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Apoptosis as a Possible Mechanism of Infertility in *Echinococcus granulosus* Hydatid Cysts

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Abstract *Echinococcus granulosus* is a parasitic cestode causing hydatidosis in intermediate hosts (human and herbivorous). Most symptoms of the disease occur by the pressure exerted on viscera by cysts that are formed upon ingestion of the parasite eggs excreted by definitive hosts (canines). Protoscoleces, the developmental form of the parasite infective to definitive hosts, are formed in the germinal nucleated layer of fertile hydatid cysts. For unknown reasons, some cysts are unable to produce protoscoleces (infertile hydatid cysts). In this study, analysis of DNA fragmentation using TUNEL and agarose gel electrophoresis showed higher levels of apoptosis in infertile cysts as compared to fertile cysts. Additionally, caspase 3 was detected both in fertile and infertile cysts; the activity of this enzyme was found to be higher in infertile cysts. We conclude that apoptosis may be involved in hydatid cyst infertility. This is the first report on the presence of programmed cell death in *E. granulosus*. J. Cell. Biochem. 100: 1200–1209, 2007.

Key words: apoptosis; *E. granulosus*; hydatid cyst; cyst infertility

Hydatidosis, a zoonotic infection with high prevalence in part of Eurasia, Africa, Australia, and South America [Eckert and Deplazes, 2004; Jenkins et al., 2005; Moro and Schantz, 2006; Torgerson et al., 2006], represents a major public health and economic burden in many countries [Eckert et al., 2000; Sadjjadi, 2006; Yang et al., 2006]. The disease is caused by larvae of the parasitic platyhelminth *Echinococcus granulosus*, whose life cycle involves two mammalian hosts. The adult cestode (a flatworm) inhabits the small intestine of a carnivore (definitive host) and produces eggs containing infective oncospheres. After oral uptake of eggs by an intermediate host animal (herbivores as well as human), the larval stage (hydatid cysts) develops in internal organs (mainly liver and lungs) to unilocular fluid-filled bladders. These consist of two parasite-derived layers, an inner nucleated germinal layer and an outer acellular laminated layer, surrounded by a host-produced fibrous capsule (adventitial layer) [Zhang et al., 2003]. Protoscoleces, the developmental forms of the parasite infective to canine, are formed in the germinal layer of hydatid cysts. Depending on growth rate, localization, and number of cysts, the infection may remain asymptomatic or may turn to symptomatic [Eckert et al., 1995]. Clinical signs in secondary hosts derive from the mechanical damage produced by hydatid cysts on target organs [Gottstein and Hemphill, 1997; McManus et al., 2003; Sayek et al., 2004].

Two types of hydatid cysts can be observed in intermediate hosts: fertile cysts, on which protoscoleces are both joined to the germinal

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layer and free in the hydatid fluid filling the cyst cavity, and infertile cysts, which do not produce protoscoleces and are therefore unable to continue with the life cycle of the parasite [Kamenetzky et al., 2000; Lahmar et al., 2004]. The biological and molecular background underlying the generation of both types of cysts have not yet been elucidated [Vatankhah et al., 2003].

Apoptosis is a type of programmed cell death that can be identified by morphological and biochemical events such as chromatin condensation resulting in nuclear picnosis, DNA fragmentation, formation of apoptotic bodies, and cysteine aspartate-specific protease activation [Gupta, 2001; Zimmermann et al., 2001]. The central component of this machinery is a proteolytic system involving a family of proteases called caspases. These enzymes participate in a metabolic cascade that is triggered in response to proapoptotic signals and culminates in the cleavage of a set of key proteins, thus resulting in disassembly of the cell [Thornberry and Lazebnik, 1998].

Many cells undergo programmed cell death during normal animal development and in most mammalian tissues this process continues throughout the entire life [Weil et al., 1996]. Cell death during embryonic development is essential for successful organogenesis and crafting of complex tissues [Weil et al., 1996]. Also, the apoptosis pathway is beneficial for multicellular organisms since it is instrumental in eliminating cells which are damaged, infected, or are simply no longer required [Zangger et al., 2002; Ho and Hawkins, 2005].

Apoptosis is an evolutively conserved pathway [Kroemer et al., 1997] that in its basic tenets appears to be operative in all metazoans. It has been observed in developmental stages of many invertebrates, such as Porifera, Cnidarians, Echinoderm, Nematode, and Arthropods [Hwang et al., 2004] but so far no evidence of its presence in *E. granulosus* has been shown.

In platyhelminthes it has been demonstrated that soluble egg antigens from *Schistosoma mansoni* induce apoptosis in human cells [Carneiro-Santos et al., 2000] while proteases from *Taenia solium* induce apoptosis in human CD4+ T cells [Tato et al., 2004]. In planarian, apoptosis-related genes with homology to caspase family members have been reported [Hwang et al., 2004]. On the other hand, antibodies elicited by immunization with cDNA from cC1 protein, reacted with the native form of that protein and induced apoptosis in the cyst wall of *T. solium* metacestode [Wang et al., 2003]. Gamma irradiation against *T. solium* metacestode provided evidence of the occurrence of apoptosis and helped to elucidate mechanisms that would be involved when gamma irradiation inhibits the normal development of the *T. solium* metacestode into the adult worm [Flores-Perez et al., 2003].

The present study was designed to ascertain whether programmed cell death occurs and plays a role in the modulation of protoscolex production at the germinal layer of *E. granulosus* hydatid cysts. Our results are consistent with the notion that apoptosis is involved in a negative regulation of protoscoleces generation, leading to hydatid cyst infertility.

MATERIALS AND METHODS

Hydatid Cysts

Fresh fertile and infertile *E. granulosus* hydatid cysts were obtained from bovine livers or lungs at abattoirs in Santiago, Chile. Cysts were processed as described elsewhere [Galindo et al., 2003; Martinez et al., 2005]. Through macroscopic observation we defined a fertile cyst when it presented both free protoscoleces in the hydatid fluid, whitish color, and a thick laminar layer [Bortoletti and Ferretti, 1978; Galindo et al., 2003; Martinez et al., 2005]. Fertile cysts were confirmed under light microscopy by the presence of buds, and both grown protoscoleces attached to the germinal layer and free protoscoleces in the hydatid fluid. Infertile cysts were those showing a brown color in the laminar layer and the absence of protoscoleces either by macroscopic or microscopic observations.

Extraction of the Inner Surface of Hydatid Cysts and Collection of Protoscoleces

The inner surface of fertile and infertile cysts containing the germinal layer and part of the cysts wall were aseptically obtained by soft scraping of the inner layer of the cysts (previously washed with 10 ml of sterile phosphate buffered saline (PBS) pH 7.4) in a laminar flow chamber. Protoscoleces were decanted by gravity from the hydatid fluid, washed in PBS pH 7.4 at 38.5°C, and treated with pepsin 0.1% in Hanks' salt solution pH 2.0 at 38.5°C for 15 min

to eliminate remnants of germinal layer. Pepsin was removed by four washings with PBS.

Agarose Gel Analysis of DNA Fragmentation

DNA was isolated following a modified protocol previously described by Das et al. [2001]. Briefly, the inner surface of fertile and infertile cysts, as well as protoscoleces, were homogenized in 1% SDS, 10 mM Tris pH 8.0, treated with 300 μg/ml of proteinase K for 2 h at room temperature, and then incubated with 200 μg/ml of RNase (Sigma) for 1 h at 37°C. DNA was extracted from these samples with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and centrifuged at 16,000*g* for 5 min at 4°C. The upper phase was mixed with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ice cold ethanol. After overnight incubation at −20°C, samples were centrifuged at 16,000*g* for 10 min at 4°C. The pellets were washed once with 70% ethanol and recovered by centrifugation. DNA was suspended in 10 mM Tris, 1 mM EDTA and its concentration was determined by measuring the optical density at 260 nm. Aliquots containing 30 μg of DNA were subjected to electrophoresis on 1.8% agarose gels, stained with ethidium bromide, visualized in a UV light box, and photographed. DNA extracted from overgrowing Jurkat cells (mainly in apoptosis) was used as a positive laddering control.

TUNEL Assay and Nuclear Staining

Pieces of germinal layer from fertile and infertile hydatid cysts and protoscoleces, were fixed in 4% (w/v) paraformaldehyde in PBS pH 7.2 at 4°C for 24 h, and then embedded in paraffin. Sections (5 μm) were made permeable with 20 μg/ml proteinase K for 10 min at room temperature and the fragmented DNA was labeled using the TdT (terminal deoxynucleotidyl transferase) reaction mixture containing fluorescein-12-dUTP for 1 h at 37°C according to supplier recommendations (DeadEnd™ Fluorometric TUNEL system, Promega). Nuclei were stained with 1 μg/ml propidium iodide (PI) or 1 μg/ml Hoechst and visualized in a Nikon Eclipse E400 epifluorescence microscope. Digital images of TUNEL (terminal deoxy uridine triphosphate nick end labeling) and nuclear morphology were obtained using a Coolpix 4500 Nikon camera. The apoptotic index was obtained by scoring TUNEL positive reaction in 500 nuclei. The statistical significance of differences between control and experimental conditions were calculated by the Student's *t*-test.

Caspase 3 Identification and Localization

Protoscoleces were lysed in 1% Triton X-100, 150 mM NaCl, 5 μg/ml Aprotinin, 2 mM EDTA, 5 mM PMSF, 2.5 mM TLCK, 30 mM Tris pH 8.0. Total proteins, as measured by the Bradford assay [Bradford, 1976], were separated by electrophoresis in 15% SDS-polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane (Bio-Rad), blocked overnight with 3% soy bean proteins in PBS [Aguillon et al., 1992], and incubated for 2 h with specific antibodies against procaspase 3 (Pharmigen N° 552037). Reactive proteins corresponding to procaspase 3 were visualized by secondary antibodies conjugated to horseradish peroxidase (Jackson N° 111-035-144) and ECL Western blotting (Amersham Biosciences) in Bio-Max film (Kodak). Vero cells were used as a control. On the other hand, histological sections of pieces of germinal layer obtained from fertile and infertile cysts fixed in 4% (w/v) paraformaldehyde in PBS pH 7.2 at 4°C for 24 h were treated for immunofluorescence. For this purpose those sections were blocked overnight with Cas-Block (Zymed) and incubated firstly for 2 h at room temperature with polyclonal antibodies against procaspase 3 (Pharmigen N° 552037) dilution 1:50 v/v and then with the secondary polyclonal antibody FITC-conjugated anti-rabbit IgG (Dako N° F0205) dilution 1:100 v/v. Finally, the samples were mounted in VECTASHIELD and visualized in a Nikon Eclipse E400 epifluorescence microscope.

Colorimetric Assay of Caspase 3 Like Activity

Samples containing the inner surface of hydatid cysts were centrifuged at 800*g* for 5 min. The sediments were resuspended in cell lysis buffer (CaspACE™ Assay System, Colorimetric, Promega) and centrifuged at 16,000*g* for 10 min at 4°C. The assay was performed by using the Caspase 3 Assay Kit (Promega) that also contains the caspase 3 inhibitor Z-VAD-FMK (Z-Val-Ala-Asp fluoromethyl ketone, Promega). Briefly, the reaction mixture contained between 200 and 400 μg of total protein, 64 μl of caspase assay buffer, 4 μl of DMSO, 20 μl of DTT

(100 mM), and 4 μl of 10 mM Ac-DEVD-pNA (*N*-Acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide). Upon cleavage by caspase 3, pNA (*p*-nitroaniline) produces a yellow color that is measured at 405 nm. Extracts from fertile and infertile hydatid cyst were coincubated with caspase 3 inhibitor Z-VAD-FMK (final concentration of 50 μM). After 24 h of incubation differences in absorbance between samples with and without caspase 3 inhibitor were considered caspase 3 like activities and expressed as specific activities (pMol pNA liberated/mg/h). Differences in specific activities of the enzyme between fertile and infertile germinal layer were considered statistically significant at $P < 0.05$ in three independent experiments performed in duplicate. In the text, a representative experiment is shown.

Detection of the Active Fragment of Caspase 3

Samples containing the inner surface of fertile and infertile hydatid cysts were treated as indicated for caspase 3 identification but a specific antibody against the active fragment of caspase 3 (Pharmigen N° 551150) was used. Reactive proteins corresponding to the active fragments of caspase 3 were evidenced as indicated for caspase 3. Vero cells treated and untreated with 10 μM helenalin, an apoptotic inducer [Jimenez-Ortiz et al., 2005], were used as a control [Dirsch et al., 2001].

RESULTS

Apoptosis Is Involved in Fertility Processes at the Germinal Layer of Hydatid Cysts

Figure 1 shows representative TUNEL/PI merged images of the germinal layer of hydatid cysts. A positive TUNEL reaction was observed in nuclei of both fertile and infertile cysts. However, the germinal layer of infertile cysts presents higher scores of apoptotic nuclei (Fig. 1A) than those from fertile cysts (Fig. 1B) and protoscoleces (Fig. 1C). Protoscoleces presenting loss of tegument continuity (Fig. 1F, thin arrow) show higher levels of apoptotic nuclei (Fig. 1C, thin arrow) than protoscoleces with intact tegument (Fig. 1C,F, thick arrows). Apoptotic indexes (n: scoring TUNEL positive reaction in 500 nuclei) for the germinal layer of fertile cysts ($3.31 \pm 2.3\%$, $n = 9$) and protoscoleces ($6.35 \pm 5.5\%$, $n = 29$) are similar. However, the apoptotic index in the germinal layer of infertile hydatid cysts was significantly higher ($40.4 \pm 16.1\%$, $n = 6$, $P < 0.05$), that is, 10- and 6-fold larger than the one observed in the germinal layer of fertile cysts and protoscoleces, respectively. Since a tissue with such a high apoptotic index has low possibilities to continue

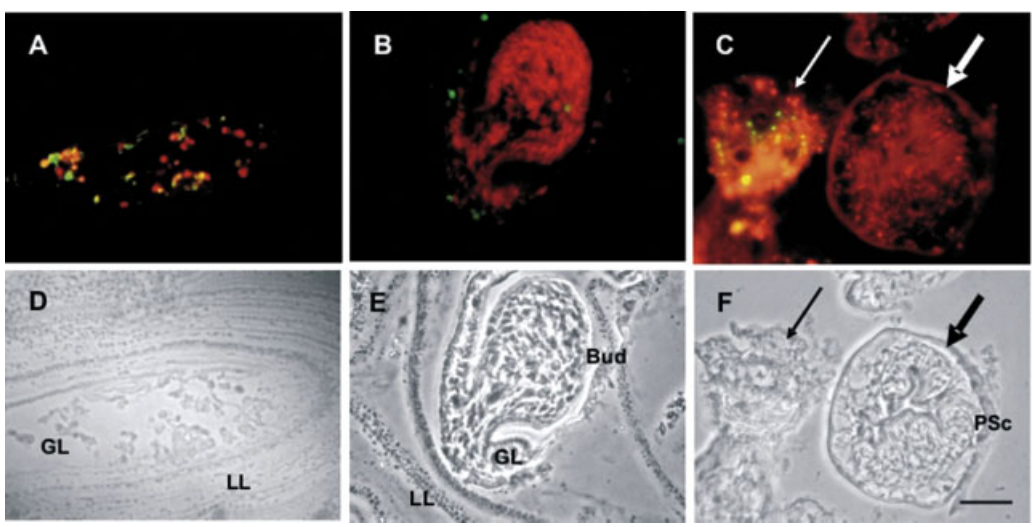


Fig. 1. Apoptosis in fertile and infertile hydatid cysts, and in protoscoleces, as determined by TUNEL assay. **A**, **B**, and **C** show positive TUNEL nuclei (green) merged with propidium iodide nuclear staining (red); yellow corresponds to colocalization of both dyes. **D**: Light microscopy image. **E** and **F:** Phase contrast. A and D: Infertile cysts. B and E: Fertile cysts and growing bud. C and F: Protoscoleces. GL, germinal layer; LL, laminated layer; PSc, protoscolex. Bar: 25 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

its development and being viable we proceeded to a more exhaustive study by applying TUNEL on histological slides of the germinal layer of infertile cysts.

The Germinal Layer of Infertile Cysts Presents Patches of Apoptosis

We observed that apoptosis in the germinal layer of infertile cysts occurs in patches. Thus, two areas of the same sample subjected to the TUNEL reaction showed either a high (71.05 %) or a low apoptotic index (1.02%) (Fig. 2B,E) despite the presence of a similar number of nuclei (Fig. 2A,D). This fact may explain the high deviations observed in the apoptotic indexes that were obtained for infertile cysts (see above) as well as the survival of the germinal layer of these cysts.

DNA Laddering

Another common approach to evidence the apoptotic process is the assessment of DNA fragmentation resulting from the action of active endonucleases. These enzymes cut DNA at internucleosomal sites, thus generating fragments of regularly variant sizes that can be observed by agarose gel electrophoresis (DNA laddering) [Huang et al., 2005]. When DNA extracted from the inner surface of infertile cysts was subjected to agarose gel electrophoresis, a clear pattern of DNA fragmentation was observed (Fig. 3, lane 2) that was absent in DNA obtained from fertile cysts or free protoscoleces (Fig. 3, lanes 3 and 4, respectively). This observation agrees with the high apoptotic index observed by TUNEL at the germinal layer of infertile cysts, as well as with the low indexes of apoptotic nuclei recorded in the germinal layer of fertile cysts and in protoscoleces. Thus, this result confirms the presence of an active apoptotic process in infertile cysts.

Presence and Activity of Caspase Like Proteins in Hydatid Cysts of *E. granulosus*

Since the metabolic pathway of programmed cell death is unknown in *E. granulosus*, we investigated the presence and localization of caspase 3, an effector molecule common to all known metabolic routes of apoptosis induction [Bayascas et al., 2002]. For this purpose a human anti-caspase 3 polyclonal antibody was

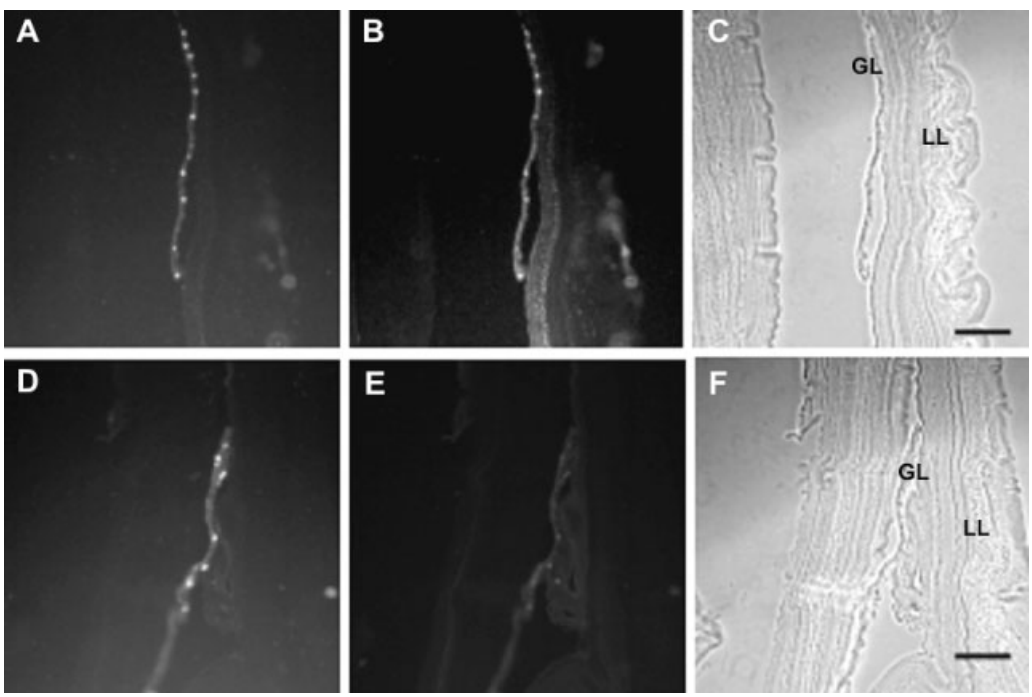


Fig. 2. Patches of apoptotic nuclei in the germinal layer of infertile hydatid cysts. Nuclei in two different areas (A/B/C and D/E/F) of a germinal layer from an infertile hydatid cyst. **A** and **D**: Hoechst nuclei staining. **B** and **E**: Positive (B) and negative (E) TUNEL nuclei. **C** and **F**: Phase contrast. Note the presence of zones with a high and a low number of apoptotic nuclei evidenced by TUNEL assay, in the same germinal layer. GL, germinal layer; LL, laminated layer. Bar: 25 μm.

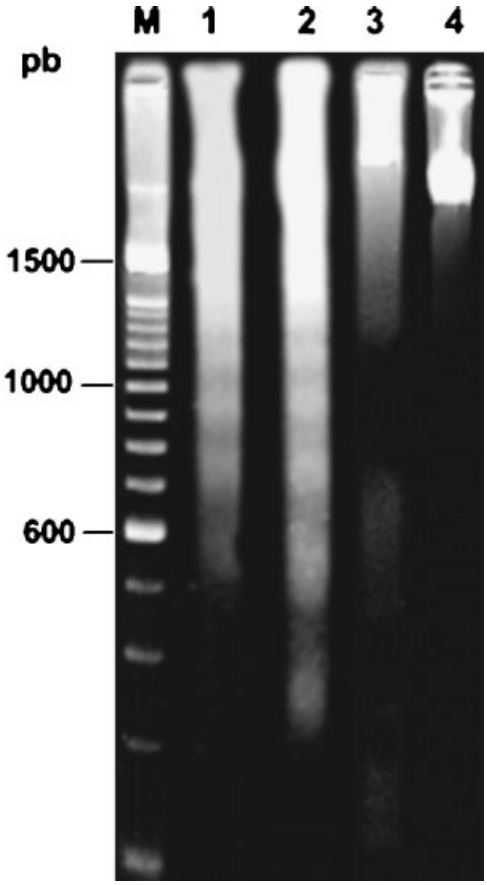


Fig. 3. Agarose gel electrophoresis of DNA extracted from the inner surface of fertile and infertile hydatid cysts and protoscoleces. M: DNA ladder; **lane 1**: DNA from Jurkat cells induced to apoptosis by overgrowing; **lanes 2** and **3**: DNA extracted from the inner surface of infertile cysts and fertile cysts, respectively; **lane 4**: DNA extracted from protoscoleces. Agarose gel (1.8%) was stained with ethidium bromide.

used. This antibody was able to recognize a protein with the expected molecular mass for procaspase 3 in monkey Vero cells (Fig. 4A, lane 1) and in *E. granulosus* protoscoleces (Fig. 4A, lane 2), this last being pure parasitic structures. The same antibody detected a putative procaspase 3 like enzyme in Vero cells (Fig. 4B,a) and in histological sections of both fertile (Fig. 4B,d) and infertile hydatid cysts (Fig. 4B,g). In all cases, the procaspase 3 like protein was distributed throughout the cytoplasm (compare Fig. 4B,a,d,g with B,b,e,h). Furthermore, the specific activity of active caspase 3 in infertile cysts was higher than in fertile cysts (Fig. 5A). In addition, by using a heterologous antibody raised against the active form of the enzyme, we also identified putative active fragments of caspase 3 in samples of the inner surface of hydatid cysts by Western blot, which are much more evident in infertile samples (Fig. 5B, lanes 1–4) than in fertile cysts (Fig. 5B, lanes 5–7). This finding is in agreement with the higher apoptotic index and the increased DNA fragmentation observed in infertile cysts.

DISCUSSION

So far, programmed cell death had not been described in *E. granulosus*. Studies in *T. solium*, a metacestode-related parasite, have shown that 0.3 kGy of radiation induce both the typical pattern of DNA laddering and TUNEL positive reaction. Growth stopped at the neck of the parasite, where the germinative zone has been described [Merchant et al., 1997], thus suggesting that this is the target for the gamma radiation. These findings indicate that irradiation inhibits the normal growth of the parasite, from the larval to the adult stage, by inducing apoptosis [Flores-Perez et al., 2003]. Moreover, excretion/secretion products containing cysteine proteases from the metacestode stage of *T. solium* induce apoptosis in CD4+ lymphocytes. This result suggests that the parasite can regulate the immunological response of the host through an apoptotic mechanism [Tato et al., 2004]. Coincidentally, a soluble antigen isolated from *S. mansoni* eggs can regulate the immune response of the host by inducing apoptosis in T cells from patients with schistosomiasis, thus determining different clinical forms of the disease [Carneiro-Santos et al., 2000]. Hwang et al. [2004] describes the occurrence of apoptotic cells during planarian regeneration and the presence of three genes with homology to caspases, named as DjClg 1, 2, and 3. Predicted amino acid sequences show a relatively short amino-terminal domain present in all caspases of different size. Phylogenetically, DjClg 1, 2, and 3 were clustered with the caspase subfamilies 3, 6, and 7, known to have a short prodomain.

In this study, we have obtained concluding morphologic and biochemical evidence that apoptosis does occur in the larval stage (hydatid cysts) of *E. granulosus*. First, picnotic nuclei and apoptotic bodies are present in the germinal layer of infertile hydatid cysts (not shown). Secondly, DNA fragmentation was clearly observed by TUNEL assay and by agarose gel electrophoresis in these parasitic structures. Thirdly, the detection of a procaspase 3 like

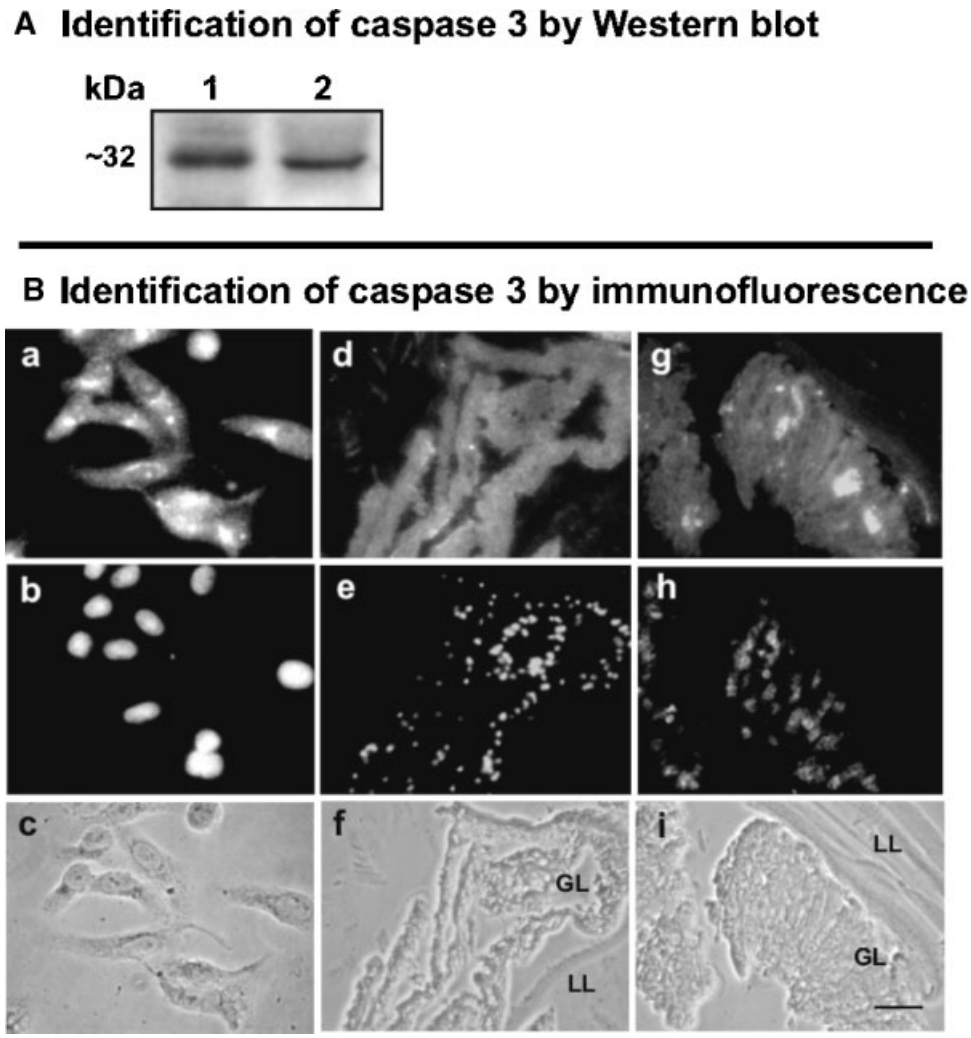


Fig. 4. Detection of a caspase 3 like protein in fertile and infertile hydatid cysts. **A**: Procaspase 3 detected in Vero cells (**lane 1**) and in protoscoleces (**lane 2**) homogenates by Western blot using a polyclonal antibody raised against the human caspase 3 and a horseradish-peroxidase-conjugated anti-rabbit antibody. **B**: Procaspase 3 in Vero cells (**a**), in the germinal layer of fertile cysts (**d**), and in the germinal layer of infertile cysts (**g**) detected by immunofluorescence, using the same antibody as in (A) and a FITC-conjugated secondary antibody. **b**, **e**, and **h**: Hoechst nuclei stain of Vero cells, fertile and infertile cysts, respectively. **c**, **f**, and **i**: Phase contrast of Vero cells, fertile and infertile cysts, respectively. GL, germinal layer; LL, laminated layer. Bar: 25 μm.

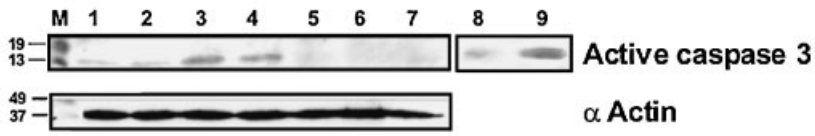
molecule in protoscolex homogenates and in the cytoplasm of the germinal layer of hydatid cysts is another evidence supporting the presence of a cystein-protease that could be involved in the apoptotic process in these parasites. Additionally, our results suggest the presence of the active fragment of a caspase 3 like enzyme in the inner surface of hydatid cysts based on a protein which is recognized by a specific antibody raised for the active fragment of caspase 3 and whose molecular weight is the one expected for this protein. Finally, we found a caspase 3 like activity in the inner surface of infertile cysts which is inhibited by a specific caspase inhibitor. All these results are unequivocal signals of the presence of an active mechanism of apoptosis in *E. granulosus*.

Interestingly, the germinal layer of infertile cysts displays apoptotic indexes as measured by TUNEL that are one order of magnitude higher than those observed in the germinal layer of fertile cysts. Likewise the germinal layer of fertile cysts, healthy protoscoleces attached to it or free in hydatid fluid show a low apoptotic index, with no evidence of DNA fragmentation, while damaged protoscoleces show a massive apoptotic process. Moreover, scraps taken from the inner surface of infertile cysts and

A Caspase 3 like activity

Cysts	pmol pNA/mg/hour
Fertile	2.53[a] ± 0.67
Infertile	4.67[a] ± 0.20

B Caspase 3 active peptide



Densitometric analysis (AU): Infertile cysts 255±122; fertile cysts 7±5

Fig. 5. Caspase 3 like activity (**A**) and presence of the active peptide derived from pro-caspase 3 (**B**) in the inner surface of hydatid cysts. A: Caspase 3 like activity was measured as the difference in absorbance between the samples with and without the caspase 3 inhibitor Z-VAD-FMK. The results correspond to the mean ± SD from three experiments in duplicate. [a]$P < 0.05$ by Student's *t*-test. B: Western blot of a caspase 3 active peptide, detected by chemoluminescense using a heterologous antibody raised against the human active caspase fragment and a horseradish-peroxidase-conjugated anti-rabbit antibody. Analysis of the gel was performed by UN-SCAN-IT gel software 4.1 version. Densitometric values in arbitrary units (AU) correspond to the caspase 3 active fragment as normalized by α-actin (mean ± SD). M, Molecular weight markers; **lanes 1–4**: Inner surface of infertile cysts; **lanes 5–7**: Inner surface of fertile cysts; **lane 8**: Stationary phase Vero cells; **lane 9**: Vero cells induced to apoptosis by 10 μM helenalin for 6 h.

containing both the germinal layer and part of the cyst wall, present both high DNA laddering and high caspase 3 like activity, as compared to fertile cysts.

Apoptosis in a platyhelminth such as *E. granulosus* should be expected. In fact, it has been suggested that the apoptotic mechanism probably takes place in normal intact flatworms as well as during tissue regeneration, thus having a role in controlling cell number, in eliminating unnecessary tissues or cells, and in remodeling the old tissues of body parts [Hwang et al., 2004]. In summary, apoptosis may be a normal program of tissue reorganization during development or it could be a response to damaging external stimuli. Moreover, parasitic flatworms may elicit programmed cell death in host cells, mainly for the regulation of host-parasite interactions [Carneiro-Santos et al., 2000; Flores-Perez et al., 2003; Tato et al., 2004].

We propose that apoptosis plays a definitive and different role in *E. granulosus*, basically by modulating the production of protoscoleces at the germinal layer of hydatid cysts. Thus, this type of programmed cell death may be a cellular mechanism underlying hydatid cyst infertility, which is observed in some herbivores and humans [Yildiz and Gurcan, 2003; Zhang et al., 2003].

Another aspect of interest that has derived from this study is the patchy distribution of the apoptotic nuclei in the germinal layer of infertile cysts. In previous works we demonstrated that particular sectors of the germinal layer show an initial increase in cell proliferation which subsequently develop to elongated buds and protoscoleces [Galindo et al., 2002, 2003]. Those sectors of cellular proliferation are patch-distributed in the fertile germinal layer of fertile hydatid cysts [Galindo et al., 2003]. It is then possible that the apoptotic mechanism is elicited only in the proliferative nuclei patches thus stopping bud generation and producing infertile cysts.

Finally, the possibility that the apoptotic mechanism here described may also be related to an effective modulation of the host response to the parasite, cannot be ruled out at present.

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