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Anti-apoptosis effects by *Eimeria tenella* infection in Madin-Darby bovine kidney cells

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Abstract

Apoptosis is a host defense mechanism that the cell uses to limit production of infectious pathogens. Although many bacteria, viruses and parasites can induce apoptosis in infected cells, some pathogens usually exhibit the ability to suppress the induction of apoptosis in the infected cells. Sophisticated evasion strategies of obligate intracellular parasites, in particular prevention of host cell apoptosis, are necessary to ensure successful replication. To study the ability of *Eimeria tenella* in this regard, *in vitro* experiments were performed applying Madin-Darby bovine kidney (MDBK) cells as host cell. We have demonstrated that productive infection of adherent cell lines by *E. tenella* resulted in an anti-apototic effect. This phenomenon was confirmed using *in situ* terminal deoxynucleotidyl transferase- mediated (TdT) deoxyuridine triphosphates (dUTP)-fluorescein nick end labeling (TUNEL) assay to detect apoptosis. Therefore, *E. tenella* could complete its cycle of productive infection while inducing anti-apoptosis in the infected cells. This finding might have implications for the pathobiology of *E. tenella* and other *Eimeria* species.

Key words : Protozoa, Apoptosis, Anti-apoptosis, Apicomplexa, *Eimeria tenella*

INTRODUCTION

Apoptosis is one type of cell death, which is classically defined by both morphological and biochemical characteristics, which are representative with cell shrinkage, condensation and fragmentation of the cell nuclei and fragmentation of chromosomal DNA into nucleosomal oligomers (Kerr and Harmon, 1991). Once apoptosis is triggered, a series of programmed events leads to the death of the cell manifested by morphological changes, activation of specific enzymes, and especially by the degradation of cellular DNA (Ellis et al, 1991). Chromosomal fragmentation, possibly caused by cytoplasmic proteases indicating endonuclease activation, has become accepted as hallmark for this form of cell death, which is detected as a ladder pattern on gel electrophoresis (Wyllie et al, 1984). Kim et al (2000) described *in situ* terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphates (dUTP)-fluorescein nick end labeling (TUNEL) assay that makes possible the demonstration of apoptosis.

Apoptosis is a host defense mechanism that the cell uses to limit production of infectious pathogen. Although many pathogens can induce apoptosis in infected cells, large DNA viruses, such as poxviruses, herpesviruses and adenoviruses, usually exhibit the ability to suppress the induction of apoptosis in the infected cells (O'Brien, 1998; Tschopp et al, 1998). There is considerable evidence that herpesviruses block apoptosis (Cheng et al, 1997; Koyama and Adachi, 1997; Leopardi and Roizman, 1996; Shen and Shenk, 1995; Sieg et al, 1996; Zhu et al, 1995). Also, these findings were reported

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during a general evasion strategy of intracellular protozoa such as *Toxoplasma gondii* (Nash et al, 1998; Goebel et al, 1999; Payne et al, 2003; Lüder and Gross, 2005) and *Neospora caninum* (Nishikawa et al, 2001; Herman et al, 2007).

Eimeria tenella (*E. tenella*) is an intracellular coccidian (phylum Apicomplexa, order Coccidia, family Eimeriidae), which affects the cecal epithelial cells of chickens. It is the asexual stages (schizogony or merogony) of the lifecycle that are pathogenic and manifest clinically as coccidiosis. The parasite is considered to be the most important poultry infection worldwide (Cox, 1998). *Eimeria* spp. are obligate intracellular parasites, which makes them challenging organisms to study. *In vitro* culture has become a useful tool by enabling us to understand aspects of their lifecycle that could not otherwise be studied (McDougald, 1978). Patton (1965) first described the *in vitro* cultivation of this parasite in Madin-Darby kidney (MDBK) cells in which he obtained first stage, asexual development.

It was recently shown that also *Eimeria* spp. in chicken induce the transcriptional factor NF-kB in the course of their development (del Cacho et al, 2004). Previously, several research groups reported permissive host cells, which allow macromeront formation *in vitro* and non-permissive cells, which allow the sporozoites to survive but prevent any further development of the parasite (Hermosilla et al, 2002; Behrendt et al, 2004). In order to trigger host cell apoptosis actinomycin D was used for the pro-apoptotic inducer (Kalousek et al, 2007; Wang et al, 2007; Saji et al, 2007).

The present study is aimed to investigate the anti-apoptotic activities by *E. tenella* infection to MDBK cells *in vitro*.

MATERIALS AND METHODS

Host cells and *Eimeria* infection

Sporozoites of *E. tenella* were excysted as described (Tomley, 1997). Briefly, after surface sterilization with bleach, the oocyst walls were broken using 0.5 mm glass beads (Biospec Products, Bartlesville, OK, USA). Thereafter, the sporozoites were recovered from sporocysts by enzymatic excystation using 0.25% trypsin (w/v) (Sigma-Aldrich, St. Louis, MO, USA), 10 mM $MgCl_2$ (w/v) and 1% sodium taurocholic acid (w/v) (Sigma-Aldrich) at 41°C for 60～90 min. The excysted sporozoites were purified by centrifugation in 60% (v/v) isotonic Percoll™ solution (density, 1.129 g/ml; Pharmacia, Stockholm, Sweden) by a 1 min spin at 10,000×g. The pelleted sporozoites were collected carefully from the bottom of the microtubes and washed three times with phosphate buffered saline (PBS) as described previously (Dulski, 1990; Tomley, 1997).

Induction of apoptosis

Actinomycin D (Sigma-Aldrich) was used as pro-apoptotic inducer. In this experiment, we used the following conditions: actinomycin D: 3.5 mg/ml, 48 hrs incubation with MDBK cell. Actinomycin D, inducer of apoptosis, was not applied to infected cells until 72 hrs because otherwise parasites were initiated to egress from the once infected host cell.

TUNEL assays

Apoptosis at single cell level was analysed by the TUNEL assay (Roche, Basel, Switzerland), which is based on the digoxigenin labelling of DNA strand breaks. Cell cultures grown on 8-well chamberslides (BD Biosciences, San Jose, CA, USA) were infected with 4×10^4 freshly isolated *E. tenella* sporozoites. Three days after infection actinomycin D were applied as described above. Following incubation, chamber slides were rinsed twice with PBS, dried at room temperature (RT) and fixed with paraformaldehyde (4% w/v, 1 hrs, RT; Sigma-Aldrich). Cells were permeabilised with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) in PBS (10 min, RT), rinsed twice with PBS, exposed to 50 ml of TUNEL reaction mixture (Roche), covered with coverslips and incubated (humidified atmosphere at 37°C, 1 hrs in the dark). Slides were then rinsed three times with PBS, and slides were incubated with anti-digoxigenin conjugated with alkaline phosphatase (Roche) and colorized with nitroblue tetrazolium (NBT) and 5-bromocresyl-3-indolyl-phosphate

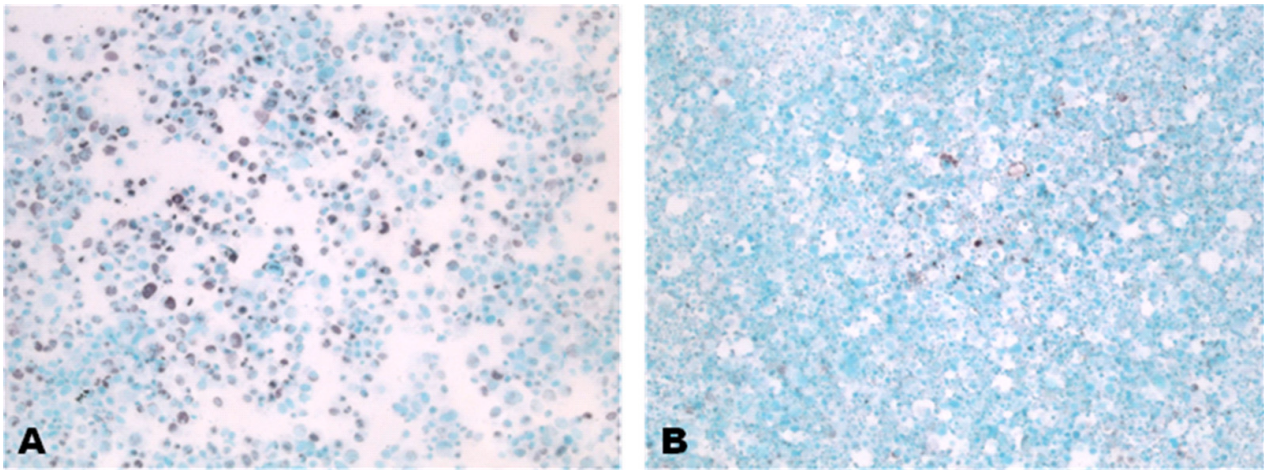

Fig. 1. Apoptosis at single cell level was analysed by the TUNEL assay. Counterstain with 0.5% methyl green (×100). (A) *E. tenella* non-infected MDBK cells with actinomycin D. (B) *E. tenella*-infected MDBK cells with actinomycin D.

(BCIP). Then, slides were counterstained with 0.5% methyl green. Specific dark blue signals were examined with light microscope (Nikon, Tokyo, Japan) and FOculus F2 Digital system (New Electronic Technology GmBH, Lerchenberg, Germerny).

The percentage of apoptotic cells was calculated as follows: % apoptosis=(Total number of cells with apoptotic signals/total number of cells counted)×100.

RESULTS

Positive apoptosis signal at single cell level by the TUNEL assay resulted in the nucleus as distinct areas of dark brown signals in MDBK cells (Fig. 1). The percentages of apoptotic cells in *E. tenella*-infected and non-infected MDBK cells with or without pro-apoptotic drug, actinomycin D, were evaluated using TUNEL assay, an assay able to directly detect DNA fragmentation at the single-cell level. In the absence of apoptotic inducer, only single cells underwent apoptosis and this was equally observed in *E. tenella*-infected and non-infected MDBK cell cultures. After treatment with the pro-apoptotic drug, the number of apoptotic cells increased strongly. The percentage of apoptotic cells was 45.2±3.35% in *E. tenella* non-infected MDBK cells with actinomycin D (Fig. 2). However, *E. tenella*-infected MDBK cells with actinomycin D showed marked decrease as 8.6±2.88% in the percentage of apoptotic cells,

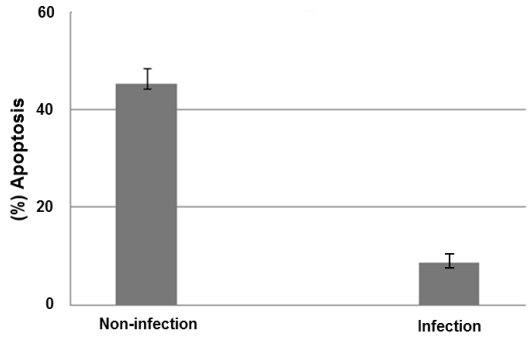

Fig. 2. Proportion of apoptotic cells detected by TUNEL assay. Non-infection: *E. tenella* non-infected MDBK cells with actinomycin D. Infection: *E. tenella*-infected MDBK cells with actinomycin D.

when compared with *E. tenella* non-infected MDBK cells with actinomycin D (Fig. 2). Results showed marked decrease in the percentage of apoptotic cells following *E. tenella* infection, when compared with *E. tenella* non-infected cells ($P<0.01$)

Thus infection obviously protected the host cells from apoptosis. Protection occurred in the infected MDBK cells and was observed.

DISCUSSION

Avian coccidiosis is of great economic impact for poultry industry and is caused by seven different species of protozoa belonging to the Genus *Eimeria*. The world-

wide economic losses due to avian coccidiosis are estimated at about $800 million (Williams, 1998, 1999; Lillehoj and Lillehoj, 2000). The increasing resistance of *Eimeria* spp. of chicken to anticoccidial drugs currently used by poultry industry has stimulated the search for new and alternative control methods for avian coccidiosis (Allen et al, 1998).

Apoptosis, or programmed cell death, is a cell suicide program characterized by chromatin condensation, DNA fragmentation, membrane asymmetry and membrane blebbing (Trump and Berezesky, 1998). A variety of pathogens have been found to induce apoptosis in infected cells (Ameisen and Capron, 1991; Sadzot-Delvaux et al, 1995; Superti et al, 1996). However, in some kinds of microbes, control of apoptosis may be critical to produce adequate levels of progenies, to spread their descendants in tissues or to facilitate their persistence (Shen and Shenk, 1995; Tropea et al, 1995).

Apart from the generally anti-apoptotic effects of intracellular parasites it should, however, not be overlooked that also an ability of apicomplexa to promote host cell apoptosis is known. As such, upon complete maturation of *E. necatrix* meronts infected host cells lack expression of anti-apoptotic factors, suggesting that the parasites then initiate host cell apoptosis in order to promote the release of merozoites (del Cacho et al, 2004). Also other apicomplexa exhibit dual ability to inhibit or to promote the host cell apoptosis in this sense, as seen for *T. gondii* and *P. berghei* (Lüder et al, 2001; van de Sand et al, 2005).

The present study demonstrates that infected host cells with *E. tenella* are protected from artificially induced apoptosis. Applying TUNEL assays, we confirmed that *E. tenella* infected host cells are protected from experimentally induced apoptosis *in vitro*. Our results do not allow us to fully understand the pathways involved in *E. tenella*-induced anti-apoptosis and further studies are necessary. Nevertheless, whatever the exact mechanism, our results could lead to adding the information about *E. tenella*-induced anti-apoptosis and to a suggestion that they could induce the anti-apoptosis of infected cells during the stage of their replication.

In this study, *E. tenella* revealed anti-apoptosis activities during the infection stage in MDBK cells. This finding might have implications for the pathobiology of *E. tenella* and other *Eimeria* species.

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