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Effects of bile acids on proliferation and production of proteinase activity of *Uronema marinum* (Ciliophora: Scuticociliatida)

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ABSTRACT: Little is known about the effects of bile acids in relation to infectivity on the biological characteristics of *Uronema marinum*, a serious opportunistic parasite of farmed olive flounder *Paralichthys olivaceus*. In this study, we examined the effects of bile acids on the proliferation of *U. marinum* and on proteinase production *in vitro*. Proliferation of *U. marinum* was significantly enhanced by lithocholic acid (LCA) at 30 and 60 µmol, and by chenodeoxycholic acid (CDCA) at 0.06 µmol. In contrast, a significant decrease in proliferation was observed with cholic acid (CA) at 30 and 60 µmol, and with deoxycholic acid (DCA) at all amounts used. Proteinase production from live *U. marinum* was significantly increased by LCA, whereas CA significantly decreased proteinase production. CDCA and DCA had no effect on proteinase production. Although the types and concentrations of bile acids in the faeces of olive flounder are not known, the present results suggest that bile acids in the culturing water might influence the proliferation and production of proteinases in *U. marinum*, resulting in an increased possibility of scuticociliatosis in olive flounder farms.

KEY WORDS: *Uronema marinum* · Bile acids · Proliferation · Proteases

INTRODUCTION

Uronema marinum is a facultative scuticociliate responsible for an increasing number of serious infections in farmed olive flounder *Paralichthys olivaceus* in Korea (Jee et al. 2001). The ciliate is characterized by its high potential for systemically invading and destroying fish tissues leading to significant mortalities of the host. However, the parasite and the host factors that determine the onset of disease remain undetermined. Several researchers have assumed that immunosuppression by various stressors on the fish including handling, environmental pollution, inappropriate diet, and wounds could make it possible for scuticociliates to cross the immunological barriers of fish (Cheung et al. 1980, Dragesco et al. 1995, Munday et al. 1997, Sterud et al. 2000, Iglesias et al. 2003). However, it cannot be excluded that the strengthening of scuticociliates infection potential is due to certain factors present in fish culture tanks and might be involved in infection by scuticociliates.

In the pathogenesis of parasitic diseases, proteinases have been shown to play important roles in the facilitation of host tissue invasion, digestion of host proteins, and protection against immunological attacks by the host (McKerrow 1989, McKerrow et al. 1993). The roles of proteinases in the virulence of *Uronema marinum* are unclear, but secretion of proteinases is thought to be necessary to break down protein barriers in the host. In addition, high density of scuticociliates in culturing tanks would increase the infection pressure.

Bile acids are steroid metabolites of cholesterol, which function as trophic factors for the gut epithelium and as detergents for the absorption of cholesterol and fat-soluble vitamins. In humans, the primary bile acids, cholic and chenodeoxycholic acids, are synthesized in the liver and are excreted into the duodenum, where they facilitate absorption of dietary lipids. Most of

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these bile acids are reabsorbed in the intestine. However, a small quantity remains unabsorbed and passes into the colon, where it is converted to secondary bile acids, deoxycholic and lithocholic acids, by enteric bacteria. Fish release bile acids into the surrounding water mainly through faeces (Zhang et al. 2001), and it has been suggested that the excreted bile acids play a role as chemical signals which can induce behavioral responses in fish (Stabell 1987, Sola & Tosi 1993, Li et al. 1995).

Studies on human colorectal cancer have shown that bile acids stimulate cell proliferation and invasion of carcinoma cells (Debruyne et al. 2001). Little is known about the effects of bile acids on the biological characteristics of *Uronema marinum* in relation to infectivity. In this study, we examined the effects of bile acids on the proliferation of *U. marinum* and on proteinase production *in vitro*.

MATERIALS AND METHODS

Culture of *Uronema marinum*. Cells of *U. marinium* isolated from the brain of infected olive flounders *Paralichthys olivaceus* in the logarithmic phase of growth were cultured in filtered and autoclaved seawater containing 0.2% yeast extract at 25°C .

Effect of bile acids on the proliferation of *Uronema marinum*. The bile acids cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid (LCA) were obtained from Sigma. The ciliate count was 10^2 in 100 µl of total volume at the start of the experiments. The ciliates were held in 96-well flat-bottomed microplates, and 5 wells comprised 1 group. The ciliates were exposed to 0 (control), 0.06, 0.6, 6, 30, and 60 µmol of each kind of bile acid, and were cultivated in a humidified chamber at 25°C. After 48 h, the ciliates were fixed in 4% formaldehyde, and the number of cells was determined with a light microscope and Neubauer haemocytometer.

Effect of bile acids on proteinase activity of *Uronema marinum*. The proteolytic activity of the live *U. marinum* was detected by incubating 5×10^4 cells of the ciliate in 10 µl filtered seawater. To this was added 10 µl of 10 µg ml^{-1} of fluorescein isothiocynate (FITC)-casein (Sigma) and 180 µl of filtered seawater (pH 7.0) containing 60 µmol of each bile acid in a black 96-well plate (Greiner Bio-One) at 25°C. Wells not supplemented with any bile acids were used as controls. In addition, wells with FITC-casein plus each bile acid without the ciliate were used to confirm whether bile acids themselves influence the fluorescence polarization (FP) values, and wells containing only 200 µl of filtered seawater were designated as blanks. The fluorescence polarization was measured in a Polarion instrument (TECAN Austria). The excitation wavelength and the emission wavelengths were 485 and 535 nm, respectively. All assays were done in 5 replicates. Readings were automatically recorded at 30, 60, 90, 150, and 180 min in millipolarization units (mP).

Statistics. The Mann-Whitney test was used to compare each amount of bile acid with the controls, i.e. ciliates cultured without bile acids. Differences were considered significant when $p < 0.05$.

RESULTS

Effect of bile acids on *Uronema marinum* proliferation

Proliferation of *Uronema marinum* was significantly enhanced by LCA at 30 and 60 µmol, and by CDCA at 0.06 µmol. In contrast, a significant decrease in proliferation was observed with CA at 30 and 60 µmol, and with DCA at all amounts used (Fig. 1).

Effect of bile acids on the production of proteinase activity

The production of proteinase activity from live *Uronema marinum* was significantly increased by LCA, whereas CA significantly decreased proteinase activity. CDCA and DCA had no effect on proteinase activity (Fig. 2).

DISCUSSION

The present results suggest that specific bile acids can exert potent effects on *Uronema marinum* proliferation. The proliferation was significantly induced by LCA at 30 and 60 µmol and by CDCA at 0.06 µmol. The effects of bile acids on cell proliferation have been well demonstrated from researches in human colon cancer (Debruyne et al. 2001). Raised levels of secondary bile acids have been reported in patients with adenomatous polyps and colon cancer (Reddy & Wynder 1977, Imray et al. 1992). Although the mechanism of this tumor promoter activity is unknown, it has been suggested that bile acids act by modifying intracellular signaling and gene expression, perhaps by altering the activity of Protein Kinase C (PKC) (Huang et al. 1992, Pongracz et al. 1995, Hirano et al. 1996, Rao et al. 1997). PKC plays a key role in the regulation of cellular processes including growth, differentiation, tumor promotion, and apoptosis (Blobe et al. 1994). Studies have shown that bile acids activate PKC in both normal colonic epithelial cells and in colon tumor cell lines

(Craven et al. 1987, Huang et al. 1992). Ciliates have well-developed signal cascade mechanisms (Csaba 1985, 1994, Kovács & Csaba 1995, Christensen et al. 1998). Increased survival and proliferation of *Tetrahymena thermophila* by treatment with phorbol 12-myristate 13-actate (PMA), a direct activator of PKC, has been reported (Straarup et al. 1997). Therefore, activation of PKC and modification of signal transduction appear to be an aspect of the effect of bile acids in the proliferation of *U. marinum*. Epidemiological studies in humans have associated the development of colorectal cancer with elevations in fecal bile acid concentration, particularly LCA (Hill 1991a,b). It has been reported that the ratio of LCA to DCA is 2-fold greater in persons with colon cancer compared to controls (Owen et al. 1986). Interestingly, in the present study, the proliferation of *U. marinum* was significantly increased by LCA but significantly decreased by DCA.

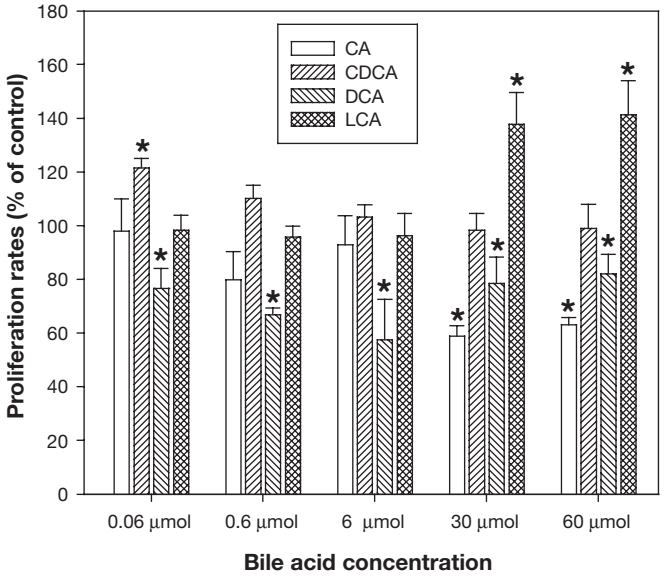

Fig. 1. *Uronema marinum*. Effect of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid (LCA) on the proliferation of *Uronema marinum* related to the control as 100%. Points represent mean values ± standard deviation. (*$p < 0.05$)

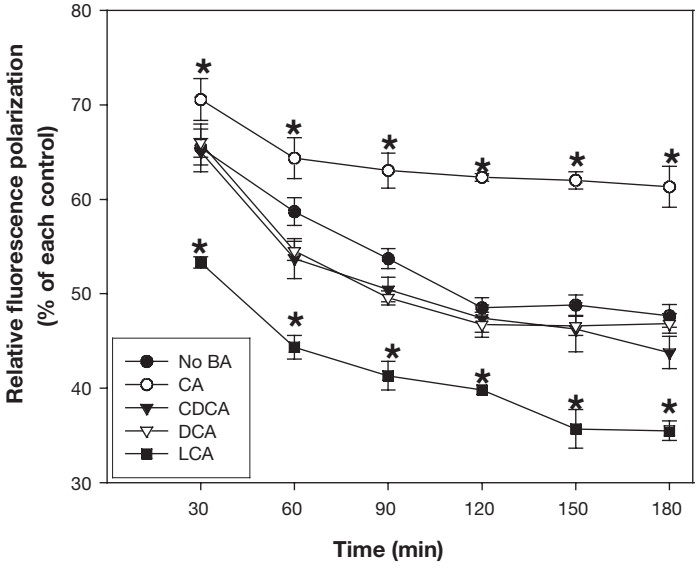

Fig. 2. *Uronema marinum*. Effect of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid (LCA) on the production of proteinase activity of *U. marinum* related to each control as 100%. Proteinase activity was measured by fluorescence polarization, and was detected using FITC-casein as a substrate at pH 7. Points represent mean values ± standard deviation. (No BA = not supplemented with any bile acids; *$p < 0.05$)

The results presented in this paper suggest that bile acids can modulate the production of proteinase activity of *Uronema marinum*. LCA significantly increased proteinase activity, whereas CA significantly decreased it. It has been reported that treatment of human colorectal cancer CaCo-2 cells by LCA resulted in an enhanced secretion of Gelatinase A or Matrix Metalloproteinase 2 (MMP-2) (Halvorsen et al. 2000). Proteolytic degradation of extracellular matrix (ECM) components by MMPs is involved in both physiological and pathological processes such as development, tissue remodeling, inflammation, tumor cell invasion, and tumor metastasis (Woessner 2002). The positive effect of PKC activation on the secretion of MMPs has been well demonstrated in cancer cell lines (Williger et al. 1999, Shum et al. 2002). Thus activation of PKC by LCA might be a cause of the increase of *U. marinum* proteinase activity in the present study.

Limited information is available on the types and concentrations of bile acids released by fish into the surrounding water. Zhang et al. (2001) reported that lake char *Salvelinus namaycushi* released 4 nmol min^{-1} bile acids per kg of body weight into the surrounding water, and faeces contained a total of 31 nmol mg^{-1} bile acids. The composition of bile acids in faeces was similar to that in bile, containing over 80% taurocholic acid, taurochenodeoxycholic acid, and a trace of taurolithocholic acid. Although the types and concentrations of bile acids in the faeces of olive flounder are not known, the present results suggest that bile acids in the culturing water might influence the proliferation and proteinase activity of *Uronema marinum*, resulting in the increased possibility of scuticociliatosis in olive flounder farms. Further research into the types and concentrations of bile acids in olive flounder faeces, and their effects on the biological characteristics of *U. marinum* may provide us with a better understanding of the causes of scuticociliatosis.

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