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Short communication

Fasciola hepatica products induce apoptosis of peritoneal macrophages

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ABSTRACT

Immunomodulatory properties have been described for *Fasciola hepatica* excretory–secretory products (FhESP), with their interaction with the innate immune cells being crucial during the early stages of infection. Previously, we demonstrated that FhESP induce eosinophil apoptosis. In this work, the ability of FhESP to induce apoptosis of peritoneal macrophages was evaluated.

These parasite products were observed to induce apoptosis in peritoneal macrophages stimulated in vitro with FhESP, as well as in cells recovered from infected mice. The ability of FhESP to modify the viability of macrophages by apoptosis induction may constitute a crucial event for extending its survival in the host.

1. Introduction

Fasciola hepatica causes liver fluke disease, and consequently is responsible for economic losses to the global agricultural community. Although fasciolosis used to be only considered of relevance in mammals such as cattle and sheep, an increasing number of reports have indicated that this parasitosis is becoming a serious public health problem in humans (McManus and Dalton, 2006). This chronic infection can persist for several years in the host, resulting in a large production of eggs and thereby contributing to the infection's transmission (Mas-Coma et al., 2005; McManus and Dalton, 2006). As with other pathogens, the innate immune system plays a crucial role in the defense mechanisms against *F. hepatica*, as a quick eosinophilia and as alternative activated macrophages (aaMΦ) (Flynn et al., 2010). Related to this, different experimental models have shown that *F. hepatica* excretory–secretory products (FhESP) are able to induce aaMΦ (Donnelly et al., 2005; Flynn et al., 2007).

The induction of apoptosis has been observed in various parasite infections (Lüder et al., 2001). Specifically, different excretory–secretory products from helminths have been able to induce apoptosis of immune cells such as T lymphocytes (Carneiro-Santos et al., 2000; Chow et al., 2000; Chen et al., 2002; Tato et al., 2004) and eosinophils (Eo) (Shin, 2000; Serradell et al., 2007), as well as of non-immune cells such as intestinal epithelial cells (Kuroda et al., 2002). In this regard, pro-inflammatory functions of MΦ can be invalidated by the induction of apoptosis (Adam-Klages et al., 2005), and in this work we describe the induction of this effect in peritoneal MΦ (pMΦ) stimulated with FhESP in vitro, and in cells derived from infected mice.

2. Materials and methods

2.1. Reagents

For cell cultures, RPMI-1640 is supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 50 μg/ml gentamycin (Sigma–Aldrich Co., St Louis, MO).

Abbreviations: aaMΦ, alternative activated macrophages; DCs, dendritic cells; Eo, eosinophils; FhESP, excretory–secretory products from *F. hepatica*; PECs, peritoneal exudate cells; pEo, peritoneal eosinophils; PI, propidium iodide; pMΦ, peritoneal macrophages; TH, total homogenate.

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Phycoerythrin-conjugated antimouse F4/80 was bought from Invitrogen (Carlsbad, CA). Propidium iodide (PI) was obtained from Sigma–Aldrich. Annexin-V was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. FhESP preparation

Live adult worms of *F. hepatica* were obtained from the bile ducts of bovine livers and then washed with phosphate buffered saline (PBS) pH 7.4, before being incubated (1 worm/2 ml of PBS) for 3 h at 37 °C. Then, the supernatant was centrifuged (10,000 rpm, 30 min, 4 °C), concentrated using a high-flow YM 10 membrane filter (Millipore-Amicon Corp., Billeria, MA), and stored at −20 °C until being used.

2.3. Mice and purification of macrophages from PECs

Six to eight-week-old female BALB/c mice were purchased from the Ezeiza Atomic Center (CNEA, Buenos Aires, Argentina), housed and cared for in the animal resource facilities of the Department of Clinical Biochemistry, Faculty of Chemical Sciences, National University of Cordoba, following institutional guidelines. All experimental protocols were approved by the Animal Experimentation Ethics Committee, Faculty of Chemical Sciences, National University of Cordoba. To obtain pMΦ, the peritoneal cavity was washed with ice-cold PBS containing 0.1% FBS and 5 mM EDTA. After determination of viability by trypan blue exclusion (cell viability was >95%), PECs were re-suspended in RPMI supplemented with 10% FBS, 2 mM glutamine and 50 μg/ml gentamycin, and adjusted to 5×10^5 cells/well in 48-well plates. The remaining adherent cells were highly enriched for MΦ, with flow cytometry analysis revealing 90% F4/80+ cells.

2.4. In vitro assays

Macrophages obtained from non-infected BALB/c mice were stimulated with FhESP at a concentration of 50 μg/ml for 24 or 48 h at 37 °C in 5% CO_2.

A similar treatment was performed with J774 cells.

2.5. In vivo assays

Mice were orally infected with 10 metacercariae of *F. hepatica* (Baldwin Aquatics Inc., Monmouth, OR). Then, forty-eight hours after being infected, peritoneal lavages were carried out and peritoneal cells were analyzed.

2.6. Cytofluorometric analysis

2.6.1. PI staining

The percentage of apoptotic cells displaying a hypodiploid DNA peak was determined using PI staining. In brief, 2×10^5 cells were fixed and permeabilized with 70% EtOH (4 °C, overnight) and then stained with PI (50 mg/ml). The fluorescence of individual nuclei was measured in a FACS flow cytometer, with cell apoptotic nuclei being distinguished by their hypodiploid DNA content.

2.6.2. Annexin-V assay

PECs were washed twice in PBS and resuspended in 100 ml of annexin-V binding buffer before being incubated with FITC-conjugated annexin-V (0.5 μg/2×10^5 cells). After 15 min at room temperature in the dark, 400 μl of annexin-V binding buffer and 5 μl PI solution (100 μg/ml in PBS) were added, and the cells were then analyzed by using flow cytometry after incubation for 5–10 min. Early apoptotic cells were stained with annexin-V alone whereas necrotic and late apoptotic cells were stained with both annexin-V and PI.

2.7. Statistical analysis

Data are expressed as means ± standard errors of the means (SEMs). The 2-tailed Student's *t*-test was utilized to determine the statistical significance, with a p value of 0.05 being considered significant. All experiments were performed in triplicate and equivalent results were obtained.

3. Results and discussion

Taking into account that innate immune cells such as peritoneal MΦ (pMΦ) and peritoneal Eo (pEo) might play a critical role in determining the outcome of *F. hepatica* infection, the induction of apoptosis of these cells could be important in modulating the immune response. Previous works in our laboratory have demonstrated that FhESP are able to induce pEo apoptosis, in a dose and time dependent way (Serradell et al., 2007). In the present study, we analyzed the cell morphology by May–Grünwald–Giemsa or by ethidium bromide staining of pMΦ incubated with FhESP for 24 or 48 h, and features of apoptotic cells such as decreased cell size, chromatin condensation and enterinuclei coagulated into small dense balls were observed (data not shown). In order to evaluate the presence of hypodiploid cells, pMΦ were stimulated with FhESP, and then the percentage of hypodiploid nuclei by propidium iodide (PI) staining was measured. A significant increase of hypodiploidy was observed in cells stimulated for 24 or 48 h with the parasite products (Fig. 1a, $^*p < 0.006$ and $^*p < 0.0003$, respectively). Moreover, this effect was also present in J774 cells stimulated with FhESP for 24 or 48 h (Fig. 1b, $^*p < 0.044$ and $^*p < 0.000077$, respectively).

Although *F. hepatica* total homogenate (TH) is able to induce aaMΦ (Hacarız et al., 2011), we did not observe any changes in the viability of cells stimulated with *F. hepatica* TH (data not shown).

Apoptosis induction has been previously observed in rat pEo during the early stages of infection, as well as after peritoneal injection of FhESP (Serradell et al., 2007, 2009). In a similar way, we observed a significant increase in the percentage of hypodiploid cells derived from the peritoneum of infected mice after 48 h of metacercarial challenge with respect to control animals (Fig. 2a, $^*p < 0.00091$). In addition, the main population of hypodiploid cells corresponded to the pMΦ (Fig. 2a, R3 population). When the presence of hypodiploid cells in the peritoneum of infected mice was evaluated 24 and 96 h after challenge, the main effect on pMΦ viability was seen to occur after 48 h of infection (data not shown). In order to confirm

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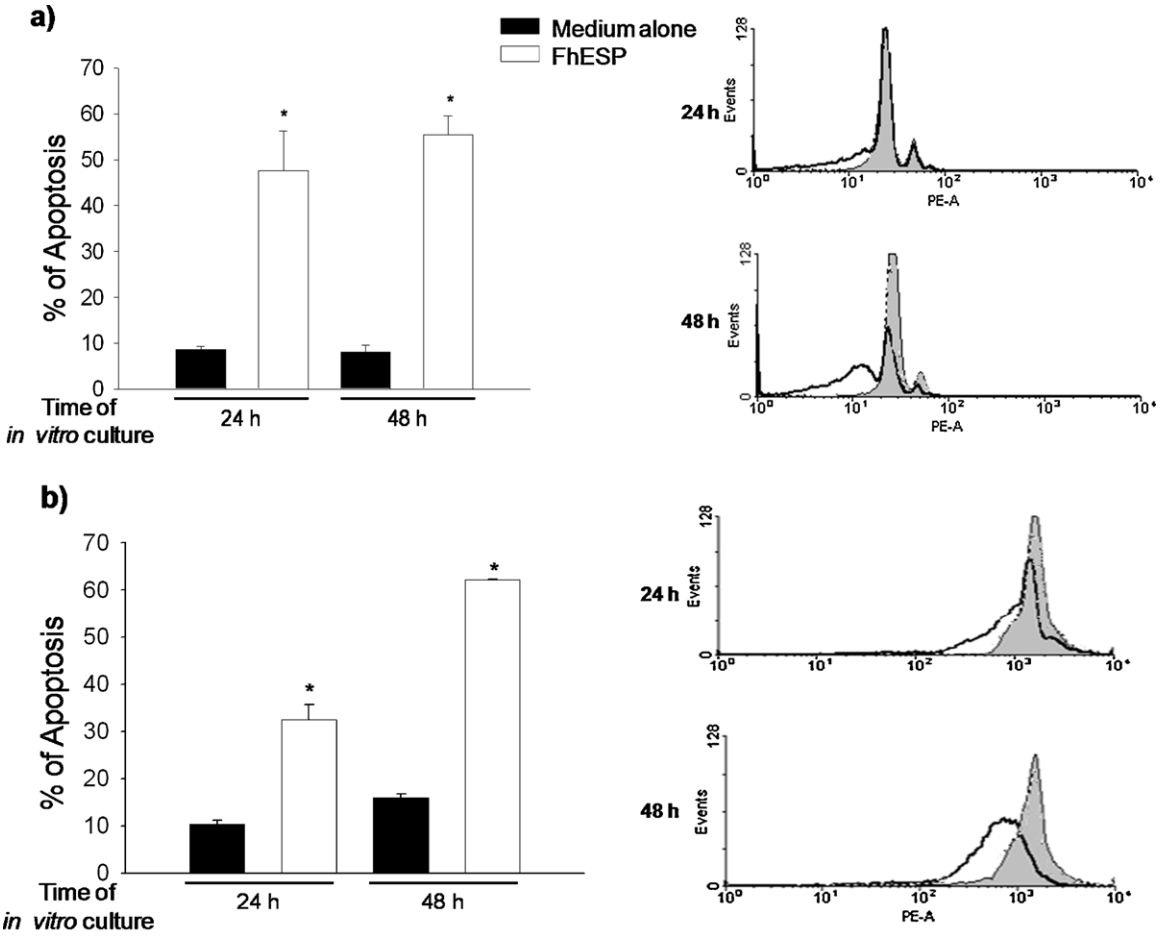

Fig. 1. FhESP induce apoptosis of mouse pMΦ. Peritoneal macrophages obtained from non-infected BALB/c mice (a) and J774 cells (b) were stimulated with FhESP (50 μg/ml) for 24 or 48 h. Apoptosis was detected by PI staining and analyzed by flow cytometry. Data are means ± SEM of 3 independent experiments, analyzed in triplicate. $^{*}p < 0.05$ (cells incubated with FhESP compared with cells in medium alone).

apoptosis induction during the early stages of infection, 48 h after metacercarial challenge the peritoneal cells were recovered and stained with FITC-conjugated annexin-V and PI. As shown in Fig. 2b, the percentage of cells that were positive for annexin-V ($^{*}p < 0.0015$), and annexin-V/PI ($^{*}p < 0.00030$) was significantly increased with respect to uninfected animals (data analyzed in the region corresponding to pMΦ).

Other helminths have also been shown to induce apoptosis of different innate immune cells. For example, *Trichomonas vaginalis* infection effectively kills MΦ (Jae-Ho et al., 2006), filarial parasites induce apoptosis of NK cells (Babu et al., 2007), and *Brugia malayi* microfilariae induce the death of DCs (DosReis et al., 2007). Furthermore, other parasites such as *Schistosoma japonicum* are able to induce Th1/Th2 cell apoptosis (Xinyu et al., 2010). Therefore, immune cell apoptosis may represent a way to restrict immunopathology in the host, as well as being a survival strategy to modulate the immune response and maintain parasite infection.

F. hepatica is able to induce apoptosis of two important innate immune cells (Eo and MΦ) which play key roles in the immune response developed during helminth infections. This seems to be a parasite strategy to avoid the host immune response, and identification of the components of *F. hepatica* responsible for the induction of apoptosis may help to clarify how this parasite directs the immune response for its own benefit. Ultimately, this knowledge could be used to enhance a protective immune response or in the application of immunosuppressive parasitic molecules as therapeutic agents.

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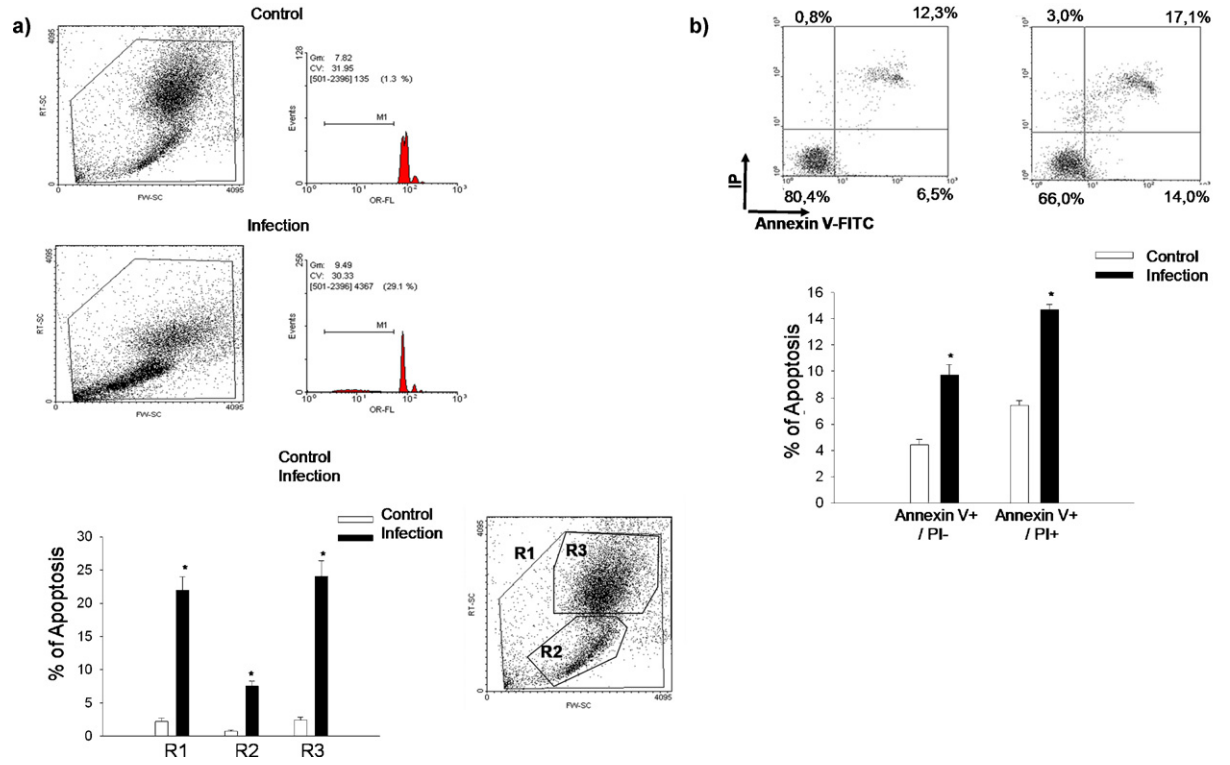

Fig. 2. *F. hepatica* induces apoptosis of mouse pMΦ during the early stages of infection. Mice were orally infected with 10 metacercariae of *F. hepatica*. Forty-eight hours after infection, peritoneal cells were recovered, and apoptosis was evaluated. The percentage of apoptotic cells displaying a hypodiploid DNA peak was determined using PI staining and flow cytometer analysis (a). Representative dot-plots and histograms of peritoneal cells (top panel), evaluation in cells gated on different peritoneal cell populations (bottom panel; R1 (PECs: 73% CD11b+; 23% F4/80+; 22% B220+), R2 (lymphocytes: 81% B220+; 20% CD11b+; 2% F4/80+) and R3 (MΦ: 83% F4/80+; 96% CD11b+; 9% B220+)). Furthermore, apoptosis was evaluated by annexin-V and PI staining and flow cytometer analysis of the MΦ population (b). Early apoptotic cells were stained with annexin-V alone whereas necrotic and late apoptotic cells were stained with both annexin-V and PI. In the infection experiment, 4 mice/group were analyzed. Data are means ± SEM of 3 independent experiments, analyzed in triplicate. $^{*}p < 0.05$ (cells from non-infected mice compared with cells from infected mice).

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