In vitro characterization of neutrophil extracellular traps (NETs) in canine neutrophils: a pilot study

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Introduction

As an essential part of the innate immune system, neutrophils are the front line of defense against invading pathogens. In addition to phagocytosis of microbes, studies in humans and mice have shown that neutrophils can produce neutrophil extracellular traps (NETs), web-like chromat lined with citrullinated histones and granular components such as myeloperoxidase and elastase. NETs have the ability to trap and kill bacteria and fungi.1,2 NETs also facilitate thrombus formation in a platelet-dependent and -independent manner resulting in microvascular thrombosis and preventing further spread of infection.3

Neutrophils play a pivotal role in tissue damage, organ dysfunction and systemic inflammation in sepsis. In human medicine, NET formation (NETosis) is associated with multiple organ failure in sepsis, but this process and its implications during sepsis remain to be understood in dogs.4,6

Methods and Materials

Neutrophils were isolated from healthy dogs using a modified protocol (Figure 1). 1x10⁶/ml of cells were incubated with 200 nM phorbol-myristate acetate (PMA) (positive control), DPBS (negative control), or 100 µg/ml lipopolysaccharides (LPS) for 30, 90, or 180 minutes at 37°C. Following incubation, samples were centrifuged at 1500 x g for 10 min at 20°C to separate cellular (c)DNA from cell-free (cf)DNA in the supernatant.

cfDNA and cDNA were purified using the QIAmp DNA blood mini kit (QIAGEN) according to the manufacturer’s instructions. Isolated DNA was quantified by spectrophotometry as previously described.3 Relative DNA was assessed as the ratio of cell-free to cellular DNA.

Live cells were stained with cell impermeant Sytox orange (Molecular Probes) for extravasated DNA and cell permeant Syto Green (Molecular Probes) for cDNA for 15 minutes prior to visualization by fluorescence microscopy.

Results

In vitro NETosis after 90-minute incubation with PMA. NET formation was characterized by extravasated DNA (red) from their nuclei (green). Magnification: 20x.

In vitro NETosis as seen by aggregated cell-free DNA (red) after extravasation from neutrophils (neutrophil nuclei were stained green). Magnification: 40x.

Discussion

Our data demonstrated that PMA stimulated NETosis in canine neutrophils in a time-dependent manner. In the presence of LPS, canine neutrophils also release similar quantity of cfDNA suggesting that NETosis may occur in dogs with sepsis. The time-dependent nature of NETosis was confirmed by spectrophotometry and fluorescence microscopy.

In vitro characterization of NETs is the first step towards a deeper understanding of NETosis and its implications in sepsis in dogs. Further study is needed to determine the significance of platelet-neutrophil interaction in NETosis. Knowledge gained from these studies could benefit humans and dogs alike.

Conclusions

• NETosis increases as a function of time.
• In the presence of LPS or PMA, in vitro NETosis occurs in canine neutrophils in a time-dependent manner.

References