EFFECT OF CURCUMIN ON PATHOGENESIS OF HAMSTER-OPISTHORCHIASIS THROUGH APOPTOSIS-RELATED GENE EXPRESSION

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Abstract. The present study investigated the effect of curcumin, a phenolic compound with yellow color from *Curcuma longa* L., on the expression of the apoptosis-related genes [BAX (Bcl-2 associated protein X), PKB, *p53*, MDM2 (mouse double minute 2), caspase 9, c-Ski, smad1 and smad4] in hamster opisthorchiasis. On *Opisthorchis viverrini* infection treated with dietary curcumin apoptosis-related gene expression profiles were similar to *O. viverrini*-infected group, but the expression levels seemed lower. Light microscopic observation revealed that aggregation of inflammatory cells surrounding the hepatic bile ducts in the groups infected with *O. viverrini* and treated with dietary curcumin was lower than in infected group. The intensity of the response is correlated with expression of the genes studied. The results suggest that curcumin reduces pathogenesis in hamster-opisthorchiasis by controlling apoptosis-related gene expression.

INTRODUCTION

The highest worldwide prevalence of *Opisthorchis viverrini* infection is in Northeast Thailand, where each year more than 6 million people are infected or become re-infected because of the consumption of uncooked cyprinoid fish (Jongsuksuntigul and Imsomboon, 1997).

O. viverrini infection is a known risk factor for cholangiocarcinoma (CCA) development (Holzinger *et al*, 1999). Treatment (killing of parasite) is achieved simply and effectively with a course of praziquantel. However, breakdown of the tegument of the parasite, induces a host immune response and aggregation of inflammatory cells around the infected area (Boonmars *et al*, 2007, 2008) which may be the trigger for CCA development after years of repeated cycle of infection. Since changing dietary behavior has met little success, preventing the genesis of CCA by reducing the pathological changes caused by *O. viverrini* infection may be the next best solution.

Several Thai medicinal plants are being studied because of their potential for disease prevention and treatment. Turmeric, *Curcuma longa*, has been used in Thai traditional medicine (Goel *et al*, 2008). Curcumin, 1,7-bis (4-hydroxy-3-methoxyphenyl) hepta-1, 6-diene- 3,5-dione, the active ingredient in turmeric, is a yellow compound present in

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the rhizomes of turmeric and has properties that contribute to wound healing (Panchatcharam *et al*, 2006), anti-inflammation, antioxidant and anti-cancer effects (Maheswari *et al*, 2006; Sharma *et al*, 2007; Goel *et al*, 2008). Johnson and Mukhtar (2007) found curcumin can prevent colon cancer, while Chuang *et al* (2000) found curcumin inhibits hepatocarcinogenesis caused by diethylnitrosamine in a mouse model. Bachmeier *et al* (2007) found that it induces apoptosis and inhibits the formation of breast cancer metastasis and Li *et al* (2008) showed that it prevents lung cancer from Quartz particles in rat lung epithelial cell lines.

Curcumin also has demonstrated antifungal, anti-viral, anti-protozoal and antinematodal properties (Negi *et al*, 1999; Cui *et al*, 2007). Reddy *et al* (2005) reported that after malaria-infected mice are administered curcumin, pathogenesis decreases. Curcumin reduces pathogenesis of opisthorchiasis and prevents oxidative and nitrative stress (Kaewsamut *et al*, 2007).

The purpose of this study was to investigate the effects of curcumin at in a hamster-opisthorchiasis model with a focus on pathological changes and apoptosis-related gene expression during the development of OV infection.

MATERIALS AND METHODS

Collection of metacercariae

Metacercariae of *O. viverrini* were obtained from naturally-infected cyprinoid fish captured from a fresh water reservoir in an endemic area of Khon Kaen Province in Northeast Thailand. Fish were minced and digested with pepsin-HCl, filtered and then washed with normal saline until clear. Metacercariae of *O. viverrini* were identified under a dissecting light microscope.

Parasite infection

Male hamsters, between 6 and 8 weeks of age, from the Animal Unit, Faculty of Medicine, Khon Kaen University, were divided into 4 groups: i) normal hamster, ii) curcumin treatment alone, iii) *O. viverrini* infection, iv) *O. viverrini* infection plus administration of dietary curcumin. In the infected group, hamsters were administered with 50 metacercariae. Hamsters were sacrificed on Days 7, 14, 21, 30, 60 and 90, whereupon their livers were harvested, photographed and then assessed through dissection for histopathological changes. The protocol was approved by the Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University, Thailand (Ethical Clearance No AEKKU0514.1.12/438).

Dietary curcumin

Curcumin (Sigma Chemical, St. Louis, MO) was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma Chemical). Curcumin (37.5 g) was mixed with hamster pellet food (15 kg) (CP, Thailand). The final concentration of curcumin in the food was 0.25% w/w (0.21- 0.42 g/kg body weight/day).

Light microscopic examination

Livers were processed for light microscopic examination according to the established protocol [fixed with 10% formalin solution and processed in a conventional manner for hematoxylin and eosin (H&E) staining].

Detection by RT-PCR of apoptosis-related genes in liver infected with *Opisthorchis viverrini*

Whole liver at the hilar's region (100 mg) from each group was used for analysis. Total RNA was isolated using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The isolated RNA was treated with DNase (RQ1 RNase-Free DNase, Promega, Madison, WI) and ri-

bonuclease inhibitor (Takara Shuzo, Kyoto, Japan) in 400 mM Tris-HCl buffer pH 7.5 containing 100 mM NaCl, 60 mM $MgCl_2$ and 20 mM ditheothreitol. The treated RNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in RNase-free water (100 µl). RNA was reverse transcribed into cDNA using Oligo (dT)15 primer (Amersham Phamacia Biotech Piscataway, NJ) and M-MLV (Invitrogen, USA) and then PCR anplified.

The PCR reaction mixture comprised 3 µl of reverse transcription product (diluted 1:10), 3 µl of 10x PCR buffer, 3 µl of 4 deoxynucleoside triphosphate (2.5 mM each), 6 µl of primer pairs (5 pM), 0.12 µl of *Taq* polymerase (5 U/ml, Takara Shuzo), and 15 µl of distilled water. PCR amplification was performed under the following conditions: 1 cycle at 95ºC for 3 minutes; 30, 35, 40 cycles (depending on primers) of 95ºC for 30 seconds, 56ºC for 30 seconds and 72ºC for 2 minutes; and a final extension cycle at 72ºC for 10 minutes. Aliquots of PCR products were analyzed by gel electrophoresis and photographed under UV light. Photographs were analyzed for density using Scion Image (Scion Frederick, MD). All PCR reactions were performed in triplicate. The amounts of BAX, PKB, *p53*, MDM2, caspase9, smad1, smad4, c-Ski were normalized relative to MG3PDH according to Boonmars *et al* (2007, 2008). The sequences of the primers used are shown in Table 1.

RESULTS

Pathological change

The gross appearance of the hamster's hepatobiliary system, monitored at Day 90,

Table 1
Sequence of primer pairs used for amplification of apoptosis-related genes.

Gene	Product length (bp)	Sequence upper line : forward primer bottom line : reverse primer
c-Ski	244	5'-CTGCGAGTGAGAAAGAGACG-3' 5'-TTTTCGTGGCTGGATACAAG-3'
Smad1	282	5'-TTGAAAACACCAGGCGACATA-3' 5'-CGAAGCTATCCGAATAGTGC-3'
Smad4	220	5'-GACAAGGTGGGGAAAGTGAA-3' 5'-CTCCACAGACGGGCATAGAT-3'
MG3PDH	218	5'-GGCATTGTGGAAGGGCTCAT-3' 5'-GACACATTGGGGGTAGGAACAC-3'
BAX	419	5'-CACCTGAGCTGACCTTGGAG-3' 5'-GAGGACTCCAGCCACAAAGA-3'
PKB (Akt)	434	5'-GCCTTGCTTCGCTCTGTGAC-3' 5'-AGCCGAGCGGTAAAGGATTC-3'
p53	232	5' AAGGCGATAGTTTGGCTCCT3' 5' CTGGGGTCTTCCAGTGTGAT 3'
MDM2	422	5'-AGGTCTATCGGGTCACAGTC-3' 5'-CTCTTTCACGCTTTCTTGG-3'
Caspase 9	474	5'-ACCAATGGGACTCACAGCAA-3' 5'-AGGATGACCACCACAAAGCA-3'

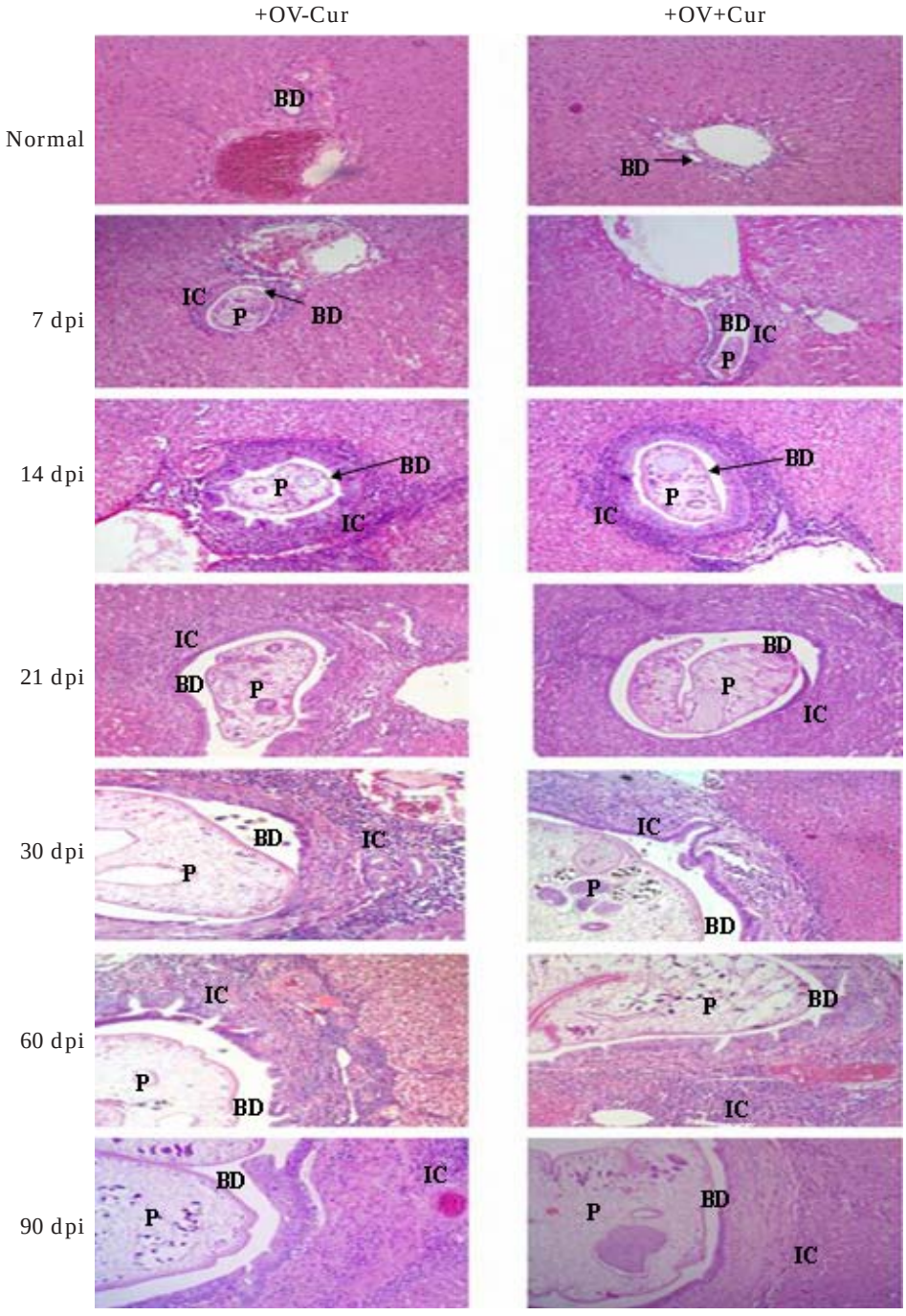

Fig 1–Hematoxylin and eosin staining of infected liver at various stages of *O. viverrini* infection. Note at 30, 60 and 90 day post-infection (dpi), both the *O. viverrini* (OV)-infected group and the *O. viverrini*-infected group administered dietary curcumin (Cur) have many inflammatory cells surrounding the bile ducts but fewer inflammatory cells in the latter. P, parasite; BD, bile duct; IC, inflammatory cells.

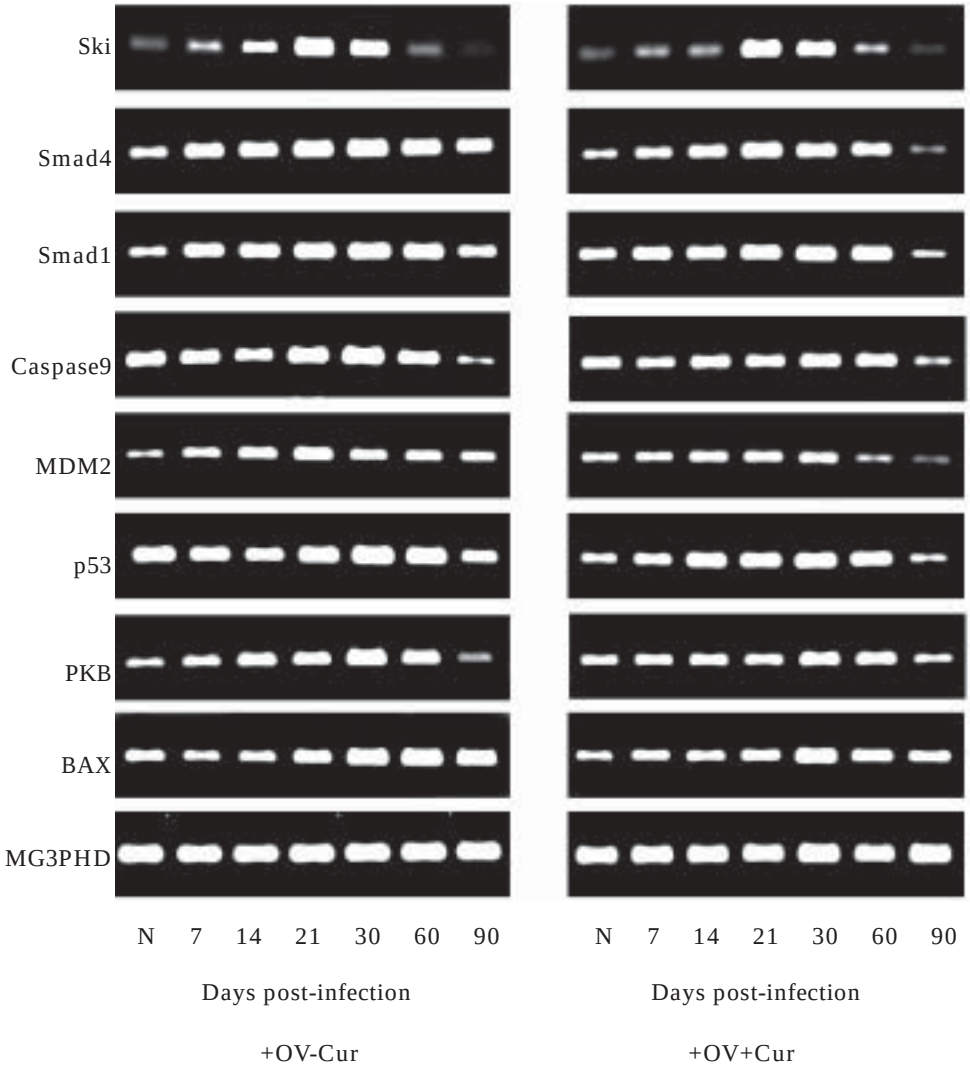

Fig 2–Expression of the apoptosis-related genes in *O. viverrini*-infected liver. Total RNA isolated from infected liver at various stages of infection [0(N), 7, 14, 21, 30, 60 and 90 days post-infection]; and the RT-PCR amplification products were analysed by agarose gel electrophoresis. OV, *O. viverrini*; Cur, curcumin.

was similar between normal control and curcumin treated group. Fig 1 shows inflammation around the parasite observed on Day 7. The severity of inflammation gradually increased and reached its maximum on Day 30, with proliferation of mononuclear cells and eosinophilic infiltrates around the intrahepatic bile ducts. The inflammatory reaction tended to decrease after 60 days of infection. Lymphoid follicles, as well as plasma infiltration, were predominant. Thickened and dilated hepatic bile ducts were clearly observed. In the *O. viverrini*-infected group administered dietary curcumin, the number of inflammatory cells around the hepatic bile duct was less (albeit not statisti-

cally significant compared with *O. viverrini*-infected group).

Apoptosis-related gene expression profile in hamster-opisthorchiasis and curcumin treatment

The housekeeping gene MG3DPH, was expressed at a similar level in all stages of infection (Fig 2). The experimental genes [BAX (Bcl-2 associated protein X), PKB, *p53*, MDM2 (mouse double minute 2), caspase 9, c-Ski, smad1 and smad4] were also expressed in all stages of infection but to different degrees.

Gene expression in curcumin treated group increased by Day 14 but was not significantly different from the normal control group (data not shown). In the *O. viverrini*-infected group a significant increase in expression of most gene expression was observed at 30 day post-infection (dpi) compared to the normal control and decreased at 90 dpi (Fig 2). In the *O. viverrini*-infected group administered dietary curcumin, most of the apoptosis-related gene expression increased after Day 14, albeit not statistically significant compared with infected group.

DISCUSSION

Curcumin, a compound from turmeric, a traditional medicine, has many properties including anti-inflammation, apoptosis, antioxidant and anti-cancer (Maheshwari *et al*, 2006; Sharma *et al*, 2007; Goel *et al*, 2008). In the present study, Syrian golden hamsters were infected with 50 metacercariae and fed a diet containing 0.25% (w/w) curcumin. Histopathology and expression of apoptosis-related genes were investigated.

Inflammation around the parasite was observed on Day 7, when the juvenile fluke's sucker caused epithelial distortion. The severity of inflammation gradually increased and reached a maximum on Day 30 with proliferation of mononuclear cells and eosinophilic infiltrates around the intrahepatic bile ducts. The inflammatory reactions tended to decrease after 60 days of infection. Lymphoid follicles, as well as plasma infiltration, were predominant. Thickened and dilated hepatic bile ducts were clearly observed, in accord with previous reports (Sripa and Kaewkes, 2002; Sripa, 2003). In the *O. viverrini*-infected group administered curcumin, inflammation was slightly lower than in the *O. viverrini*-alone-infected group. These results show that curcumin at 0.25% (w/w) mixed in food had an anti-inflammatory effect. Our data corroborate a previous study suggesting that curcumin exhibits anti-inflammatory activity perhaps related to its ability to inhibit up-regulation of COX-2 (Goel *et al*, 2001).

Gene expression results indicated expression of BAX, PKB, *p53*, MDM2, caspase9, c-Ski, smad1 and smad4 in all samples but with varying degrees. These genes increased from Day 14 post-infection, became predominant at Day 30, and then gradually decreased from Day 60 and to near normal control levels by Day 90 post-infection. These results agree with previous reports (Boonmars *et al*, 2007, 2008) and suggest that the parasite and its excretory-secretory antigens induce the host's immune response, which causes cell damage.

Recently investigators have reported that opisthorchiasis predisposes to an increase in free radicals, such as nitrogen radicals and cytokines, which cause damage to DNA (Pinlaor *et al*, 2004) leading to cell death. Boonmars *et al* (2004) reported an increase in apoptotic genes during encapsulation, due to inflammation caused by *Trichinella spiralis* larvae-infected mice. The expression of apoptotic genes during the pathogenesis process of other parasitic infection, *viz. Trichomonas vaginalis* infection inducing vagina apoptosis (Chow *et al*, 2000), *Toxoplasma gondii* infection inducing eye

apoptosis (Hu *et al*, 2001), *Schistosoma mansoni* and *Necator americanus* infection inducing T cell apoptosis (Chow *et al*, 2000; Chen *et al*, 2002), have been reported. Though the techniques for apoptotic detection were different in each report, it seems reasonable that any disease that can cause cell damage will exhibit apoptotic expression. Apoptosis-related gene expression profiles in human and animal models of opisthorchiasis have not yet been reported. This is the first study to show that apoptosis-related gene expression appears to be correlated with the severity of pathogenesis (intensity of inflammation) during hamster-opisthorchiasis model.

In summary the present study demonstrated that opisthorchiasis can induce apoptosis-related gene expression in hamster liver, which may depend on the severity of pathogenesis (inflammation). Curcumin can act as an anti-inflammatory antioxidant leading to reduced pathology from opisthorchiasis, through control of apoptosis-related gene expression. Humans infected or re-infected with *O. viverrini* may benefit from dietary curcumin.

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