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Short communication

Fasciola hepatica induces eosinophil apoptosis in the migratory and biliary stages of infection in sheep

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ABSTRACT

The aim of the present work was to evaluate the number of apoptotic eosinophils in the livers of sheep experimentally infected with *Fasciola hepatica* during the migratory and biliary stages of infection. Four groups ($n = 5$) of sheep were used; groups 1–3 were orally infected with 200 metacercariae (mc) and sacrificed at 8 and 28 days post-infection (dpi), and 17 weeks post-infection (wpi), respectively. Group 4 was used as an uninfected control. Apoptosis was detected using immunohistochemistry with a polyclonal antibody against anti-active caspase-3, and transmission electron microscopy (TEM). Eosinophils were identified using the Hansel stain in serial sections for caspase-3, and by ultrastructural features using TEM. At 8 and 28 dpi, numerous caspase-3$^+$ eosinophils were mainly found at the periphery of acute hepatic necrotic foci. The percentage of caspase -3$^+$ apoptotic eosinophils in the periphery of necrotic foci was high (46.1–53.9) at 8 and 28 dpi, respectively, and decreased in granulomas found at 28 dpi (6%). Transmission electron microscopy confirmed the presence of apoptotic eosinophils in hepatic lesions at 8 and 28 dpi. At 17 wpi, apoptotic eosinophils were detected in the infiltrate surrounding some enlarged bile ducts containing adult flukes. This is the first report of apoptosis induced by *F. hepatica* in sheep and the first study reporting apoptosis in eosinophils in hepatic inflammatory infiltrates *in vivo*. The high number of apoptotic eosinophils in acute necrotic tracts during the migratory and biliary stages of infection suggests that eosinophil apoptosis may play a role in *F. hepatica* survival during different stages of infection.

1. Introduction

Fasciola hepatica causes liver fluke disease in temperate climates and is responsible for major economic losses in animal production (Spithill et al., 1999). This disease is also considered a serious public health problem in humans (McManus and Dalton, 2006). *F. hepatica* often causes chronic infection, which implies the development of strategies by the parasite to modulate/suppress the host immune response (Dalton et al., 2013; Morphew et al., 2013). The parasite modulation of the host immune response is a serious obstacle to obtaining protective vaccines against *F. hepatica* in ruminants (Toet et al., 2014; Molina-Hernández et al., 2015).

In helminth infections, eosinophils play important roles either in developing tissue pathology (Cadman et al., 2014) and in the host effector response by releasing cytotoxic granule proteins and various lipid mediators (Klion and Nutman, 2004; Cadman et al., 2014). An effective response in rats infected with *F. hepatica* has been associated with a significant increase in eosinophil infiltration of the gut lamina propria in early post-infection stages (Van Milligen et al., 1998, 1999). In *F. hepatica* infected rats, peritoneal eosinophils producing very high levels of nitric oxide (NO) have been reported (Jedlina et al., 2011). In sheep, peritoneal eosinophils and macrophages, as well as mammary gland eosinophils from *Fasciola gigantica* resistant Indonesian thin-tail (ITT) sheep were able to kill juvenile *F. gigantica in vitro* by antibody-dependent cytotoxicity, but they did not kill larvae of *F. hepatica*, suggesting that eosinophils are important effector cells involved in the resistance of sheep to *F. gigantica* (Piedrafita et al., 2007). Previous studies have reported that bovine eosinophils incubated with bovine serum were unable to damage juvenile *F. hepatica*, but major basic protein

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(MBP) obtained from bovine eosinophils was able to kill juvenile *F. hepatica* at very low concentration (Duffus et al., 1980).

Apoptosis of effector cells such as eosinophils has been reported as a mechanism of immunosuppression during helminth infections (Shin, 2000; Yan et al., 2008; Zepeda et al., 2010). It has been reported that *F. hepatica* induces eosinophil apoptosis in the hepatic inflammatory infiltrate in rats *in vivo* (Serradell et al., 2007). *In vitro* studies in the rat model have demonstrated that secreted excretory products of *F. hepatica* (FhESP) are able to induce apoptosis in eosinophils and peritoneal macrophages, suggesting that apoptosis in effector cells may play a role in the host immune evasion/suppression induced by *F. hepatica* infection (Serradell et al., 2007; Guasconi et al., 2012). Although the mechanisms of immune modulation/suppression induced by *F. hepatica* are a serious obstacle to developing protective vaccines in ruminants (Molina-Hernández et al., 2015), to date, apoptosis has not been investigated in ruminants infected with *F. hepatica*.

Several markers of apoptosis in tissue sections have been investigated, among them activated caspase-3 immunohistochemistry is considered an easy, sensitive, and reliable method for detecting and quantifying apoptosis in histological sections (Duan et al., 2003; Resendes et al., 2004). Transmission electron microscopy is also a useful technique to detect apoptotic cells and to identify eosinophils based on the morphological features of their cytoplasmic granules (Duffin et al., 2009) and it allow to evaluate ultrastructure of apoptotic eosinophils (Balic et al., 2006).

The aim of the present work was to evaluate the presence and number of eosinophils undergoing apoptosis in hepatic inflammatory infiltrates from sheep experimentally infected with *F. hepatica* during the migratory (8 and 28 days post-infection—dpi) and biliary stages of infection (17 weeks post-infection—wpi). Activated caspase-3 immunohistochemistry was used for quantifying eosinophil apoptosis and transmission electron microscopy was used to evaluate ultrastructure of apototic eosinophils.

2. Materials and methods

2.1. Experimental design

Twenty 7-month-old female Merino-breed sheep were used for this study. All animals were obtained from a liver fluke-free farm. Animals were purchased at 1 month of age and housed indoors in the experimental farm of the University of Córdoba until they reached the appropriate age for challenge. All animals were tested monthly for parasite eggs by fecal sedimentation; the results were negative. In addition, all animals were tested for serum IgG specific for *F. hepatica* cathepsin L1 by ELISA prior to challenge and all of them were negative. The sheep were allocated into four groups of five animals each: groups 1, 2 and 3 were orally infected with one dose of 200 metarceriae (Ridgeway Research Ltd., Gloucestershire, UK); group 4 was used as the uninfected negative control. Animals were sacrificed by intravenous injection of thiobarbital at different stages of infection. Groups 1 and 2 were sacrificed at 8 and 28 days post-infection (dpi) and the animals of group 3 were sacrificed at 17 weeks post-infection (wpi). The experiment was approved by the Bioethics Committee of the University of Cordoba (No. 1118) and was performed taking into account European (2010/63/UE) and Spanish (RD 1201/2005) directives on animal experimentation.

2.2. Histopathology and fluke burdens

At necropsy, the duodenum was ligated proximally and distally to the ductus choledochus (8–10 cm) and the liver was photographed on the visceral and diaphragmatic aspects for gross evaluation. Liver tissue samples showing hepatic lesions were collected and fixed in 10% neutral buffered formalin for 24 h, then routinely processed and embedded in paraffin wax. Four micron-thick tissue sections were stained with hematoxylin and eosin (H&E) for histopathology. Fluke burden was conducted in animals from group 3 as follows: gallbladders were opened and flukes were recovered, then the bile ducts were carefully opened and flukes were removed with blunt forceps. Finally, the livers were cut into small pieces (1 cm^3) and washed in hot water to collect the remaining flukes.

Hansel's stain was used in liver tissue sections to identify the presence of eosinophils. Two 4 μm-thick tissue serial sections were obtained. All these tissue sections were deparaffinized and dehydrated through a graded ethanol series. The first section was stained with Hansel's stain, the second by immunohistochemistry for active caspase-3. For Hansel's stain, tissue sections were incubated for 1 min with eosin, washed in distilled water and incubated for 1 min in methylene blue, then washed in distilled water, dehydrated and mounted.

2.3. Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded sections and the avidin-biotin-peroxidase method (Zafra et al., 2013b) were used for the IHC study. A polyclonal (pAb) rabbit anti-human caspase-3 antibody (RP096, Diagnostic BioSystems, Pleasanton, USA) was used as a marker for apoptosis. The primary antibody reacts with cleaved activated caspase-3 but does not recognize full-length caspase-3 or other cleaved caspases; according to the manufacturer, this antibody cross-reacts with activated ovine caspase-3. Liver tissue sections from 8 dpi in which apoptosis had been detected by transmission electron microscopy were used as positive controls.

2.4. Transmission electron microscopy (TEM)

For the ultrastructural study, fresh liver samples from groups 1, 2 and 4 were used. In groups 1 and 2, samples were collected from whitish tortuous tracts, cut into 1-mm cubes, fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide and embedded in Epon 812. Thin sections (50 nm) were stained with uranyl acetate and lead citrate and examined and photographed using a Jeol Jem 1400 transmission electron microscope.

2.5. Gross lesions evaluation and cell counting

Gross pictures of livers were used to evaluate gross damage (hemorrhages and necrotic tracts at 8 and 28 dpi, and fibrosis and scars at 17 wpi) as reported Zafra et al. (2008) using the Image Pro 6.0 software (Media Cybernetics, Silver Spring, USA). The perimeter of the diaphragmatic and visceral aspects of the liver was delineated and the total area obtained, then, the perimeter of the damaged areas was delineated and the area obtained. The percentage of damaged area was then expressed as mean value ± SD per group.

Cell counting was carried out in 10 fields of 0.08 mm^2 per animal randomly selected at the periphery of necrotic foci (8 dpi), necrotic foci and granulomas (28 dpi) and inflammatory infiltrate surrounding large bile ducts with erosive cholangitis (17 wpi). Cell counting was carried out in tissue sections stained with Hansel's technique (eosinophils), and IHC (caspase-3^+ eosinophils and apoptotic bodies). Results are given as mean ± SD per group.

2.6. Statistical analyses

The Kolmogorov–Smirnov test was applied to decide whether distributions were parametric. Comparison between pairs of