Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 148 (2007) 318–324
veterinary parasitology
www.elsevier.com/locate/vetpar

In vitro activity of the nonsteroidal anti-inflammatory drug indomethacin on a scuticociliate parasite of farmed turbot

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Received 15 March 2007; received in revised form 8 June 2007; accepted 14 June 2007

Abstract

The scuticociliatosis produced by the endoparasite *Philasterides dicentarchi* is a severe parasitic infection of farmed turbot (*Scophthalmus maximus*) characterized by several histopathological effects including extensive inflammation. Indomethacin is a nonsteroidal anti-inflammatory drug that specifically inhibits synthesis of the proinflammatory mediator prostaglandins. The effect of indomethacin on the *in vitro* growth of *P. dicentrarchi* was investigated. *In vitro* growth of the scuticociliate was significantly inhibited by treatment with 100 μM indomethacin for 48 h. Higher concentrations of indomethacin (mM levels) did not affect the gelatinolytic activity of the cysteine proteinases of *P. dicentrarchi*. *In vitro* treatment with 25, 50 or 100 μM indomethacin for 3 days did not significantly affect the enzymatic activity of cysteine proteinases, as assayed with *p*-nitroanilide as substrate. Immunoblot analysis with anti-cysteine proteinase antibodies revealed an increase in proteinase expression (molecular weights of 80, 32 and 40–45 kDa) in parasite lysates originating from *in vitro* cultures incubated with 25 μM indomethacin for 72 h. Degradation of genomic DNA of the ciliates was observed in cultures incubated with 100 μM indomethacin for 1, 3 and 7 days. The results suggest that indomethacin is capable of inhibiting *in vitro* growth of the scuticociliate *P. dicentrarchi* by a mechanism related to the induction of programmed cell death, without affecting the enzymatic activation of parasite proteinases, which demonstrates the potential therapeutic use of this drug in the control of turbot scuticociliatosis.

Keywords: *Philasterides dicentrarchi*; Turbot; Indomethacin; Cysteine proteinases; Apoptosis

1. Introduction

Philasterides dicentrarchi is an emerging histophagous endoparasitic scuticociliate that causes severe systemic infections in farmed flatfish in several regions of the world (Iglesias et al., 2001; Kim et al., 2004; Jung et al., 2005). In turbot (*Scophthalmus maximus*) the histopathological effects of infection are characterized by severe encephalitis and meningitis, necrosis of the hepatic parenchyma, degeneration of muscle fibres, hyperplasia of the branchial epithelium, severe oedema of the intestinal wall and abdominal distension caused by accumulation of ascitic fluid in the body cavity, which indicates the existence of a strong inflammatory response (Iglesias et al., 2001).

We have recently demonstrated high levels of the proinflammatory eicosanoid prostaglandin E_2 (PGE_2) in

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doi:10.1016/j.vetpar.2007.06.018

the ascitic peritoneal fluid of turbot infected with *P. dicentrarchi* and have suggested that the presence of PGE_2 may play a central role in innate immune regulation, as well as in the pathogenesis of this disease or in the survival of the parasite (Paramá et al., in press). We have also demonstrated that *P. dicentrarchi* produces proteinases, of the cysteine proteinase type, which may play a role in evasion of the turbot immune response via induction of apoptosis in leucocytes (Paramá et al., 2007) and also in the invasion and dissemination of the parasite in host tissues (Paramá et al., 2004). Cysteine proteinases from *P. dicentrarchi* also provoke an increase in the intracellular production of superoxide anion (O_2^-) in turbot pronephric leucocytes, which may be deleterious to the host cells (Paramá et al., in press).

Indomethacin is a nonsteroidal anti-inflammatory drug, which, in mammals provokes inhibition of the synthesis of prostaglandins via inhibition of cyclooxygenase activity (Tanaka et al., 2002). Indomethacin has also demonstrated an inhibitory effect on prostaglandin synthesis in turbot leucocytes stimulated with β-glucans (Paramá et al., in press). It has been suggested that indomethacin may play a role in protecting host cells from damage by free radicals (Hrabák et al., 2001), and in inhibiting cysteine proteinase-mediated degradation of cytoskeletal protein (Banik et al., 2000). Release of various eicosanoids has also been demonstrated for a number of protozoan and metazoan endoparasites and these substances appear to play a role in penetration, immune suppression, inflammation and modulation of haemostasis, which enables invasion and establishment of parasites (Daugschies and Joachim, 2000). The protective role of indomethacin in several parasitic infections has been shown to be due to the effects of the drug on production of prostaglandins generated during the disease (Terrazas et al., 1999; Martín Pérez-Santos and Talamás-Rohana, 2001; Guimaraes et al., 2006), or due to a direct effect on the metabolism of the parasites (Kovács and Csaba, 1997; Pfaff et al., 2005).

Although indomethacin is known to have some inhibitory effects on the ciliates *Tetrahymena* and *Paramecium* (Miglietta and Nelson, 1988; Kovács and Csaba, 1997; Kovács and Pállinger, 2003), there is no information about the effect of the drug on fish scuticociliates. The aim of the present study was to investigate the effect of indomethacin on the growth rate of *P. dicentrarchi* in culture, to determine the potential inhibitory capacity of this molecule on the proteolytic activity of the cysteine proteinases secreted by the parasite, and also to analyze the effect of indomethacin on the induction of apoptosis in parasite trophozoites.

2. Materials and methods

2.1. Parasite cultures and preparation of lysate

The scuticociliate *P. dicentrarchi* (Iglesias et al., 2001) was obtained from ascitic fluid of naturally infected turbot and was maintained in culture, as previously described (Iglesias et al., 2003).

Parasite lysate was obtained as described by Paramá et al. (2004). Briefly, the ciliates were washed three times by centrifugation at 650 × *g* for 5 min in 0.25 M sucrose, disrupted ultrasonically, homogenized in a tight-fitting Dounce homogenizer, centrifuged at 15,000 × *g* for 15 min and the supernatant fractions stored at −80 °C. The protein concentration in the extract was determined with a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Germany), and with serum albumin bovine (Sigma–Aldrich, Spain) as standard.

2.2. Effect of indomethacin on the growth rate of *P. dicentrarchi*

Ciliates in the late exponential phase or early plateau phase of culture were concentrated by centrifugation at 650 × *g* for 5 min and then resuspended in fresh culture medium to a final cell density of 10^5 cells/ml. Aliquots (100 μl) of ciliate suspension containing 10^4 ciliates were added to each well of 24-well polystyrene plates containing 900 μl of culture medium with the required dose of indomethacin (Sigma–Aldrich). The final doses of indomethacin tested (25, 50 and 100 μM) were prepared in culture medium from a 100 mM stock solution diluted in ethanol (and obtained from an stock initial solution of 1 M indomethacin dissolved in dimethyl sulphoxide (DMSO; Sigma–Aldrich)). Control cultures were prepared by adding the same concentrations of ethanol and DMSO, present in the 100 μM dose of indomethacin to the culture medium. Cultures were prepared in triplicate and incubated for 8 days at 18 °C. The numbers of cells in formalin-fixed samples (0.5 ml) after 0–4, 7 and 8 days of culture were counted with a haemocytometer.

2.3. Proteinase purification

P. dicentrarchi proteinases were purified on a bacitracin-sepharose affinity column, as previously described (Paramá et al., 2007). Briefly, heat-treated bacitracin (Sigma–Aldrich) in 0.1 M $NaHCO_3$, pH 8.3, was coupled to BrCN-activated Sepharose 4B (GE Healthcare, USA) by incubating for 1 h at ambient temperature, and the excess reactive groups were