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Short Communication

Retinoic acid induces apoptosis in activated canine neutrophils

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ABSTRACT

Activated neutrophils live longer, produce toxic metabolites and cause considerable tissue injury, which is central to the pathogenesis of many inflammatory conditions. Retinoids are a class of lipophilic compounds with anti-inflammatory effects. We examined the effect of retinoic acid on apoptosis in normal and activated neutrophils. Our results showed that treatment with 1 μg/ml *Escherichia coli* lipopolysaccharide (LPS) for 12 and 36 h delayed the spontaneous neutrophil apoptosis compared to untreated cells. But exposure of LPS-treated cells to retinoic acid (1 and 5 μM) abolished the inhibitory effects of LPS on neutrophil apoptosis in a concentration-dependant manner based on annexin V staining, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, light and electron microscopy. These results show that retinoic acid increases apoptosis in activated canine neutrophils and this effect could enhance the resolution of inflammation in vivo.

1. Introduction

Neutrophils are the most abundant white blood cells and they play an important role in the innate immune system (Appelberg, 2007). Although neutrophils are critical for host defence, extracellular release of proteases and reactive oxygen species at the site of inflammation leads to considerable tissue damage, which has been considered central to the pathogenesis of inflammatory diseases including those of the lung (Smith, 1994). Neutrophil depletion reduces severity of lung injury (Folz et al., 1999). Neutrophils are terminally differentiated cells and their short life span ends through triggering of spontaneous apoptosis driven by constitutive expression of pro-apoptotic proteins and lack of anti-apoptotic proteins (Akgul et al., 2001). However, apoptosis of neutrophils is delayed at the site of inflammation by a number of pathogen and host derived inflammatory mediators (Lee et al., 1993). This leads to accumulation and prolonged activation of neutrophils which further aggravates neutrophil-induced tissue injury (Mecklenburgh et al., 1999). Because apoptotic neutrophils protect from lipopolysaccharide-induced sepsis (Ren et al., 2008), it is important to find ways to induce apoptosis in activated neutrophils for therapeutic purposes.

Retinoids are a group of compounds that include vitamin A as well as its biological and synthetic derivatives. Retinoids possess anti-inflammatory properties through their inhibitory effects on transcription factors such as nuclear factor kappa-B (NF-κB) and activator protein-1 (Kuenzli et al., 2004). Retinoic acid is the active metabolite and biologically the most active retinoid, which binds to retinoid receptors to regulate gene expression (Evans, 1988; Kliewer et al., 1992). Retinoic acid deficiency causes abnormal expansion of myeloid cells due to impaired apoptosis in neutrophils and associated neutrophilia (Kuwata

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et al., 2000). However, there are no data on the effect of retinoic acid on apoptosis of either resting or activated canine neutrophils.

Dogs are susceptible to several infectious inflammatory diseases in which neutrophils and neutrophil-derived mediators play a pivotal role (Krogsgaard Thomsen, 1991). In addition, dogs are increasingly being used as animal models for study of human diseases such as inflammatory disorders, autoimmune diseases and cancer (Chapman, 2008; Chase et al., 2006). Therefore, the objective of this study was to determine the effect of retinoic acid on spontaneous apoptosis of resting and activated canine neutrophils.

2. Materials and methods

2.1. Blood collection and neutrophil isolation

The experiments described here were approved by the Research Ethics Board of the University of Saskatchewan's Animal Care Assurance Committee in accordance with the guidelines of the Canadian Council on Animal Care. Blood samples were collected from clinically healthy dogs and diluted by mixing 1.5 ml of blood with 0.5 ml of normal saline just before the isolation.

Neutrophils were isolated as per the previously described method with few modifications (Comazzi et al., 2001). Briefly, equal volumes of isotonic percoll solutions of different densities (1.096 and 1.072) were overlaid on each other. Blood was carefully layered over percoll gradients and centrifuged at 400 × *g* for 20 min. A band of neutrophils formed between two percoll gradients was collected and contaminating erythrocytes were lysed by adding 5 ml of sterile distilled water for 1 min and isotonicity was restored by adding 2.5 ml of 0.3 mol/l NaCl in 0.0132 mol/l phosphate buffer pH 7.2. The cell suspension was centrifuged at 400 × *g* for 12 min and washed twice with phosphate buffer saline. Neutrophils were resuspended in 1 ml RPMI 1640 media and counted to determine the cell number and the cell viability was assessed by Trypan blue exclusion. The cell suspension was cytocentrifuged, stained with May-Grunwald Giemsa and differential cell count performed to determine the purity of cells. The purity of neutrophils was always >95%.

2.2. Cell treatments

Neutrophils were suspended (2×10^6 cells per ml) in RPMI 1640 (Sigma–Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were treated with media alone or with LPS (1 μg/ml). To determine the effects of retinoic acid, some groups were incubated with both LPS and retinoic acid (1 and 5 μM). Recognizing that there are differences in the toxicity of various forms of retinoic acids (Breitman et al., 1980), we used *all-trans* retinoic acid (Sigma–Aldrich). Sham controls of only retinoic acid (1 and 5 μM in dimethyl sulfoxide), a vehicle group (dimethyl sulfoxide), and as positive control, camptothecin (10 μM) were also used. Cells were incubated with these reagents at 37 °C in a humidified 5% CO_2 incubator for 12 and 36 h. Flow cytometry was performed on neutrophils incubated for 8 h.

2.3. Flow cytometry

For flow cytometry, isolated cells were incubated for 8 h with either LPS (1 μg/ml) alone or LPS plus retinoic acid (1 and 5 μM), and the assay was done using FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Mississauga, Canada) as per manufacturer's instructions. Briefly, after the treatment period, cells were washed twice with PBS and suspended in annexin V binding buffer (0.1 M Hepes/NaOH, 1.4 M NaCl; 25 mM $CaCl_2$). Cells were stained with propidium iodide and FITC-annexin V for 15 min at room temperature and analyzed by flow cytometry (FACS Calibur, BD Biosciences, Canada) within 1 h.

2.4. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL assay)

TUNEL assay was done using In Situ Cell Death Detection Kit (Roche Applied Science, Laval, Quebec, Canada) as per the manufacturer's instructions. Briefly, cytospin slides were fixed with 4% paraformaldehyde, and cells were permeabilized with 0.1% Triton X in 0.1% sodium citrate for 2 min at 4 °C. Afterwards, the slides were incubated with TUNEL reaction mixture for 1 h at 37 °C in a humidified chamber. The slides were incubated with converter-AP for 30 min at 37 °C. Finally, the slides were incubated with substrate solution (NBT/BCIP) for 10 min at room temperature in the dark. The slides were mounted with PBS/glycerol and 500 cells were counted and differentiated as positive and negative cells based on nuclear staining. Cell numbers were converted to percent apoptotic cells for statistical analysis.

2.5. Light microscopy

After the indicated treatments, cells were pelleted, washed twice with PBS and cytocentrifuged. The cytospin slides were stained with May-Grunwald Giemsa stain and 500 cells were counted in a blinded fashion, differentiating cells as apoptotic or normal cells based on nuclear morphology. Cell numbers were converted to percent apoptotic cells for statistical analysis.

2.6. Electron microscopy

After the treatment period, cells were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 3 h at 4 °C and then post fixed in 0.1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at room temperature. The cells were dehydrated with a graded series of alcohol embedded in Epon/Araldite and polymerized overnight at 60 °C. Cells were differentiated as normal or apoptotic based on nuclear morphology. The apoptotic cells were distinguished based on the previously described features such as condensed nuclei and apoptotic bodies in the cytoplasm (Kerr et al., 1972b; Wyllie et al., 1980).