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Constitutive apoptosis in equine peripheral blood neutrophils in vitro

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ABSTRACT

The aim of this study was to characterise constitutive apoptosis in equine peripheral blood neutrophils, including assessment of factors that potentially modulate neutrophil survival through alteration of the rate of constitutive apoptosis. Cells underwent spontaneous time-dependent constitutive apoptosis when aged in culture for up to 36 h, developing the structural and functional features of apoptosis observed in many cell types, including human neutrophils. Neutrophils undergoing apoptosis also had diminished zymosan activated serum (ZAS)-stimulated chemiluminescence, but maintained responsiveness to phorbol myristate acetate (PMA). The constitutive rate of equine neutrophil apoptosis was promoted by lipopolysaccharide (LPS), tumour necrosis factor α and phagocytosis of opsonised ovine erythrocytes, while it was inhibited by dexamethasone and ZAS (a source of C5a). Formyl-Met-Leu-Phe, leukotriene B_4, platelet activating factor and PMA had no demonstrable effect on equine neutrophil apoptosis. There was a difference between equine and human neutrophil apoptosis in response to LPS and the time-dependence of the response to dexamethasone.

Introduction

Equine neutrophils play a key role in host defence, but also contribute to host tissue injury through secretion of pro-inflammatory and histotoxic agents (Morris, 1991; Moore et al., 1995; de la Rebière de Pouyade and Serteyn, 2011). This has prompted investigation into the role of neutrophil apoptosis in equine pulmonary (Turlej et al., 2001; Brazil et al., 2005; Lavoie-Lamoureux et al., 2012), gastrointestinal (Krista, 2012) and orthopaedic (Kim et al., 2003) diseases. Neutrophils are programmed to undergo constitutive apoptosis, which, in contrast to necrosis, locks the cell into a non-secretory mode and initiates rapid engulfment and removal by phagocytes without inducing an inflammatory response, thereby limiting host tissue injury (Mecklenburgh et al., 1999).

The aims of this study were to characterise constitutive apoptosis of equine peripheral blood neutrophils when these cells were aged in culture for up to 36 h and to determine the effects of various stimuli on the rate of constitutive apoptosis in vitro. Stimuli used were phorbol myristate acetate (PMA), zymosan activated serum (ZAS, a source of C5a), the bacterial product formyl-Met-Leu-Phe (fMLP), leukotriene B_4 (LTB_4), platelet activating factor (PAF), tumour necrosis factor α (TNF-α), lipopolysaccharide (LPS), dexamethasone (DEX) and phagocytosis of opsonised ovine erythrocytes.

Materials and methods

Isolation and culture of peripheral blood neutrophils

Venous blood was collected from clinically healthy horses into 1:10 V/V 3.8% sodium citrate (Phoenix Pharmaceuticals), with ethical approval granted under UK Home Office Licence. For some experiments, potential inter-species differences were investigated by parallel examination of human peripheral blood neutrophils. Blood was collected from healthy human volunteers with ethical approval (Lothian Health 1702/95/3/11; date of approval 9 August 1995). Fresh peripheral blood neutrophils were isolated using discontinuous plasma/Percoll gradients (Brazil et al., 1998), suspended at 5×10^6/mL in Iscove's modified Dulbecco's medium (Gibco) supplemented with 10% autologous serum, 100 U/mL penicillin (Sigma) and 100 μg/mL streptomycin (Sigma), and aged by culturing for up to 36 h in flat-bottomed polypropylene flexiwells (Becton Dickinson) at 37 °C in a humidified 5% CO_2 atmosphere (Brazil et al., 2005).

Morphology

After culture, apoptosis was assessed by light and transmission electron microscopy (Savill et al., 1989; Brazil et al., 2005). For light microscopy, apoptotic neutrophils were defined as cells containing one or more darkly staining pycnotic nuclear remnants (Fig. 1).

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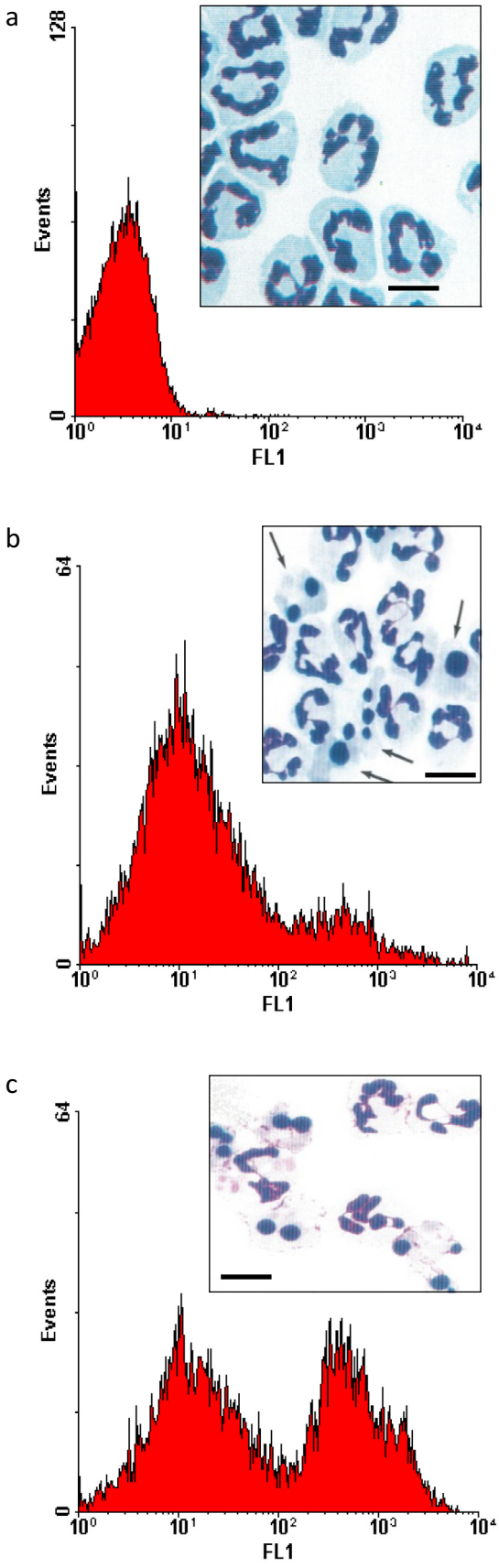

Fig. 1. Annexin V binding (10,000 events) and light microscopic morphology (Diff-Quik, insert panels) of equine peripheral blood neutrophils aged in culture for (a) 0, (b) 8 and (c) 20 h. Arrows indicate cells with apoptotic morphology. Scale bar = 10 μM.

Chromatin fragmentation

DNA of cells undergoing apoptosis fragments in a characteristic internucleosomal pattern that is recognised as a 'ladder' pattern on electrophoresis of DNA. Neutrophils (4×10^6) were aged in culture, harvested, centrifuged at 2000 *g* for 2 min and lysed in 0.5 mL lysing solution containing 6 M guanidine hydrochloride, 20 mM Tris pH 8.0 and 0.1% N-lauryl sarcosine (Sigma). Tris-Cl pH 8.0-buffered phenol/$CHCl_3$ (1:1) was added and the organic phenol/$CHCl_3$ phase was separated by centrifugation at 11,300 *g* for 10 min. The upper DNA-containing phase was removed and precipitated by adding 1:1 V/V propan-2-ol and 1:10 V/V 3 M sodium acetate. DNA was separated by centrifugation at 11,300 *g* for 5 min and dissolved in a 100 μL solution containing 10 mM Tris-Cl, 1 mM EDTA-Na (pH 8.0) and 50 μg/mL RNase A (Sigma). After incubation at 37 °C for 15 min, DNA was separated by electrophoresis on 1.6% agarose gels at 100 V for 2 h through 0.5× buffer containing 89 mM Tris, 89 mM boric acid and 5 mM ethylene diamine tetraacetic acid (EDTA). Gels were stained with 0.5 μg/mL ethidium bromide and photographed over an ultraviolet (UV) transilluminator. A 1 kilobase pair DNA ladder marker (Life Technologies) was included.

Annexin V binding

Exposure of phosphatidylserine on outer membrane leaflets of cells undergoing apoptosis can be detected by annexin V binding (Homburg et al., 1995). Neutrophils were harvested and incubated on ice for 10 min with fluorescein isothiocyanate (FITC)-labelled annexin V diluted 1:4 in annexin V buffer (Biowhittaker) and 10,000 events were analysed by FACScan flow cytometry (Becton Dickinson).

In situ identification of chromatin condensation and DNA fragmentation

To facilitate simultaneous identification of chromatin condensation and DNA fragmentation in cytospin preparations of apoptotic neutrophils, terminal 3'-OH groups of fragmented DNA were labelled in situ by terminal deoxynucleotide transferase (Tdt)-mediated UTP nick end-labelling (TUNEL, FragEL DNA Fragmentation Detection Kit, Calbiochem; Brazil et al., 2005).

Receptor-mediated respiratory burst activity

Freshly isolated neutrophils mount a respiratory burst in response to ZAS and PMA (Brazil et al., 1998). To investigate whether this capacity was maintained as cells underwent apoptosis and whether different mechanisms of cellular activation (receptor-mediated through ZAS vs. direct activation of protein kinase C by PMA) were important, superoxide anion generation in response to ZAS (10% V/V; Sigma) and PMA (100 ng/mL; Sigma) was assessed using lucigenin dependent chemiluminescence (LDCL) before and after 20 h in culture (Brazil et al., 1998).

Constitutive apoptosis of equine and human neutrophils

Equine and human peripheral blood neutrophils (625,000 cells/135 μL) were aged in culture for up to 36 h with either medium (control) or test agent (15 μL), namely LPS from *Escherischia coli* serotype 0111:B4 (Sigma; 0.1 ng/mL-10 μg/mL; from 1 mg/mL stock in phosphate buffered saline, PBS; disaggregated by sonication), PAF (Sigma; 1 μM; from 10 mM stock in ethanol), fMLP (Sigma; 1 μM; dissolved in dimethyl sulphoxide, DMSO, then diluted to 1 mM in PBS), PMA (Sigma; 0.1–10 ng/mL; dissolved in DMSO then diluted to 1 mM in PBS), LTB_4 (kindly donated by Professor A. Rossi, University of Edinburgh, UK; 100 nM; from 1.49×10^{-4} M stock in ethanol), equine recombinant TNF-α (erTNF-α, kindly donated by Dr M. Barton, University of Georgia, USA; 0.1 pg/mL-1 ng/mL in PBS), DEX (Sigma, 0.1 nM-1 μM; from 8.33 mM stock solution in Iscove's modified Dulbecco's medium; Gibco) or 10% V/V ZAS.

To investigate the effect of LPS on neutrophil apoptosis, neutrophils were aged in the presence of other forms of LPS (a different batch of *Escherichia coli* O111.B4 and rough mutant *Salmonella enterica* serovar Typhimurium LPS Ra 60 (kindly supplied by Professor I. Poxton, University of Edinburgh, UK). In additional, the effects of equine TNF-α neutralising antibody (kindly donated by Professor R. MacKay, University of Florida, USA) or control murine isotype-matched monoclonal antibody (MAB002, R&D Systems) on the pro-apoptotic effect of LPS was determined. Apoptosis was assessed by observation of light microscopic features and confirmed by DNA fragmentation studies.

Effects of phagocytosis on the rate of constitutive equine neutrophil apoptosis

Equine neutrophils were cultured with opsonised ovine erythrocytes (OsRBC) or control non-opsonised ovine erythrocytes (sRBC) at a ratio of 1:3. OsRBC were prepared freshly by centrifuging (800 *g*, 10 min) 10 mL ovine blood containing 3.8% sodium citrate, harvesting and washing 2 mL erythrocyte pellet three times in PBS, resuspending in PBS to a haematocrit of 1% and incubating (37 °C, 30 min) with a sub-aggregating concentration (1/40) of canine anti-ovine erythrocyte antibody (VMRD). Cells were washed twice, then resuspended in Iscove's modified Dulbecco's medium. Control sRBC were prepared identically, except with PBS incubation alone. Proportions of apoptotic and phagocytic neutrophils were determined by light microscopy.