Veterinary Immunology and Immunopathology 144 (2011) 487–492

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Short communication

The role of apoptosis in immunosuppression of dogs with demodicosis

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ARTICLE INFO

Article history:
Received 28 February 2011
Received in revised form 6 August 2011
Accepted 10 August 2011

Keywords:
Apoptosis
Demodicosis
Dogs
Immunosuppression
Leukocytes

ABSTRACT

The aim of the present study was to evaluate the status of apoptosis in peripheral blood leukocytes of dogs with demodicosis. A total of 26 dogs suffering from demodicosis, and positive for *Demodex canis* mites by skin scraping, participated in the study, 13 with localized demodicosis (LD) and 13 with generalized demodicosis (GD). A further 13 clinically healthy dogs, all of whom were negative for mites upon skin scraping, were used as controls. The dogs with GD revealed significantly higher ($P \leq 0.0001$) percentage of leukocytes with externalization of phosphatidylserine (PS) and depolarized mitochondrial membrane potentials ($\Delta\Psi$m) as compared with the dogs with LD and healthy controls. These dogs also revealed significantly lower values ($P \leq 0.0001$) of hematological parameters viz. hemoglobin, total erythrocytes count total leukocytes count, lymphocytes, monocytes and neutrophils. Significantly higher ($P \leq 0.0001$) percentages of leukocytes with externalization of PS and depolarized $\Delta\Psi$m were also found in dogs with LD as compared with the healthy controls. These dogs also revealed significantly lower values of Hb ($P \leq 0.0001$), TEC ($P = 0.025$), TLC ($P \leq 0.0001$), lymphocytes ($P = 0.008$), monocytes ($P \leq 0.0001$) and neutrophils ($P = 0.03$). It is concluded that premature apoptosis of PBL may be implicated in the immunosuppression of the dogs with demodicosis.

1. Introduction

Canine demodicosis is one of the most common and severe skin diseases of dogs, caused by the proliferation of *Demodex canis* mites in hair follicles and sebaceous glands (Scott et al., 2001). Despite the high prevalence and severity of the disease, many aspects of canine demodicosis remain poorly understood. *D. canis* is considered to be a normal inhabitant of canine skin (Ravera et al., 2011) and the disease is thought to be the consequence of a genetically mediated specific immunodeficiency that allows the proliferation of the *Demodex* mites (Scott et al., 2001; It et al., 2010). Albeit, the disease is associated with immunosuppressive disorders (Jones et al., 1997) the predisposing factors for the development of generalized demodicosis (GD) are not yet fully understood. Some of the most important factors are known to include the immune status of the animal, the breed (Plant et al., 2011) and breeding line, the age (Ghubash, 2006), while genetics, nutritional status and oxidative stress (Dimri et al., 2008), length of hair coat, stage of estrus cycle, parturition, endoparasitism, and debilitating diseases are also implicated. Of these, the immune status is thought to be the most significant (De Bosschere et al., 2007). Juvenile-onset localized demodicosis resolves spontaneously in 90% of cases, whereas in some patients administration of topical or oral antibiotics is needed in order to control the secondary bacterial skin infections (Ghubash, 2006). Conversely, generalized

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doi:10.1016/j.vetimm.2011.08.008

demodicosis is considered a severe and potentially life-threatening disease requiring prolonged treatment (Fourie et al., 2007).

Detrimental effects caused by apoptosis can be triggered by parasitic infection, depending upon the specific host–parasite situations (Bienvenu et al., 2010). The parasites have evolved a variety of strategies to modulate the host cells apoptosis. An apoptosis can be either initiated or down-regulated by the parasite, thereby contributing to dissemination within the host, inhibiting or modulating host immune responses, or facilitating the intracellular survival of the pathogen. An increased apoptosis of non-parasitized red blood cells in experimentally malaria parasite infected mice has been reported (Totino et al., 2010). Previously we have demonstrated that the dogs with GD have remarkably lower number of CD4+ T cells than dogs with LD and healthy controls, and we hypothesized that the phenomenon may be the consequences of either specific down regulating activity of *D. canis* mites on CD4+ T cells or increased rate of apoptosis or immunological exhaustion of these cells or their combination (Singh et al., 2010). There are no scientific reports regarding determination of apoptosis in peripheral blood leukocytes of the dogs with demodicosis to the best of the author's knowledge in published electronic data. Detection and quantification of apoptotic leukocytes in response to the *D. canis* mite infestation may possibly provide a useful tool for understanding immune system homeostasis. It is important to understand the role of apoptosis during the course of demodicosis in dogs, for instance localized and generalized demodicosis. Therefore, in the present study we investigated apoptotic response in peripheral blood leukocytes of both the dogs with LD and GD.

2. Materials and methods

2.1. Animal selection criteria and design of the study

All dogs included in the study were presented to Referral Veterinary Polyclinic for clinical and dermatological examination. Skin scrapings, hair plucks and swabs of exudate were obtained. Dogs positive for *D. canis* mites with skin lesions distributed over more than 50% of the body surface, and involvement of two or more feet were classified as cases with generalized demodicosis (GD). Dogs with small alopecic, erythematous, scaly and hyperpigmented skin lesions over the face and forelegs covering less than 50% of the total body area were classified as cases with localized demodicosis (LD) (Gortel, 2006). Skin swabs were smeared on a clean glass slide and kept for microscopic examination for the overgrowth of secondary invaders. None of the dogs had been treated with ectoparasiticides or steroidal anti-inflammatory drugs in the last 30 days before the obtaining the blood samples. On microscopic examination of stool samples, all enrolled dogs were negative for intestinal parasites. The diseased dogs were also free from ecto-parasites apart from the *D. canis* infestation. The dogs were allocated into two groups (groups A and B), of 13 dogs in each group. Group A consisted of dogs with GD (5 intact males and 8 intact females aged 31.38 ± 23.89 months; 4 German shepherds, 3 Labrador retrievers and 2 each of Pomeranians, Pugs and Crossbreds), while group B consisted of dogs with LD (7 intact males and 6 intact females aged 26.76 ± 18.30 months; 6 German shepherds, 3 Labrador retrievers, 2 Doberman pinschers and 1 each of Pomeranian and Pug). Another 13 age-matched dogs (6 intact males and 7 intact females aged 34.15 ± 25.69 months; 5 Labrador retrievers, 2 each of Pomeranians, Pugs and Crossbreds and 1 each of Doberman pinscher and German shepherd), clinically healthy and free of *D. canis* mites, were used as controls. Approximately 2 ml of blood was obtained from each dog, placed into a tube containing EDTA and used for the apoptosis assay and hematology.

2.2. Apoptosis assay

2.2.1. Peripheral blood leukocytes (PBL) isolation

The aliquot of 400 μl of whole blood samples were placed in appropriate tubes, and centrifuged at $200 \times g$. The supernatant plasma was discarded and sediment cells containing buffy coat were washed with phosphate-buffered saline (PBS; pH 7.4). After removing the PBS, the samples were incubated in NH_4Cl buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$ [pH 7.4]) for 7–10 min at 4 °C to destroy the erythrocytes. For complete lysis of erythrocytes, the treatment was repeated once again, followed by 2 washes in Ca^{2+}–Mg^{2+}-free PBS (PBS-A) supplemented with 0.035% (w/v) EDTA, and centrifugation at $400 \times g$ for 10 min at 4 °C. Finally, isolated PBL were kept on ice until further processing.

2.2.2. Flow cytometric analysis of cell death by apoptosis and necrosis

For quantification of apoptotic and dead cells, dual-parameter analysis of Annexin V-EGFP detection kit (GenScript, Centennail Ave., Piscataway, USA) was used (Vermes et al., 1995). Briefly, isolated PBL (0.5×10^6) were washed twice with PBS by centrifuging at 2000 rpm for 5 min and cells were resuspended in 500 μl binding buffer, and kept at 4 °C for 30 min. Finally, 5 μl of annexin V-EFGP and 5 μl of propidium iodide (PI) were added, and samples were incubated at room temperature for 10-15 min, away from light after thorough mixing. Final analysis of annexin V-EGFP was performed in flow cytometer ($E_x = 488$ nm; $E_m = 530$) using the FITC signal detector (FL 1), and PI staining by the phycoerythrin emission signal detector (FL 2). For fluorescence balance normal unstained cells were used as control.

2.2.3. Determination of depolarization mitochondrial membrane potential ($\Delta\Psi m$)

For determination of depolarization of the mitochondrial membrane potential, Mitochondrial Apoptosis Detection kit (JC-1) (GenScript, Centennail Ave., Piscataway, USA) was used (Adrie et al., 2001). Depolarization of $\Delta\Psi m$ was measured by using the lipophilic cationic dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, commonly known as JC-1) which selectively enters mitochondria. Briefly, isolated PBL (0.5×10^6) were washed twice with PBS by centrifuging at 2000 rpm for 5 min. 1 μl of JC-1 was added to 500 μl of pre-warmed 1× incubation buffer,