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A deficiency in the in vivo clearance of apoptotic cells is a feature of the NOD mouse

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Abstract

Deficiencies in apoptotic cell clearance have been linked to autoimmunity. Here we examined the time-course of peritoneal macrophage phagocytosis of dying cells following the direct injection of apoptotic thymocytes into the peritoneum of NOD mice and BALB/c controls. Macrophages from NOD mice demonstrated a profound defect in the phagocytosis of apoptotic thymocytes as compared to control macrophages. Nonobese diabetic mice also demonstrated a decrease in the clearance of apoptotic cell loads following an apoptotic stimulus to thymocytes (dexamethasone) when compared to BALB/c or NOR controls. Further, NOD mice demonstrated an increase in apoptotic cell load following an apoptotic stimulus to keratinocytes (ultraviolet light, UVB) when compared to control strains. Animals deficient in macrophage phagocytosis of apoptotic debris often manifest an autoimmune phenotype characterized by the production of antinuclear autoantibodies (ANA). We determined whether increased apoptotic cell loads (through repeated exposure to UVB irradiation) could accelerate such autoimmune phenomena in young NOD mice. Following repeated UVB irradiation, NOD mice, but not BALB/c or NOR controls, developed ANA. We propose that abnormalities in apoptotic cell clearance by macrophages predispose NOD mice to autoimmunity.

Keywords: Clearance; Macrophages; Autoimmunity; Diabetes; Apoptosis

1. Introduction

The nonobese diabetic (NOD) mouse spontaneously develops Type 1 diabetes mellitus as a consequence of an autoimmune process that culminates in the destruction of the insulin-producing pancreatic β cells [1]. Nonobese diabetic mice are also susceptible to the development of other autoimmune disorders. Senescent NOD mice develop antinuclear autoantibodies (ANA), hemolytic anemia, and other characteristics of non-organ specific autoimmunity while age-matched non-autoimmune BALB/c mice do not [2]. Interestingly, prevention of diabetes in NOD mice can precipitate a systemic lupus erythematosus (SLE)-like disease. Administration to NOD mice of a heat-killed bacillus Calmette–Guerin (BCG, *Mycobacterium bovis*) vaccine arrests diabetes development [3] and subsequently treated mice develop rheumatic autoimmune responses typical of SLE, including the production of ANA [4,5]. This reciprocal switching between two autoimmune phenotypes by a single environmental stimulus has led

Abbreviations: NOD, nonobese diabetic; NOR, nonobese resistant; ANA, antinuclear autoantibodies; Dex, dexamethasone; SLE, systemic lupus erythematosus; KC, keratinocyte; UVB, ultraviolet B.

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to the hypothesis that genetic susceptibility for Type 1 diabetes and SLE may be conferred by a single collection of genes in the NOD mouse [6].

Deficiencies in apoptotic cell clearance by macrophages have been linked to both autoimmunity and inflammation [7–9]. Animals defective in macrophage phagocytosis of apoptotic debris manifest an autoimmune phenotype characterized by the production of autoantibodies to DNA and the onset of glomerulonephritis [10,11]. We previously reported that macrophages from diabetes-prone rodents demonstrate impaired phagocytosis of apoptotic thymocytes and transformed β cells in vitro [12,13]. Similarly, macrophages from SLE patients exhibit deficiencies in the clearance of apoptotic cells [10].

Macrophages have been reported to play a major role in progression towards Type 1 diabetes. Over-expression of granulocyte macrophage-colony stimulating factor (GM-CSF) within β cells results in the recruitment of macrophages to the pancreas and the prevention of immune-mediated low dose streptozotocin-induced Type 1 diabetes [14]. These results suggest that efficient macrophage clearance of apoptotic β cells may prevent autoimmunity. We hypothesize that decreased macrophage clearance of apoptotic debris during the neonatal period may be responsible for the initiation of β-cell directed autoimmunity in Type 1 diabetes [8].

To further elucidate the role of macrophage clearance in the initiation of autoimmunity in the NOD mouse, we have investigated macrophage phagocytic ability in vivo following the injection of apoptotic thymocytes into the peritoneum. To determine if macrophage function in NOD mice was deficient in situ, we examined the incidence of apoptotic cells after discrete apoptotic stimuli. To establish if enhanced in situ cell death may generate autoimmune sequelae in NOD mice, we induced keratinocyte cell death with ultraviolet B (UVB) irradiation. Nonobese diabetic mice were found to have deficient clearance of apoptotic cells in vivo. Further, this phenomenon is of physiologic relevance as repeated exposure to UVB irradiation resulted in the induction of ANA in NOD mice.

2. Materials and methods

2.1. Animals

For this study, 5–6-week-old female BALB/c, NOD, and nonobese resistant (NOR) mice were used (Taconic, Germantown, NY). BALB/c mice were chosen as a non-autoimmune control strain as they share both a class I MHC haplotype with NOD mice (H-2K^d) and a light color coat for the purposes of UVB irradiation. All animals were purchased from the supplier and housed in our animal care facilities. Maintenance and experimental manipulation of the animals were performed in accordance with the guidelines and regulations of the Canadian Council on Animal Care and were approved by Animal Care Committees at Simon Fraser University and University of British Columbia (Vancouver, BC).

2.2. In vivo phagocytosis assays

For the induction of thymocyte apoptosis, single cell thymocyte suspensions were irradiated with ultraviolet light for 10 min and then cultured for 3 h at 37 °C/5% CO_2. Cell death was assessed after staining with fluoroisothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI, purchased from Molecular Probes, OR) and observation under a fluorescent microscope. Forty million (4×10^7) early apoptotic (Annexin V^+/PI^-) thymocytes in 0.5 ml PBS were then injected into the peritoneal cavity of BALB/c and NOD mice. At different time points (15, 30, 60, and 120 min), peritoneal exudate cells were collected by lavage using ice-cold RPMI 1640 medium, cytospun onto glass slides, and fixed using 2% paraformaldehyde (ACROS, NJ) for 30 min. Cells were stained with rat monoclonal antibody to mouse F4/80 (Caltag, CA) and counterstained with Harris' hematoxylin (Sigma, MO). Phagocytosis was evaluated by counting 1000 F4/80$^+$ cells (macrophages) on each slide at 1000× magnification. Macrophages were considered to have phagocytosed apoptotic cells only when thymocytes were visible wholly within the perimeter of the macrophage.

2.3. Induction of apoptosis in situ

To induce thymocyte apoptosis in situ, mice were injected intraperitoneally with 0.2 mg dexamethasone (Dex; Sigma–Aldrich, ON) in sterile PBS. Background levels of thymocyte apoptosis were determined by injecting controls with an equal volume of sterile PBS. Thymi were removed at 8, 24 and 48 h after Dex treatment and were fixed (using 25% v/v formaldehyde, 5% v/v glacial acetic acid, 70% v/v dH_2O) for 48 h. Tissue was then embedded in paraffin using standard histological techniques.

To induce keratinocyte (KC) apoptosis, mice were anesthetized by intraperitoneal injection of ketamine (Ketaclean; MTC Pharmaceuticals, ON) plus xylasine (Rompun; Bayer, ON) and irradiated with 4500 J/m^2 UVB as measured by a UVB meter (National Biologics Corporation, ON) delivered to the shaven dorsal surface. The UV source was a bank of four FS40TL12 lamps (National Biological Corp., Twinsburg, OH) with an emission peak at 310 nm (in the UVB range). The spectral emission of the UV source was filtered to specifically remove UVC using a Kodacel filter (Eastman Kodak, Rochester, NY). Mice were killed at 6, 12, 24, and 48 h and dorsal skin biopsies were fixed in 10% neutral buffered formalin and were paraffin-embedded within 24 h.

Sections (4 μm thick) of thymi and skin were cut and collected onto microscope slides such that each section was separated from the next by at least 40 μm. The terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-nick end labeling (TUNEL) method for labeling DNA strand breaks [15] (ApopTag kit, InterGen, NY), was used to quantify the number of apoptotic thymocytes or KCs. Cells with brown nuclei (TUNEL positive) and exhibiting the morphological changes characteristic of apoptosis (condensation or fragmentation of nuclear chromatin) were