Lupus nephritis: consequence of disturbed removal of apoptotic cells?

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ABSTRACT

In the last decade it has become clear that systemic lupus erythematosus (SLE) is an autoantigen driven T cell dependent autoimmune disease. The nucleosome has been identified as a major autoantigen. Nucleosomes are generated during apoptosis. Either an increased or delayed apoptosis or a reduced clearance of apoptotic cells (which are not mutually exclusive) leads to an increased exposure of (modified, more immunogenic) nucleosomes to the immune system. This generates the formation of nucleosome specific T cells and antinucleosome autoantibodies. After complex formation of antinucleosome or anti-double-stranded (ds)DNA antibodies with nucleosomes, these autoantibodies are targeted to basement membranes, especially the glomerular basement membrane (GBM). This nephritogenic potential is due to the binding of the positively charged histone components of the nucleosome to the negatively charged heparan sulphate (HS) within the GBM. This incites glomerular inflammation.

INTRODUCTION

Formation of antinuclear autoantibodies, especially against double-stranded (ds)DNA, is a hallmark of systemic lupus erythematosus (SLE). Lupus nephritis is one of the most serious complications in SLE, occurring in up to 60% of the patients with SLE. Traditionally, it was thought that lupus nephritis was initiated by the glomerular deposition of DNA/anti-DNA complexes.[1] However, DNA/anti-DNA complexes are hardly nephritogenic.[2]
In 1995 we presented a new hypothesis for the development of lupus nephritis[3] depicted in *figure 1*. The importance of apoptosis and the phagocytosis of apoptotic cells in SLE was recently supported by various observations. In this review we will discuss the support for the different parts of the hypothesis outlined in *figure 1*.

APOPTOSIS AND SLE

The first notion that abnormal apoptosis was associated with lupus came from the discovery that MRL/lpr lupus mice had a functional Fas deficiency. Binding of the Fas ligand to the Fas receptor (CD95), present on activated T and B cells, leads to apoptosis. Also deficiency of the Fas ligand as in gld mice, leads to the same lupus phenotype as in MRL/lpr mice. Transgenic correction of these deficiencies prevented the development of autoimmunity. In patients with SLE, Fas-related defects in apoptosis are less clear. Patients with a Fas or Fas-ligand deficiency develop an autoimmune lymphoproliferative syndrome (ALPS). This condition is characterised by lymphadenopathy, splenomegaly, haemolytic anaemia and thrombopenia. Only a quarter of the patients develop antinuclear antibodies and glomerulonephritis is rarely seen. So, unlike the animal models, in human SLE no clear-cut genetic defects in the Fas pathway have been detected so far.[4] From these data it is difficult to draw an unequivocal conclusion at this moment on the role of apoptosis in human SLE. It seems likely that in SLE the delicate balance regulating apoptosis is lost, resulting in apoptosis which occurs at the wrong time-point and/or in the wrong micro-environment.[5] Abnormal apoptosis in itself does
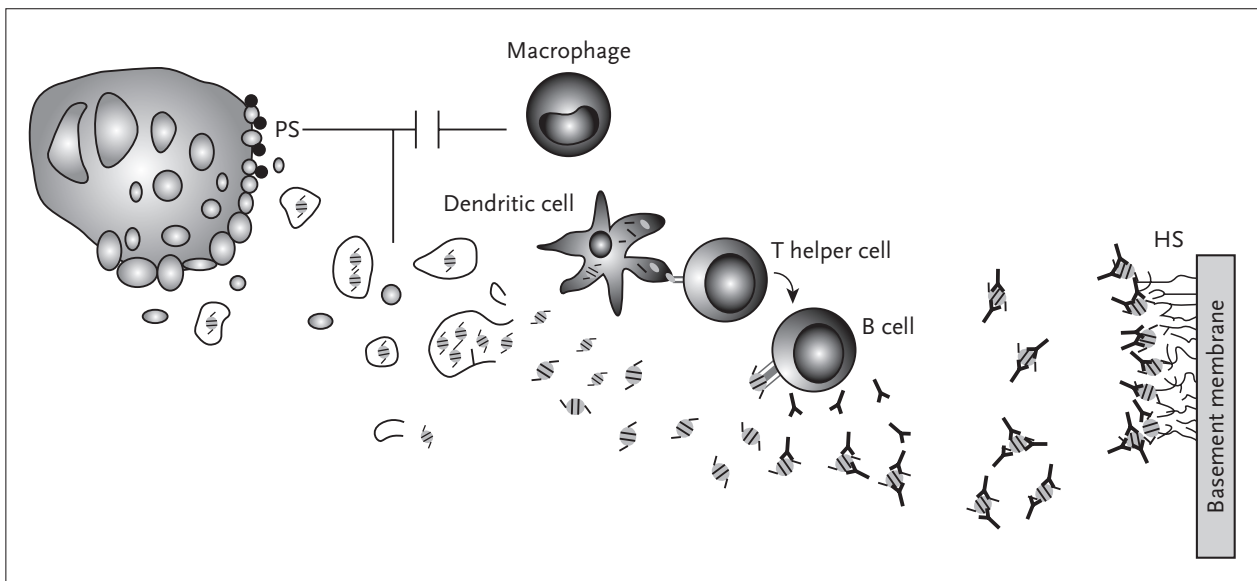
Figure 1

Hypothesis for the development of lupus nephritis

Abnormal apoptosis (in the wrong micro-environment or at wrong time-point) can lead, especially if the phagocytic capacity for apoptotic cells is inadequate or overflown, to the release of modified autoantigens including nucleosomes. These (modified) nucleosomal antigens are presented by dendritic cells to T cells, which provide help for autoreactive B cells. Via this T cell dependent autoantigen driven response various antichromatin autoantibodies are formed including antinucleosome, anti-dsDNA and antihistone antibodies. Nucleosome autoantibody complexes are targeted to the glomerular basement membrane (GBM). This binding to the GBM is the result of binding of the strongly cationic parts of the core histones within the nucleosomes to the anionic heparan sulphate within the GBM (reprinted with permission from J Nephrol).

not need to be detrimental if the removal of apoptotic cells is rapid and complete. Therefore, impairment of apoptotic cell removal is probably more important then disturbances in apoptosis itself.

PHAGOCYTOSIS OF APOPTOTIC CELLS IN SLE

The discovery that autoantigens are clustered in surface blebs, after induction of apoptosis of keratinocytes with UV light, opened new ideas for the pathogenesis of SLE.[6] In fact all autoantigens targeted in SLE can be found at the surface of apoptotic cells. The smaller blebs contain SS-A (52kD), ribosomal P protein, α-fodrin and Jo-1, while the larger apoptotic bodies contain nucleosomes, SS-A (60kD), SS-B, Sm, SnRNP complexes, PARP and other autoantigens. Since several mechanisms can alter these autoantigens during apoptosis, making them more immunogenic,[7] it is of utmost importance that these apoptotic cells are removed swiftly and adequately. It is beyond the scope of this review to describe in detail the process of phagocytosis of apoptotic cells, since excellent reviews are available.[8] In brief, induction of the apoptotic process leads, after intranucleosomal cleavage of chromatin

and nuclear condensation, to a number of surface changes, most notably the expression of phosphatidylserine (PS), which is normally present at the inside of the cell membrane. This and other cell surface changes provide 'eat-me' signals to neighbouring cells and macrophages. Via a large number of receptors including scavenger receptors, the LPS receptor (CD14), the C1q receptor, vitronectin receptor, other β-integrins and lectins, the apoptotic cell binds to the macrophage and is subsequently internalised and degraded. For some of these receptors 'bridging' molecules are necessary such as C1q, β_2-glycoprotein I (which binds to PS), thrombospondin, C-reactive protein (CRP) and serum amyloid P protein (SAP). The ligands which bind to these bridging molecules or receptors are for the larger part putative.

Because all autoantigens targeted in SLE are either located in small or apoptotic blebs or at the surface of apoptotic cells, it has been postulated that a defective phagocytosis of apoptotic cells may be a pivotal feature in the generation of the autoimmune response. This impaired phagocytosis may lead to the release of nuclear antigens including nucleosomes, since the major pathway for the generation of nucleosomes is apoptosis. In fact, circulating nucleosomes have been found in SLE patients and lupus mice. Indeed,

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a defective removal of apoptotic cells has been documented in patients with active SLE.[9] However, this defect can be secondary to the disease, since autoantibodies could potentially inhibit binding and/or engulfment of apoptotic cells. Therefore, we analysed the phagocytic capacity for apoptotic cells in lupus mouse strains with a sensitive technique.[10] In prediseased mice no constitutive defect was found,[11] while in animals with clinical overt disease, phagocytosis of apoptotic cells was impaired. This defect resided in the plasma and was either a shortage of a critical plasma component (complement?) or the presence of an inhibitor (autoantibody?) (Licht *et al.*, unpublished observations).

Recently, a number of studies with well-defined knock-out mice (C1q, SAP, Dnase I) have been reported, documenting the utmost relevance of proper removal of apoptotic cells.[12] In all these knock-out mice three features were observed: 1) an impairment of apoptotic cell removal; 2) generation of antinuclear autoantibodies most notably against nucleosomes; and 3) development of glomerular deposits containing immunoglobulins and complement factors and in some models histological signs of glomerulonephritis. These observations show that inadequate removal of apoptotic cells and/or chromatin may lead to lupus.

IMMUNOGENICITY OF NUCLEOSOMES

Since naked dsDNA has long been regarded as the major autoantigen in SLE, many attempts have been made to immunise with dsDNA in all sorts of forms and conditions. However, these procedures failed to induce anti-dsDNA antibodies with lupus specific characteristics. The first positive result was obtained after immunisation with dsDNA complexed to histone-like DNA-binding proteins from either viral or protozoal origin. The antibodies formed were directed against dsDNA and nucleosomes.[13] Seminal studies by Datta and colleagues showed that in the SNF1 murine lupus model 50% of the pathogenic T helper cells were directed against nucleosomes. These T helper cells did not only provide help for the production of nucleosome specific antibodies, but also for anti-dsDNA and antihistone antibodies, a phenomenon known as antigen spreading.[14] This observation shed new light on the initiation of the anti-dsDNA antibody response: not dsDNA but the nucleosome is the driving autoantigen in SLE. Subsequently, similar observations have been reported in human SLE.[15,16] These nucleosome specific T cells respond to histone epitopes on MHC class II molecules presented after processing of nucleosomal material by antigen presenting cells (APC).[17,18] So, these data indicate that T cells towards nucleosomal epitopes

are present in both human and murine lupus. In fact, in murine lupus this T cell reactivity can be demonstrated long before any serological or clinical sign of the disease.[14] These observations posed the question whether anti-nucleosome antibodies are present in SLE. This anti-nucleosome reactivity was first demonstrated for monoclonal antibodies derived from lupus mice.[19] Subsequently, they were also detected in the great majority of lupus mice[20,21] and patients.[22,23] From these studies, it also appeared that the formation of antinucleosome antibodies preceded that of other antinuclear specificities such as anti-dsDNA and antihistone. From subsequent studies it became clear that measurement of antinucleosome reactivity is preferable and more specific than anti-dsDNA. Moreover, it was recently demonstrated that anti-dsDNA reactivity, as measured with the gold standard, the Farr assay, was for a large part due to histone containing immune complexes.[25]

NUCLEOSOME-MEDIATED AUTO-ANTIBODY-BINDING TO THE GBM

Nucleosomes are not only important for the induction of the autoimmune response but also play a decisive role in the development of tissue lesions, in particular lupus nephritis. The first clue for this notion came from the observation that anti-dsDNA antibodies could cross-react with an intrinsic component of the GBM, namely heparan sulphate (HS).[26] HS is the strongly anionic side-chain of agrin, the major heparan sulphate proteoglycan (HSPG) of the GBM.[27] HS determines the charge-dependent permeability of the GBM. Injection of monoclonal anti-HS antibodies instantly induces a massive proteinuria.[28] So far, in various proteinuric diseases a number of HS alterations have been identified.[29] The binding of anti-nuclear antibodies to HS was not due to cross-reactivity, as thought initially, but was mediated by nucleosomes.[30] If monoclonal anti-dsDNA antibodies, which reacted in ELISA with HS, were treated with DNAse and were subsequently purified under high salt conditions on a protein-A column, all HS reactivity was lost. Addition of the protein A column effluent restored the binding to HS. Subsequent analysis revealed that histone/DNA complexes (i.e. nucleosomes) were responsible for the binding to HS. Also *in vivo*, in renal perfusion studies in the rat, nucleosomes could mediate the binding to the GBM, while noncomplexed antinucleosome and anti-dsDNA antibodies did not bind.[31] This nucleosome-mediated binding occurred via binding of the cationic N terminal tails of the core histones to the strong anionic charges of HS. This was deduced from a number of observations. First, removal of HS, by prior intrarenal perfusion of heparanase (which cleaves HS), strongly reduced the binding to the

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GBM of subsequently perfused nucleosome/autoantibody complexes.[31] Second, binding of antihistone antibodies to the N terminal parts of the core histones to a great extent prevented the binding of nucleosome/antihistone complexes to the GBM. In contrast to this, complex formation of nucleosomes with either anti-dsDNA or antinucleosome autoantibodies created a nephritogenic complex.[32] Because the epitopes of antihistone antibodies are mainly localised on the N terminal regions, their binding masks the positive charges on these histone tails, thereby preventing the binding to anionic HS. These positive charges on the N termini of histones can not be neutralised by the binding of anti-dsDNA or antinucleosome antibodies. In fact, their binding to the nucleosome has the opposite effect, since they neutralise in part the anionic charges of dsDNA, which makes the complex even more nephritogenic. Third, once we realised the importance of the cationic regions on the core histones for the binding to HS, we argued that neutralisation of these positive charges on the histone tails with an HS 'look a-like' molecule could perhaps prevent binding to HS. Since heparin is such an HS decoy molecule, the effect of heparin was analysed in different ways. *In vitro*, heparin could inhibit dose-dependently in ELISA the binding to HS of nucleosome complexed anti-dsDNA or antinucleosome antibodies.

In the renal perfusion system addition of heparin to nephritogenic nucleosome/autoantibody complexes completely prevented GBM-binding. Based on these observations the protective effect of daily heparin injections on lupus nephritis was analysed. Treatment of MRL/lpr mice from week 8 onwards with heparin or noncoagulant heparinoids prevented the development of proteinuria and 80% of the animals had no histological signs of glomerulonephritis at the age of 22 weeks. This is in contrast to PBS-treated MRL/l mice, 80% of which developed proteinuria and glomerulonephritis. The mechanism behind this renoprotective effect was revealed by immunofluorescence. In PBS-treated mice extensive deposits along the glomerular capillary loops and in the mesangium were seen while in heparin(oid)-treated mice only mesangial deposits were observed.[33] So, neutralisation of the positive charges of the histones within the nucleosome/autoantibody complex by heparin prevented binding of these complexes to HS in the GBM.

If nucleosomes are important for the targeting of autoantibodies to the GBM, they should be present in glomerular deposits in murine and human lupus nephritis. Using polyclonal and monoclonal antibodies to the various components of the nucleosomes as probes, nucleosomes were indeed identified in human lupus nephritis, predominantly in the diffuse proliferative form (WHO class IV).[34] With elution studies of isolated glomeruli from MRL/lpr mice antinucleosome antibodies were identified.[35]

A time study revealed that these antinucleosome antibodies were deposited first, with subsequent deposition of anti-dsDNA antibodies. This sequence suggests that after the nucleosome lodges in the GBM, it acts as a planted antigen for subsequent binding of anti-dsDNA. In concordance with the above-mentioned studies that nucleosome/anti-histone complexes are less nephritogenic, the amount of antihistone antibodies was low and did not increase when the severity of the glomerular lesion progressed. An analysis of various glomerular diseases with monoclonal antibodies against HS and the HSPG core protein revealed an almost complete absence of HS staining in the GBM in 90% of the biopsies from patients with proliferative lupus nephritis.[36] A similar observation was made in murine lupus nephritis,[37] which allowed a further analysis of the responsible mechanism. In a time study in MRL/lpr mice an inverse correlation was found between HS staining and albuminuria and between HS staining and immunoglobulin deposits in the GBM. This suggested masking of HS by immune complexes. Measurement of the HS content in isolated glomeruli from these mice showed a normal amount of HS, which indicated that the decrease in HS staining was not due to a reduction in the HS content in the glomerulus. Further proof for the masking of HS by deposited glomerular immune complexes came from three observations: 1) *in vitro* pre-incubation of HS-coated ELISA plates with nucleosome/autoantibody complexes inhibited the binding of the anti-HS monoclonal to HS dose-dependently;[37] 2) heparin treatment of MRL/lpr mice not only prevented glomerular deposition as mentioned before, but also prevented the loss of HS staining;[33] and 3) in human lupus nephritis an inverse correlation was seen between GBM-HS staining and the amount of nucleosome deposits.[34] So, this decreased HS staining in lupus nephritis indicates, although indirectly, the presence of nucleosome/Ig complexes in the GBM. Taken together, these results document the presence of nucleosomes, antinucleosome antibodies and nucleosome/autoantibody complexes in glomerular deposits in lupus nephritis. They underline the relevance of nucleosome-mediated targeting of autoantibodies to the GBM, as identified in experimental animal studies.

Because of their nephritogenic potential, it could be helpful to identify nucleosome/autoantibody complexes in the circulation of SLE patients. Using an ELISA, plasma samples of SLE patients were screened for anti-HS reactivity. Onset[38] or exacerbation[39] of lupus nephritis was indeed associated with higher anti-HS reactivity. Using a GBM-based ELISA similar results were found.[40] With a more direct approach, by measuring nucleosome/Ig complexes, an association was found in MRL/lpr mice between these nucleosome/autoantibody complexes and development of proteinuria.[41]

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NOTE

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