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CONCISE REPORT

Cellular localization of nuclear antigen during neutrophil apoptosis: mechanism for autoantigen exposure?

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Juvenile-onset systemic lupus erythematosus (JSLE) is a multisystem autoimmune disease characterized by hyperactive B-cells producing auto-antibodies directed against nuclear antigens. A potential source of these antigenic components is apoptotic cells. We have previously demonstrated increased dysregulated neutrophil apoptosis in JSLE patients. Here we investigate autoantigen expression on JSLE neutrophils during apoptosis. Neutrophils from non-inflammatory controls and JSLE patients were incubated with JSLE and control serum. Apoptosis and dsDNA expression was measured using flow cytometry and confocal microscopy. Increased neutrophil apoptosis and dsDNA expression was observed in JSLE and control neutrophils incubated with JSLE serum. During neutrophil apoptosis nuclear material was exposed on the cell surface rather than within the cell as seen with viable neutrophils. The increased neutrophil apoptosis induced by JSLE compared with control serum resulted in increased surface expression of nuclear antigens. This may provide an additional mechanism leading to the generation of autoantibodies in JSLE. *Lupus* (2011) **20,** 641–646.

Key words: apoptosis; autoantigen exposure; juvenile-onset SLE; neutrophil

Introduction

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease with a wide spectrum of clinical and immunological abnormalities.[1] Although the aetiology and disease process remains unclear, the presence of autoantibodies, particularly antibodies directed against double-stranded DNA (dsDNA), is closely correlated with disease activity and is of diagnostic and even prognostic value. Autoantibodies are raised against autoantigens and a potential source of these components are apoptotic cells.[1] Apoptosis or programmed cell death is a physiological process of self destruction for cells that are damaged or no longer needed.[2] Defects in apoptosis or the uptake of dying cells by phagocytosis has been shown to play a role in several diseases including cancer and autoimmunity.[3] During apoptosis, various potential autoantigens, often components of complex particles, become modified, which may result in an increased immunogenicity of these components.[4,5]

Neutrophils are essential components of host defence yet can cause tissue injury due to their cytotoxic content and have been implicated in a wide range of non-infectious inflammatory conditions including glomerulonephritis, vasculitides and rheumatoid arthritis.[6] Neutrophils undergo rapid constitutive apoptosis, and because of their vast number, represent an enormous potential apoptotic cell burden. We have previously demonstrated an imbalance in both pro-apoptotic and anti-apoptotic factors in both neutrophils and sera from patients with juvenile-onset SLE resulting in increased neutrophil apoptosis, which is further exacerbated by the observed pro apoptotic serum and correlates with biomarkers of disease activity.[7] Here we aim to investigate whether apoptotic neutrophils are a source of autoantigens in juvenile-onset SLE.

Materials and methods

Patients and controls

This study was approved by Liverpool Paediatric Research Ethics Committee. Written informed

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patient/parental assent/consent was obtained from all participating subjects. All juvenile-onset SLE (JSLE) patients fulfilled the revised American College of Rheumatology (ACR) criteria for the diagnosis of SLE[8] before the age of 17 years. Paediatric non-inflammatory controls were defined as children investigated for non-inflammatory musculoskeletal symptoms or attending for elective surgery where no inter-current infection was present. All paediatric patients were recruited from outpatient clinics and inpatient wards at Alder Hey Children's NHS Foundation Trust, Liverpool, UK. Clinical data and laboratory results were blinded to all investigators until final experiments were completed.

Cell preparation

Neutrophils were isolated from fresh heparinized blood by one-step centrifugation through Polymorph prep (Axis Shied, UK) following the manufacturer's instructions. Contaminating erythrocytes were removed by hypotonic lysis. Purity was confirmed using morphological analysis of cytospin preparations. Purified neutrophils were re-suspended at a concentration of 2×10^6 cells/ml in RPMI1640 medium (Sigma Aldrich) supplemented with 10% JSLE or control serum and incubated at 37°C and 5% CO_2. Apoptosis and necrosis were measured by annexin V and propidium iodide (PI) staining using flow cytometry. dsDNA expression was also measured via antibody staining and flow cytometry.

Annexin V FITC and PI staining

Neutrophils were removed from culture and re-suspended in HBSS without phenol red (Invitrogen). Annexin V-FITC (Sigma-Aldrich) was added at a 1/100 dilution and cells were incubated at 4°C for 15 min, followed by incubation with 10 μg/ml PI for 10 min at room temperature. Cells were pelleted at $400 \times g$ and re-suspended in HBSS before analysis by flow cytometry using a FC500 MPL flow cytometer (Beckman Coulter).

Light microscopy

Isolated JSLE neutrophils (5×10^4) that had been incubated with JSLE serum to induce apoptosis were centrifuged onto a microscope slide at $500 \times g$ for 10 min. Any morphological changes in the neutrophils during apoptosis were visualized using light microscopy techniques. Apoptosis was confirmed by annexin V staining and flow cytometry.

Immunofluorescence

To visualize CD16b and dsDNA staining by immunofluorescence, cells were centrifuged onto a microscope slide at $500 \times g$ for 10 min. Cells were fixed with 10% neutral buffered formalin (Sigma Aldrich) and incubated for 30 min with 0.05 μg of the primary antibody mouse monoclonal anti dsDNA (Santa Cruz Biotechnology). Following incubation cells were washed with 1 × Tris buffered saline (TBS) buffer and incubated for 30 min with the secondary antibody Texas red labelled goat anti-mouse immunoglobulin G (abcam). Following incubation the cells were once more washed with 1 × TBS and incubated for a further 30 min with the FITC labelled goat anti rabbit anti CD16b (as a neutrophil marker). DNA was stained with 1 μg/ml 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (Sigma-Aldrich), for 5 min. Cells were washed with 1 × TBS and viewed with a confocal laser-scanning microscope (Leica DM2500). Images were taken with a 40 × objective and detection settings were kept constant between conditions to compare fluorescence intensities. LSM images were exported as tifs and assembled using Adobe Photoshop.

dsDNA staining using flow cytometry

Neutrophils from healthy donors ($n = 8$) and JSLE patients ($n = 4$) were incubated with 10% JSLE or control serum for 2 h. The cells were then washed by centrifugation in 2% FCS phosphate buffered saline (PBS), the supernatant was poured off, and the cells were resuspended in the residue, to which 0.05 μg primary antibody mouse monoclonal anti dsDNA was added and incubated at room temperature for 30 min. The cells were then washed again by centrifugation in 2% FCS PBS and incubated with the secondary antibody FITC labelled goat anti mouse IgG (Caltag) for a further 30 min. All cells were washed twice in 2% FCS PBS before resuspension in 500 μl 0.5% formaldehyde for flow cytometry.

Measurements

Statistical analysis was performed using the Friedman non-parametric test for multiple-related groups. If a significant value was obtained ($p < 0.05$), further statistical analysis was carried out on the data. To compare between paired groups, the Wilcoxon test was performed. For unrelated groups, the Mann–Whitney test was performed.

Results

Patient demographics

Eight JSLE patients were studied at a mean age of 15.75 (range 14–18) years old of whom two (25%) were male and all were White-British. Eight paediatric non-inflammatory controls were studied with mean (range 13–16) age of 14.75 years of whom one (12.5%) was male and all were White-British. Patients had generally mild-moderate disease activity at the time of sampling (Global BILAG 2004 score: mean 2, range 0–6), and a range of standard disease-modifying agents were being used to treat them.

Increased dsDNA expression and exposure in neutrophils incubated with JSLE serum

Increased apoptosis (as measured by annexin V staining) was observed in neutrophils incubated with JSLE serum compared with control serum ($p < 0.05$; Figure 1A). Annexin V binds to phospholipids normally present in the inner leaflet of the cell membrane, exposed during early apoptosis. However, other processes (such as activation) that alter membrane structure may alter annexin V binding. In our study neutrophil activation as measured by CD11b expression following granulocyte macrophage colony stimulating factor (GM-CSF) stimulation did not lead to increased annexin V staining (see Supplementary data). JSLE serum-induced apoptosis was further confirmed using TUNEL staining (see Supplementary data). This increased neutrophil apoptosis was associated with increased dsDNA expression as measured by flow cytometry ($p < 0.05$; Figure 1, B and C).

Microscopy techniques enabled the visualization of morphological changes in neutrophils during apoptosis. Neutrophils could clearly be observed to be 'blebbing' following incubation with JSLE serum to induce apoptosis (Figure 2, A–C). Confocal microscopy was used to visualize both DAPI (blue; nuclear stain, Figure 2, B–E) and more specifically dsDNA antibody staining (red; Figure 2, F and G) and demonstrated that during neutrophil apoptosis, nuclear material is exposed on the cell surface rather than within the cell as seen with viable neutrophils (Figure 2,B–G).

Discussion

We have previously demonstrated increased neutrophil apoptosis in JSLE that correlates with biomarkers of disease activity.[7] Here we report that JSLE serum-induced apoptotic neutrophils may be a potential source of nuclear self antigens.

The formation of apoptotic blebs is one of the characteristic cellular features during apoptosis and although the concept it is widely accepted (reviewed in Kaplan[9]), there are very few studies demonstrating which apoptotic cells may be a potential source of autoantigens in SLE. One previous study has shown that autoantigens involved in SLE are clustered in the blebs of apoptotic keratinocytes,[10] and these blebs are specifically recognized by autoantibodies. Here we demonstrate blebs being formed in JSLE neutrophils undergoing apoptosis. Further analysis of these neutrophils by flow cytometry showed both increased apoptosis and dsDNA expression in neutrophils incubated with JSLE serum compared with non-inflammatory

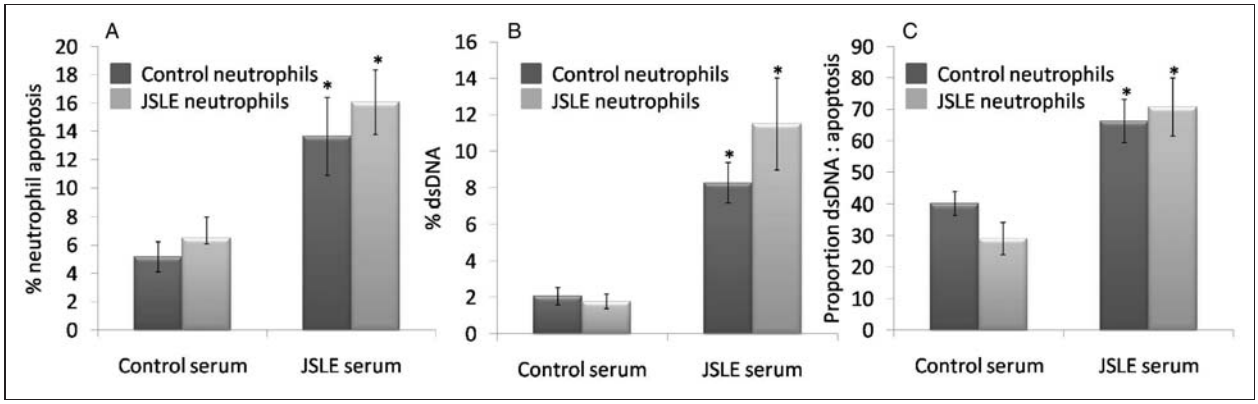

Figure 1 Neutrophils from healthy controls ($n = 8$) and JSLE ($n = 4$) patients were incubated with JSLE or control serum for 2 h. The % apoptosis (A) and % of neutrophils staining positive for dsDNA (B) as measured by flow cytometry were higher in both control and JSLE neutrophils that had been incubated with JSLE serum ($p < 0.05$) compared with controls. The proportion of apoptotic neutrophils that were positive for dsDNA expression was also higher following incubation with JSLE serum ($p < 0.05$; C).

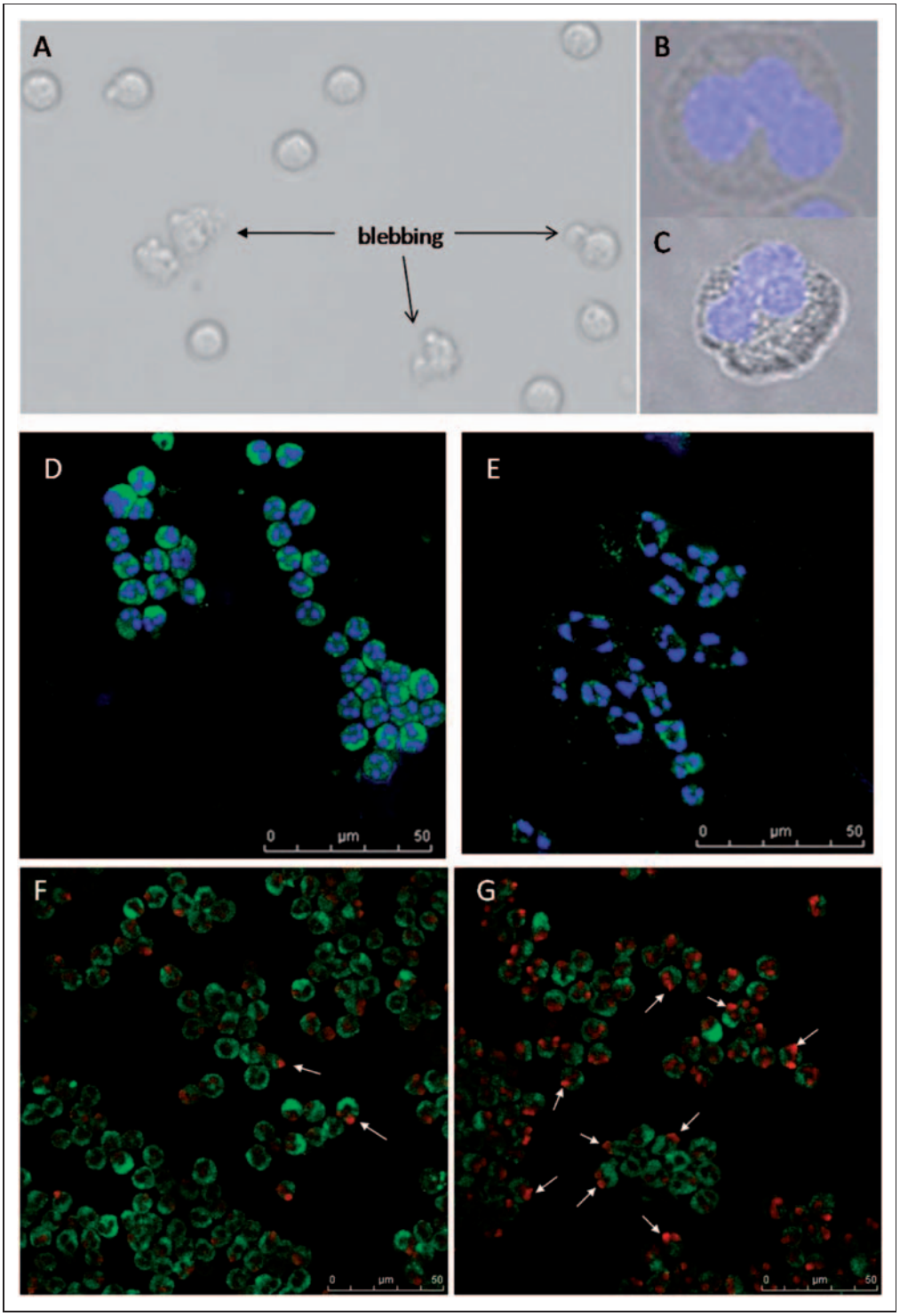

Figure 2 Neutrophils isolated from JSLE patients ($n = 4$) were incubated with JSLE or control serum for 2 h to induce apoptosis. Neutrophils (5×10^4) were centrifuged onto a microscope slide at $500 \times g$ for 10 min. Using light microscopy ($40 \times$ magnification) neutrophils can be observed to be blebbing during apoptosis (A). Confocal microscopy was used to visualize neutrophils stained with DAPI (B–E, blue; nuclear stain) and/or FITC labelled CD16b (D and E, green; as a neutrophil marker). Neutrophils were incubated with either control (B and D) or JSLE serum (C and E). During neutrophil apoptosis nuclear material is exposed on the cell surface (C and E) rather than within the cell, as seen with viable neutrophils (B and D). Confocal microscopy was also used to visualize neutrophils stained with dsDNA antibody (F and G; red). During neutrophil apoptosis dsDNA is exposed on the cell surface (F and G; labelled with a white arrow) rather than within the cell as seen with viable neutrophils. Neutrophils incubated with JSLE serum were also observed to have increased dsDNA expression (G) compared with control serum (F). The data shown is representative of those obtained in four separate experiments.

control serum. Cellular localization of nuclear antigen during neutrophil apoptosis resulted in exposure of dsDNA as observed by confocal microscopy. These results support the previous study of keratinocytes demonstrating that apoptotic blebs are a potential source of autoantigens and may play a key role in the development of SLE.

SLE is a multi-system disorder, not only involving the skin, and therefore demonstrating apoptotic neutrophils as a potential source of autoantigens is of particular importance. In nearly all autoimmune diseases, neutrophils can be considered critical effectors of inflammation.[11] Both adult-onset and juvenile-onset SLE are characterized by an over-representation of apoptotic neutrophils, and may reflect a disease-specific defect in the clearance of apoptotic cells. The effect of concomitant medication on apoptosis must also be considered. Although it is considered that corticosteroids may induce apoptosis in some immune cells[12] studies have also suggested that neutrophils are less sensitive to corticosteroids than other cell types and have been shown to inhibit apoptosis in neutrophils.[13-14] In data we have previously published we did not find a difference in the amount of neutrophil apoptosis in JSLE patients on corticosteroids compared with those not on corticosteroids.[7]

PI staining was not demonstrated in neutrophils incubated with either control or JSLE serum (data not shown). Viable cells with intact membranes exclude PI. Therefore increased dsDNA expression in the JSLE serum-induced apoptotic neutrophils was not due to the JSLE serum causing permeabilization of cells, allowing the incorporation of the dsDNA antibody.

A novel antimicrobial mechanism of neutrophils has been recently described.[15] Neutrophil extracellular traps (NETs) are extracellular structures that are composed of chromatin, with specific proteins from the neutrophilic granules attached. It has been proposed that a source of antigens in SLE could be increased NETs production, not dismantled efficiently.[16] Cell death that results in the formation of NETs has been shown to be different from necrosis and apoptosis.[17] Our study does not focus on NETs; it does, however, demonstrate that nuclear material including dsDNA is exposed during neutrophil apoptosis induced by JSLE serum. We therefore believe that, in our model, apoptotic neutrophils may be a potential source of auto antigens in SLE. Whether these actually go on to act as immune stimulators remains to be determined.

Animal models have demonstrated the potential of neutrophil apoptosis modulation in effecting disease activity in autoimmune disease.[18-19] The results from our study further highlight the importance of determining the mechanism and role of neutrophil apoptosis in the pathogenesis of SLE as a means of determining potential therapeutic targets.

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Conflict of interest statement

None declared.

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