These antigens show high affinity for the anionic site of the GBM and can act as planted target antigens for a subsequently injected antibody. The molecular mass (>40 kd) and isoelectric point (>8.5) of the antigen determine its affinity for the GBM [5]. Histones not only possess regions of positive charge [6], but also aggregate spontaneously to form stable complexes [7], making them excellent candidates for inducing immune-complex formation in the renal glomerulus.

Ubiquitin is a small protein known to be involved in the stress response system and possibly in DNA repair [8]. In the nucleus, ubiquitin attaches to histone H2A and H2B, forming branched molecules [9]. Levels of histone ubiquitination change as cells progress through the cell cycle or respond to stress. Muller et al. [10] suggested that the immune response to ubiquitin occurs because the local ubiquitin concentration increases when DNA repair systems are triggered by various factors, such as infection, UV irradiation, stress and metabolic changes. They also suggested that ubiquitin, associated with various nuclear, cytoplasmic and membrane components, has been found in every cell type examined, which is consistent with the extremely diverse non-organ-specific immune response found in SLE patients.

On the basis of these implications, we hypothesized that histones and ubiquitin deposits would be found in the glomeruli of SLE patients, and that the deposits would be related to clinical data and histological activity. The purpose of this study was to test this hypothesis and
to determine what relationships, if any, exist between these glomerular deposits and disease manifestations.

### Materials and Methods

#### Patients

Renal biopsy tissues from a total of 53 patients diagnosed in our renal division as having SLE and fulfilling four or more of the ARA [11] criteria for lupus nephritis were used in this study. WHO criteria, as outlined by Churg and Sobin [12], were followed in assigning the histopathological classification: 15 patients had minor glomerular abnormalities (MGA) (WHO I), 21 patients had mesangial lupus nephritis (MesLN) (WHO II), 12 had diffuse proliferative glomerulonephritis (DPLN) (WHO IV), and 5 had membranous lupus nephritis (MLN) (WHO V). Renal tissues from 30 non-SLE patients with primary glomerulonephritis served as controls; 10 of these patients had IgA nephropathy, 10 had membranous nephropathy, and the remaining 10 had membranoproliferative glomerulonephritis.

Preparation of anti-histone antibodies

Rabbits were immunized with histone subfractions (H1, H2A, H2B, H3 and H4) (Boehringer Mannheim, Mannheim, Germany) complexed to keyhole limpet hemocyanin (KLH) (Sigma Chemical Company, St. Louis, MO, USA) in complete Freund’s adjuvant (CFA) (Sigma). The histone subfractions were extracted from calf thymus and 300 µl of one of these histone subfractions (12 mg/ml in PBS) was mixed with 100 µl of KLH (5 mg/ml in PBS) at 4°C and incubated at 20°C for 30 min; thereafter, 400 µl of CFA was added and the mixture was emulsified. Rabbits were injected subcutaneously with the mixtures three times at two-week intervals, for a total of 3 injections in 6 weeks, and bled two weeks after the last injection. Sera were tested for antihistone antibodies by Western blotting.

**SDS-PAGE electrophoresis**

Total histones were diluted with sample buffer (0.01 M Tris buffer, 1 mM EDTA, 1% SDS, PH 8.0) without 2-mercaptoethanol, boiled for 5 min, and analyzed on 4-20% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Pharmacia-LKB Biotechnology, Uppsala, Sweden).

Individual histones were used to confirm of the responsibility of individual anti-histone antibodies.

**Western blot procedure**

After electrophoresis, the histones were transferred to nitrocellulose membranes (Bio Rad, Richmond, CA, USA) with a pore size of 0.2 µm by electrotransfer. The nitrocellulose membrane was incubated in 10% skimmed powdered milk/PBS solution, cut into 1-cm wide strips and the strips incubated overnight in a 1:1000 dilution of the rabbit sera in skimmed milk/PBS plus 0.1% Tween; this was followed by several washings in PBS/Tween. Thereafter the strips were incubated with a 1:5000 dilution of the peroxidase-labeled goat anti-rabbit IgG (Cappel Lab., Durham, NC, USA) for 2 h, followed by washing in PBS/Tween. The strips were developed with diaminobenzidine (10 mg diaminobenzidine (Sigma) + 5 µl H2O2 in 50 ml 0.05 M TBS buffer (0.05 M tris buffer + 0.15 M NaCl, PH 7.6)) for about 30 minutes. The reaction was stopped in PBS.

**Immunohistochemical staining**

Indirect immunofluorescence staining was performed on 4-µm-thick fresh frozen sections. Sections were air-dried and fixed in cold acetone. After the sections were washed with PBS, nonspecific binding sites were blocked for 10 minutes with 10% skimmed milk/PBS. Subsequently, they were incubated with rabbit anti-histone antibodies and anti-ubiquitin antibody (Sigma) for 2 h in a moist chamber at 37°C. The sections were then washed with PBS and incubated for 30 min in the moist chamber at 37°C with fluorescein isothiocyanate-conjugated anti-rabbit Ig (Cappel) as the second antibody.

**Statistical analysis**

All values were expressed as mean ±SEM. Wilcoxon’s U-test and student’s t-test were used to compare between means; differences were considered significant if p was less than 0.05.

### Results

#### Characterization of the polyclonal anti-histone antibodies

In the Western blot staining with the anti-histone antibodies, a single band in the anti-histone H2A and H4 antibodies, and two bands in the anti-histone H3 antibody were recognized (Fig. 1). We used individual histones in the Western blot to confirm each band. In lane 6, the larger band was histone H1 and the smaller band was histone H3 (data not shown). Therefore, we designated this polyclonal antibody as anti-histone H1 + H3 antibody. Normal rabbit sera showed no anti-histone reactivity.

**Immunofluorescence studies on renal biopsy material**

In kidney biopsy tissues from SLE patients, the positive incidence of glomerular deposition of histone was 32–49% and that of ubiquitin was 23.1%. The pattern of staining against histone H4 and ubiquitin was generally granular in the capillary and mesangial areas (Figs. 2 and 3). The positive staining pattern of histone H1 + H3 and H2A was similar to the staining pattern of histone H4 and ubiquitin (no photos shown). In light microscopy, diffuse proliferation of mesangial cells was seen in the patient shown in Figs. 2 and 3 (Fig. 4). The positive incidence of glomerular deposition of histone H1 + H3
and ubiquitin in DPLN (WHO class IV) was significantly higher compared with that in MGA (WHO class I) (p < 0.05, Table 1). Glomerular depositions of histone and ubiquitin were not seen in any of the kidney biopsy tissues from non-SLE primary glomerulonephritis patients.

Figure 5 shows an example of staining for IgG (A) and C3 (B) in the same patient as shown in Figs. 2–4. The staining pattern of IgG and C3 was similar to the pattern of histones and ubiquitin, with granular glomerular deposits seen in the mesangial area and along the capillary wall.

Comparison of clinical data in SLE patients with or without deposition of histone and ubiquitin

SLE patients with depositions of histone H1 + H3 and
ubiquitin had significantly lower CH50 levels than patients without histone H1 + H3 and ubiquitin (20.1 vs 32.6, \( p < 0.001 \); 17.9 vs 28.8, \( p < 0.01 \), respectively) (Table 2). Patients showing depositions of histone H1 + H3 also had significantly higher anti-DNA antibody levels than did those without deposition of histone H1 + H3 (41.2 vs 17.6, \( p < 0.05 \)). There were no correlations between urinary proteins and depositions of histone and ubiquitin (Table 2).

**Correlations between histological activity and chronicity indices, and depositions of histone and ubiquitin**

The positive incidence of glomerular deposition of ubiquitin was significantly (\( p < 0.05 \)) correlated with the activity index (Table 3). There were no significant correlations between chronicity index and the positive incidence of histone or ubiquitin deposition.

**Discussion**

In the present study, evidence for the presence of histone H2A, H1 + H3, H4 and ubiquitin in the glomeruli...
of SLE patients was obtained. We also demonstrated that depositions of histone H1 + H3 and ubiquitin were related to the levels of anti-DNA antibody and complement and that the deposition of ubiquitin was correlated with the activity index.

It is generally assumed that anti-DNA antibodies are involved in the induction of lupus nephritis [13], because elevated levels of circulating anti-DNA antibody in SLE are often followed by active manifestation of renal disease [14]. The presence of anti-DNA antibodies has been demonstrated in renal and glomerular eluates obtained from SLE patients and lupus mice [15, 16]. It has also been reported that histones have a high affinity for the glomerular basement membrane (GBM) and deposited histones mediated the binding of DNA fragments to the GBM [17–19].

Histones and ubiquitin were detected in kidney biopsy specimens taken from humans [1] and from lupus mice [2]. Schmiedeke et al. [2] reported that histones H2A and H3 were detected in glomeruli of 8 out of 12 protein-uric NZB/W F1 mice, while Stockl et al. [1] reported that positive glomerular depositions of histone H2A, H3 and ubiquitin were detected in 3/4 of their SLE patient population.

Anti-ubiquitin antibody was reportedly present in the serum of about 80% of the SLE patients in a study by Muller et al. [10]. In a study by Kohda et al. [20], the positive incidence of anti-histone antibodies in serum from SLE patients was 78.3%, and the positive incidence of anti-histone H2B antibody was highly correlated with the renal histological and clinical activity of the disease. In lupus mice, histones are frequently recognized in the serum and anti-histone antibody regularly appears several weeks before the onset of glomerulonephritis [2].

Although we attempted to make anti-histone H1, H2A, H2B, H3 and H4 antibodies, we succeeded only with anti-histone H2A, H1 + H3 and H4 antibodies, which were used in the present study. The histone H1, H2A, H2B and H4 were pure and only a single band was seen in SDS-PAGE, but histone H3 was not pure. The composition of histone H3 from Boehringer Mannheim was 90% consisting of histone H3 and 10% of other histones; and the polyclonal antibody to histone H3 reacted with histone H3 and H1.

Stockl et al. [1] reported that positive staining of synthetic peptide consisting of segments 1–21 of histone H3 and segments 22–45 of ubiquitin and ubiquitinated histone H2A was found in 65%, 29% and 54%, respectively, of patients with SLE. They used synthetic peptides of histones [21] for antigens, and suggested [2] that only the N-terminal regions of histones H3 (1–21) and H2A (1–9, 1–15) were exposed in glomerular deposits, and that the other histones and other regions of histones H3 and H2A were negative. In human SLE, only one patient showed positive deposition of segment 1–15 of histone H2A, although 26 of 48 patients had positive deposition of the branched region of ubiquitinated histone H2A [22]. In the present study, we used whole histone molecules as the antigenic protein, which may explain the differences between our data and those of Stockl et al. [1].

The data in the present study clearly support the hypothesis that glomerular deposits of ubiquitin and histones H2A, H1 + H3 and H4 are present in a significant proportion of SLE patients (Table 1), but not in biopsy tissues from non-SLE glomerulonephritis patients. Further, glomerular deposition of histone H1 + H3 and ubiquitin is correlated with the level of CH50, and histone H1 + H3, and with the titer of anti-DNA antibody (Table 2), although only glomerular deposition of ubiquitin is correlated with the histological activity index (Table 3). These data support the notion that histones and ubiquitin may play an important role in the induction of lupus nephritis.

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References

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