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Liver fluke-induced hepatic oxysterols stimulate DNA damage and apoptosis in cultured human cholangiocytes

Apinya Jusakul · Watcharin Loliome · Nisana Namwat · W. Geoffrey Haigh · Rahul Kuver · Somkid Dechakhampu · Pradit Sukontawarinsap · Somchai Pinlaor · Sum P. Lee · Puangrat Yongvanit

ABSTRACT

Oxysterols are cholesterol oxidation products that are generated by enzymatic reactions through cytochrome P450 family enzymes or by non-enzymatic reactions involving reactive oxygen and nitrogen species. Oxysterols have been identified in bile in the setting of chronic inflammation, suggesting that biliary epithelial cells are chronically exposed to these compounds in certain clinical settings. We hypothesized that biliary oxysterols resulting from liver fluke infection participants in cholangiocarcinogenesis. Using gas chromatography/mass spectrometry, we identified oxysterols in livers from hamsters infected with Opisthorchis viverrini that develop cholangiocarcinoma. Five oxysterols were present: 3-bromo-3,5-diene (76S), 3-bromo-3,7-diene (76R), 3,7-diene-3,5-diene (76S), 3-bromo-3,7-diene (76R), and cholest-3-en-5-one (76R). These oxysterols were found in significantly higher levels in the livers of hamsters with O. viverrini-induced cholangiocarcinoma. We therefore investigated the effects of 76S and 76R on induction of cholangiocarcinogenesis using in vitro human cholangiocyte culture models. 76S and 76R-treated cells induced cellular apoptosis. Moreover, oxysterols induced significantly increased levels of ROS and decreased levels of GSH. In these cells, increased oxidative stress from oxysterols was found following treatment with 76S and 76R. 76S and 76R also induced formation of the DNA adducts 1,3-dihydroxy-2-(3H)-dehydrosphinganine, 2,4-dihydroxy-2-deoxyerythroside, and 8-hydroxy-2-deoxyguanosine in cholangiocytes. These data suggest that 76S and 76R cause DNA damage via oxidative stress. Chronic liver fluke infection increases production of the oxysterols 76S and 76R in the setting of chronic inflammation in the biliary system. These oxysterols induce apoptosis and DNA damage in cholangiocytes. Insufficient and impaired DNA repair of such mutated cells may enhance clonal expansion and further drive the change in cellular phenotype from normal to malignant.

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1. Introduction

Oxysterols are the oxygenated derivatives of cholesterol derived from either enzymatic or non-enzymatic reactions. The production of oxysterols through the enzymatic pathway involves CYP450 family enzymes; this mechanism is integral to the conversion of cholesterol into bile acids. In contrast, non-enzymatic mechanisms involve reactive oxygen and nitrogen species (ROS/RNS). Numerous studies describe biological effects of oxysterols in areas such as cholesterol catabolism and cholesterol homeostasis. Pathological effects of oxysterols have also been described, including in cardiovascular disease, Alzheimer’s disease, osteoporosis, and cancer. Several studies describe the effects of oxysterols on various types of cancer, including those of the lung, breast, colon, and bile ducts. Oxysterols may contribute to tumor initiation, promotion, and progression. Oxysterols including 76S induced increased numbers of revertants in Salmonella typhimurium and increased the frequency of chromosomal aberrations in CHO cells through the induction of ROS production.

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that 7-ketocholesterol could damage mitochondrial DNA. Oxysterols also have pro-inflammatory and pro-inflammatory effects. 7α-, 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol-5α-epoxide, 7-ketocholesterol-5α-epoxide, 22R-, 22S-, and 22α-22β,27-hydroxysterols act as pro-inflammatory and pro-inflammatory molecules in U937 human promonocytic leukemia cells, and induce pro-inflammatory cytokines such as interleukin-8 (IL-8) and IL-1β. Osteoblast-derived oxysterols trigger the migration of murine cancer cells toward bone. Carcinogenic effects of oxysterols have been recently reviewed by Jusakul et al.

Several reports show a role for oxysterols in the development of cholangiocarcinoma (CCA). A malignant tumor of bile duct epithelium. Chronic inflammation caused by Opisthorchus viverrini (OV) infection can induce the expression of inducible nitric oxide synthase in bile duct epithelia and can subsequently cause oxidative damage to DNA, suggesting a potential role for OV infection in CCA development. The expression of cytosolic oxysterol-binding protein is increased in OV-induced hamster CCA. Certain oxysterols can stabilize cyclooxygenase-2 (COX-2) mRNA via a p38 MAPK-dependent mechanism resulting in COX-2 protein accumulation in CCA cells. Increased concentrations of oxysterols have been reported in infected human hepatic bile. Suggesting that biliary infection is involved in the biosynthesis of oxysterols in bile through the production of reactive oxygen species by activated leukocytes.

Taken together, this body of evidence suggests that over-production of oxysterols during chronic biliary inflammation contributes to the genesis and progression of CCA. In the present work, we determined which types of oxysterols are involved in cholangiocarcinogenesis and what roles they play using in vivo and in vitro methods.

3. Materials and methods

2.1. Animals

10 and 36B were purchased from Charles River (BR, USA). Bacterial antibiotics (E. coli, 1,000 IU/ml) and ECO-1, 1,000 IU/ml were used to promote cell survival. The NIBH mouse hepatocarcinoma cell line (HepG2) was obtained from the National Institute of Biomedical Research, Japan. All mice were reared in an air-conditioned, light-controlled environment. The percentage of intercalating agents was determined using Ethidium Bromide (EB) and examined at room temperature. The extraction and isolation procedure were performed as described above.

2.2. Animal protocol and tumor induction

The animal experimental protocol was conducted according to guidelines of the animal ethical committee of the University of Medicine, Louis Pasteur University, Thailand. Animal induction was done according to Elson et al. In brief, Syrian golden hamsters (Animal Unit, Faculty of Medicine, Iran) aged 5–6 weeks were divided into 5 groups: (a) Untreated control; (b) OV-induced; (c) 10 or 100 mg/kg (ID) NDBM-treated; and (d) OV plus NDBM-treated. Fifty 40-μm macrocarcins were injected into each hamster via intragastric intubation, and NDBM was administered in drinking water (12.5 mg/ml) provided ad libitum for 2 months. Animals were sacrificed on weeks 3, 3.5, 4.5, 12, 24, and 48. At the end of treatment, cells were maintained and washed with medium. Cells pellets were collected and the lipid fraction extracted with chloroform/methanol (2:1) containing 0.05% BSA and reconstituted as an isotonic solution at room temperature. The extraction and isolation procedure were performed as described above. The percentage of intercalating agents was determined using Ethidium Bromide (EB) and examined at room temperature.

2.3. Detection of apoptosis cells using Annexin V/FITC labeling solution (Roche, Penzberg, Germany) and incubated at room temperature for 15 min. At least 10,000 cells were counted and analyzed with a flow cytometer (Cytometric FC500, Beckman Coulter, USA).

2.4. DNA fragmentation assay

Briefly, 10% cells were lysed with 1 ml lysed buffer (1% w/v) Triton X-100, 0.5% Na deoxycholate, 5 mM KCl, 10 mM TRIS- HCl, and centrifuged for 20 s at 10,000 × g. Cell pellets were collected and treated with 200 μl of cDNA reaction solution (15 μM dNTPs, 5 mM MgCl2, 0.25 mM TRIS-HCl, 10 μM TRIS-HCl), and 8 μl of 10 μg/ml Proteinase K was added to the sample and incubated for 1 h at 55 °C. Then, 300 μl of 2× DNA solution (7 μM NaCl, 20 mM EDTA) was added to the mixture and incubated for 2 h at 37 °C. After centrifugation, the pellet was mixed with 1× SDS, washed, and centrifuged. The pellet was dissolved and resuspended with 100 μl of Tris-EDTA (10 mM Tris-EDTA) and 1× DNA loading dye and loaded onto a 10% agarose gel. Electrophoresis was done at 50 V for 1 h and the gel was stained with ethidium bromide for 15 min. Visualization was by ultraviolet 400 (CE Healthcare Bio-Science, UK).

2.5. Western blot analysis

Cells were treated with 50 μM 3-methyladenine (3MA) or 4, 2, 15, 24 hours. The protein was then extracted by RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, and 0.1% SDS) and electrophoresed, and then blotted. Antibodies were probed with 1:100 anti-actin antibody (Roche, Penzberg, Germany), 1:100 rabbit anti-actin-2 (1:1) antibody (Santa Cruz Biotechnology, USA), and 1:200 mouse-
2.1.9. Immunocytochemical detection of DNA adducts

Staining of DNA adducts including cEB and B-oxide was performed as previously described. Briefly, MNN1-1 cells were treated with Triol or 3K4 at the designated concentrations in slide chambers (Lab-Tek II chamber slide, Nalgene Nunc, USA). Slides were fixed with acetone for 10 min at -20°C, then dipped in PBS for 10 min and the endogenous peroxidases quenched with 0.3% H2O2 in absolute methanol for 10 min. Slides were incubated in 1% bovine serum albumin in PBS solution for 30 min at room temperature. Slides were incubated with PBS and double distilled water, respectively, followed by washing with double distilled water and PBS. Non-specific binding sites were blocked with 10% skim milk (w/v) or 0.05% Tween-20 (v/v) and 0.05% Triton X-100 (v/v) for 20 min at room temperature. Slides were incubated with primary monoclonal antibodies EBA-A1 against cEB and EBA -1 against B-oxide at 1:10 dilution, and primary monoclonal antibody B-oxide-1D at 1:200 dilution at 4°C overnight. Detection of cEB and B-oxide was by incubation with anti-mouse/anti-rabbit polymer conjugated with HRP (Dako, Glostrup, Denmark). For B-oxide-1D detection, slides were incubated with rabbit anti-mouse (1:400 in PBS) for 1 h. Visualization was with DAB. After stopping the reaction, counterstaining was performed with hematoxylin. Slides were dehydrated by increasing the concentration of ethanol and mounted with Kaiser’s glycerin. Results were analyzed by counting more than 500 cells.

2.1.1. Statistical analysis

The results were presented as a mean ± SD for at least three individual experiments. The level of significance in hamster tissue was statistically analyzed using Student’s t-test. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Oxysterol levels in 3K4-induced CCA hamster liver

Histopathological changes in hamster liver tissues are shown in Fig. 1. A. There was no pathological change observed in the control group, whereas the livers of the NDMA-treated group developed mild peritubular inflammation and a low degree of bile duct proliferation and cholangiofibrosis, which was seen at week 12. In the NDMA-treated group, the early pathological changes consisted of acute inflammatory changes observed around the bile ducts where liver flukes reside. The highest degree of inflammation was observed at week 3. As liver flukes developed into adult worms, they induced hyperplasia of the bile duct epithelia. In the chronic phase, a decrease in the number of inflammatory cells and the thickness of fibrosis were observed. In the NDMA plus NDMA-treated group, most of the liver alterations were similar to those found in the NDMA alone group, with the exception of a greater degree of inflammation. In addition, cholangiocarcinoma was observed at week 24, with inflammatory cells still observed in areas with tumors. Alterations in Triol and 3K4 levels during cholangiocarcinogenesis in hamsters are shown in Fig. 1. B and C, respectively. Besides 3K4 and Triol, several other oxysterols were found in hamster livers, specifically 7K0, 2K7, and 3K0. There were no significant changes in these oxysterols in all treatment groups and time points compared to controls (data not shown). The increase in both Triol and 3K4 levels in hamster livers was observed in the 7K0 infected-group.
Fig. 3. (A) In vitro effects of Triol and 3K4 on the induction of MMNK-1 cell death. MMNK-1 cells were cultured with Triol and 3K4 at the designated concentrations ranging from 1 to 100 μM for 24 h. After 48 h, the cellular proteins of viable cells were measured using the CellTiter-Blue assay. Values are expressed as the mean ± standard deviation of percentage of control cells incubated with 0.5% ethanol for three individual experiments. (B) Intracellular oxysterol uptake was analyzed by GC/MS at 0, 3, 6, 12, 24, 48 h after treatment with 20 μM Triol and 20 μM 3K4. The integrators are expressed as a percentage of the total amount of oxysterol added to the incubation medium. Significant higher levels of intracellular oxysterol compared to control were found at 6, 12, and 24 h of incubation. Standard deviations were calculated from three biological experiments. Significantly (*P < 0.05): higher level of Triol compared to 3K4.

Mutually, significantly increasing levels of 3K4 in the Ov infected-group were found at week 3 (*P < 0.05 versus control). In the NDMA group, the levels of Triol and 3K4 were low and no significant differences were seen compared to the control group at all time points. Levels of Triol in the Ov plus NDMA group were statistically significantly higher than those in the control group (*P < 0.05) from week 1 until week 24 after treatment. Moreover, levels of 3K4 tended to be higher at week 1 and 2, although these differences did not reach statistical significance. Increasing levels of 3K4 were found at week 3, 4, 12, and 24 (*P < 0.05 versus control). Increased levels of oxysterols in Ov plus NDMA-treated hamsters were observed at an early stage of carcinogenesis (week 4) until tumor development (week 24). We therefore selected Triol and 3K4 for further studies aimed at understanding molecular mechanisms involved in the development of CCA using in vitro methods.

3.2. Effects of Triol and 3K4 on MMNK-1 cholangiocytes

The effects of Triol and 3K4 on MMNK-1 cell survival were studied. Oxysterols were added to cells at concentrations ranging from 1 to 100 μM for 48 h. A shows the effects of Triol and 3K4 on the induction of cell death, which occurred in a dose-dependent manner. The concentrations at which Triol and 3K4 inhibited cell growth to 50% of control (IC50) were 27.5 ± 3.0 and 63.3 ± 5.8 μM, respectively. Triol had a greater inhibitory effect on cell growth than did 3K4. We therefore used higher concentrations of 3K4 than Triol in subsequent experiments.

3.3. Time course of oxysterol uptake

Intracellular oxysterol uptake by MMNK-1 cells (A) was determined by GC/MS. Low level uptake of Triol and 3K4 was found at 1 h after treatment (4.2% and 2.8% of levels in media, respectively). Mean Triol uptake was 6.5%, 13.7%, 19%, and 22%, whereas mean 3K4 uptake was 5.5%, 6.5%, 10.1% and 16.2% of the total amount added at 6, 12, 24, 48 h, respectively. Notably, higher Triol uptake was seen when compared to 3K4 (*P < 0.05).

3.4. Triol and 3K4 induce MMNK-1 cell apoptosis

We next investigated the mechanism of oxysterol-induced MMNK-1 cell death using flow cytometry. Cells were treated with 10, 20, 30, and 40 μM Triol, or 10, 20, 40, 80 μM 3K4 for 48 h. Oxysterol-treated cells were stained with annexin V and PI and then analyzed by flow cytometry. * shows the effects of Triol and 3K4 on the induction of MMNK-1 cell apoptosis. Increasing numbers of apoptotic cells (as determined by annexin V/PI and annexin V/PI*) were observed with increasing concentrations of Triol and 3K4. Significant numbers of apoptotic cells were found following treatment with 30 and 40 μM Triol compared to the control (P < 0.05). However, only 80 μM 3K4 induced significant apoptosis (P < 0.05).

We further clarified the effect of oxysterols on the induction of apoptosis using a DNA fragmentation technique involving agarose gel electrophoresis. Cells were treated with Triol and 3K4 at the designated concentrations for 48 h then DNA was extracted. Oligonucleosomal cleavage was measured and maximally elevated following treatment with 30 and 40 μM Triol, respectively (*A). In contrast, treatment with 40 μM 3K4 slightly increased DNA fragmentation. A marked increase in DNA laddering was found when cells were treated with 80 μM 3K4 (*B).

3.5. Activation of apoptosis-related proteins by Triol and 3K4

To investigate the molecular pathways of apoptosis induced by Triol and 3K4, we identified the induction of apoptosis-related proteins in MMNK-1 cells using western blot analysis. Cells were treated with 30 μM Triol or 80 μM 3K4 and proteins were extracted at 0, 3, 6, 12, 18, and 24 h. Oligonucleosomal cleavage of cytochrome c in Triol- and 3K4-treated cells (A and B, respectively) increased levels of Bax were found in both Triol and 3K4-treated cells. Conversely, decreasing levels of Bcl-2 were found in Triol- and 3K4-treated cells. Thus, both Triol- and 3K4-induced apoptosis involve mitochondrial-dependent mechanisms.

3.6. Triol and 3K4 induce DNA damage in MMNK-1 cells

We investigated the effects of Triol and 3K4 on the induction of DNA damage using immunocytochemistry. DNA adducts were detected at 48 h after incubation. We used specific monoclonal antibodies against etheno-DNA base adducts called EMA-1 and EM-C-1 raised against eda and edc, respectively. A specific antibody against the free radical-derived DNA adduct, 8-oxoG-C, was also used.

shows staining for eda, edc and 8-oxoG-C induced by Triol. The stains for these DNA adducts were located in the nuclei of MMNK cells by hematoxylin staining. No staining for eda and edc was observed in control groups. Significant increases in eda, edc and 8-oxoG-C levels were found when cells were treated with low concentrations of Triol (10 μM) (P < 0.05) compared to control...
groups. **A.** Beyond these concentrations of Triol (10–30 μM), DNA adduct formation gradually increased.

Staining for 8-oxOA, 8-oxCA, and 8-oxoG was also found in 3K4-treated cells (1). Small numbers of positive cells were found in control groups. We found 20 μM and 30 μM 3K4 significantly increased the levels of 8-oxOA and 8-oxCA (P<0.05). The lowest concentration of 3K4 (10 μM) significantly increased the level of 8-oxoG (P<0.01) as shown in 3K4. Thus, both Triol and 3K4 increased the formation of 8-oxOA, 8-oxCA, and 8-oxoG in MMN1-1 cells.

### 4. Discussion

The discovery of increased expression of oxysterol-binding proteins in hamsters with UV-induced CCA led us to hypothesize that oxysterols participate in the processes that initiate and promote the development of malignancy in the biliary epithelium. We thus analyzed oxysterols in the liver of hamsters induced to develop UV-associated CCA. Five oxysterols were identified: 7K, 3K4, 3K7, 3K6, and Triol. These oxysterols were found in both normal liver and tumor tissues. Notably, only Triol and 3K4 showed

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**Fig. 3.** MMN1-1 cells were treated with 10–40 μM Triol (A) and 10–30 μM 3K4 (B) for 48 h. After incubation, harvested cells were stained with annexin V and propidium iodide (PI). Stained cells were analyzed for apoptosis by flow cytometry. Live cells stained annexin V−PI− (left bottom panel) and apoptotic cells stained annexin V+PI− or annexin V−PI+ (right bottom and right top panels). These cells were distinguished by flowcytometry annexin V (phosphatidylserine) and PI (condensed or fragmented) in hypotonic lysis. Bar graphs show mean ± SD of 5 apoptotic cells induced by Triol (C) and 3K4 (D) compared to controls which were 0% ethanol-treated cells. *P<0.05.

**Fig. 4.** DNA fragmentation in Triol (A) and 3K4 (B)-treated MMN1-1 cell apoptosis analyzed by 3% agarose gel electrophoresis. Cells were treated with 10–40 μM Triol and 10–30 μM 3K4 for 48 h. Final toner were 100 bp DNA ladder. 0.5% ethanol-treated cells were used as control.
Fig. 3. Effects of Triol and Triol on the activation of apoptosis-related proteins. MKN-45 cells were treated with 30 μM Triol or with 30 μM Triol for 3, 6, 12, 18, and 24 h. Protein extracts were analyzed by western blot with specific antibodies against cytochrome c, Bax, and Bcl-2. Bar graphs show relative densitometric values of western blot of Triol and Triol on induction of cytochrome-c protein expression (C). The ratios of Bax/Bcl-2 protein expression (D) were analyzed at 3, 6, 12, 18, and 24 h after treatment. Values were normalized to GAPDH and expressed as mean ± SD from three independent experiments. *P<0.05 compared to the result of 6 h.

Fig. 6. DNA adducts formation in Triol-treated MKN-45 cells. Cells were treated with 10, 20, and 30 μM Triol for 48 h and immunocytochemical staining was used to detect 8-oxo-dG (A), 8-oxo-dG (B), and 8-oxo-dG (C). The production of DNA adducts increased after incubation with increasing concentrations of Triol as compared to 0.5% ethanol-treated cells (control). Magnification 400×.
significantly increased levels in tumor tissue compared to normal liver. A previous study reported that the same two oxysterols were present in hepatic bile of patients with biliary tract disease and pigment gallstones, with their levels correlating with serum C-reactive protein levels. TRIOL and 3K4 were also found in higher levels in human gallstones. We propose that TRIOL and 3K4 are involved in inflammation-associated diseases in the biliary tract, including Ox-induced CCA. The histopathological changes in livers of treated hamsters are similar to those described in previous reports. There were no significant histological changes in untreated hamsters at any time point examined, whereas mild degrees of inflammation were observed in the livers of hamsters treated solely with NDMA. The histological changes in both untreated and NDMA-treated groups corresponded to oxysterol levels in our experiment. There were no significant differences in oxysterol levels between untreated and NDMA-treated groups. Nevertheless, the inflammatory response corresponded to the accumulation of inflammatory cells in Ox-treated hamsters. The highest numbers of inflammatory cells were observed at week 1 and markedly decreased from week 2 until week 24. There was no significant difference in TRIOL levels between Ox-treated hamsters and the control group. Notably, significantly increased levels of 3K4 were seen at week 3 compared to the control group, with TRIOL plus NDMA-treated hamsters, abundant inflammatory cells were detected at week 3. In the chronic phase, decreased inflammatory cells were observed. However, accumulation of mononuclear cells and lymphoid follicles were also reported. The most prominent histological changes at these time points were periductal fibrosis, ductal dilation and granulomatous inflammation. Along with rising levels of oxysterols in the Ox plus NDMA group, production of TRIOL and 3K4 showed an upward trend in the acute phase (week 1–3 after treatment). However, high levels of both TRIOL and 3K4 were found in the chronic phase when decreased inflammation was reported. Thus, we postulated that oxysterols may be involved in the inflammatory process but the level of oxysterols may not correlate linearly with the degree of inflammation.

We studied the role of TRIOL and 3K4 on induction of cholangiocarcinogenesis using the immortalized human MMNK-1 cholangiocyte line. Both TRIOL and 3K4 induced cell death in a dose-dependent manner, although the doses at which cell death was induced differed. The difference in IC50 between TRIOL and 3K4 may be explained by their structures. We also determined the time course of oxysterol incorporation into MMNK-1 cells. As shown in B, we found higher accumulation of TRIOL than 3K4 in MMNK-1 cells. We propose that the cytotoxic effect of oxysterols may relate to different rates of cellular uptake as determined by oxysterol.
structure. Insertion of Triol (which is more polar than 3K4) into cell membranes could more readily cause defects in membrane structure and packing, resulting in increased membrane permeability and membrane dysfuncion. Moreover, differences in oxysterol metabolism may alter cytoplasmic oxysterol levels that could in turn influence its cytotoxicity.

Concentrations of oxysterols used in our studies (1-100 μM) were of the same order of magnitude used in previous studies.

There is little information regarding oxysterol concentrations in various organs systems in vivo. Triol in human hepatic bile ranged from 16 to 367 nM, and 3K4 in gallbladder bile ranged from 218 to 10,784 parts per million. Concentrations of oxysterols in our study were higher. We justify the use of higher concentrations because under pathological conditions, such as inflammation, the production of oxysterols is markedly increased through non-enzymatic reactions involving ROS/RNS.

This is supported by data from Haigh et al. who showed that infection and inflammation can lead to oxysterol production, including species known in man in cattle.

Secondly, cholangiocytes are exposed to bile containing oxysterols over a long time course in the setting of chronic inflammation induced by 0v infection. Such long-term, low dose experiments would not be feasible in a laboratory setting, hence the need to accelerate and amplify the effects of oxysterols with higher doses.

Several studies have described the mechanisms of oxysterol-induced apoptosis in different cell systems and models, including neurons, gallbladder epithelial cells, colon adenocarcinoma cells, monocytes and hepatoma cells. No previously reported study used human cholangiocytes. We also elucidated the mechanisms by which oxysterols induced cell death in MNNK-1 cells. Both Triol and 3K4-treated cells exhibited increasing levels of apoptosis with increasing concentrations of oxysterols. The apoptosis-inducing effects of Triol and 3K4 were confirmed by DNA fragmentation analysis. Both Triol and 3K4 induced formation of DNA ladders. Activation of Bax, a pro-apoptotic protein was found in Triol and 3K4-treated cells. We also found decreasing levels of the anti-apoptotic protein, Bcl-2, along with enhanced cytochrome c release. These results indicate that Triol and 3K4 trigger apoptosis via mitochondrial-dependent mechanisms: activated Bax interacting with Bcl-2 resulting in Bcl-2 inactivation, followed by the disruption of mitochondrial membrane permeability resulting in cytochrome c release, activation of caspase proteins, and degradation of DNA.

DNA damage is theorized to be the mechanism by which chronic inflammation induced by Ov infection leads to cancer.

Overproduction of ROS and RNS generates DNA adducts. For example, 8-oxoG and 8-nitroguanine were found in bile duct epithelia of Ov-infected hamsters. Higher 8-oxoG levels were reported in Ov-infected patients compared to healthy subjects.

Ov-infected patients were found to have the lipid peroxidation (LPO) of etheno-dNA adducts edna and edc in white blood cell DNA and urine. Accumulation of these etheno-dNA adducts correlated with cancer development in hamster bile ducts.

Based on this body of evidence, we decided to investigate whether Triol and 3K4 can induce DNA damage. We found that Triol triggers a significant increase in the production of edna, edc, and 8-oxoG even at low concentrations (e.g., 10 μM). edna, edc, and 8-oxoG were also induced by 3K4 but at higher concentrations (e.g., 20 μM) than Triol.

Effects of oxysterols on oxidative DNA damage have been described in human monocytic leukemia cells, 7-ketocholesterol and 7β-hydroxycholesterol, found at high levels in atherosclerotic lesions, induced 8-oxoG formation in U937 cells.

Oxysterols induced lipid peroxidation through increased oxidant/nitride and 4-hydroxy-2-nonenal levels. Triol also induced chromosomal aberrations and ROS in Chinese hamster ovary cells. Based on these previously published data and our results, we propose that oxysterols are not only directly involved in the production of free radical-derived DNA adducts through the induction of ROS/RNS, but can also induce the formation of LPO-derived DNA adducts.

Thus, the proposed mechanisms whereby oxysterols induce apoptosis involve oxidation and inflammation. Pro-oxidative effects of oxysterols involve overproduction of free radicals. Oxysterols increase intracellular ROS levels by inducing NADPH oxidase. Oxysterol-induced ROS decrease transmembrane mitochondrial potential and intracellular levels of antioxidants such as glutathione. Reduction of glutathione with corresponding overproduction of ROS leads to caspase activation. Oxysterols that activate nuclear factor kappa B through ROS-mediated mechanisms enhance pro-inflammatory cytokines such as IL-1β and IL-8, leading to the recruitment of immune competent cells.

Persistent production of pro-oxidative molecules such as ROS/RNS induced by oxysterols perturb cellular stress responses. ROS/RNS and nitric oxide can impair DNA repair and inhibit apoptosis. Excess nitric oxide may play an important role in cholangiocarcinogenesis. Apoptosis induction by oxysterols may stimulate some cells to become resistant to apoptosis and thereby enhance the carcinogenic process. This could occur.
when DNA repair systems or apoptosis are not sufficient to counteract it. The imbalance of DNA repair mechanisms was proposed to be an important mechanism in DNA damage–enhanced carcinogenesis. Thus, oxygen–induced impairments in the DNA repair system needs further investigation. Low concentrations (10 μM of triol and 2464) of oxygenates induce high levels of DNA-adducts but low cell death (-), supporting the concept that certain cells may become resistant to apoptosis. Chronic exposure of hiliar epithelial cells to pathogenic oxygenates may allow selective growth of apoptosis-resistant cells. This hypothesis has been previously described in bile acid–induced gastrointestinal cancer. Long-term exposure to bile acids allowed selected cells to become resistant to apoptosis in vitro and in vivo.

Other concurrent causes may enhance cholangiocarcinogenesis. Increased production of proinflammatory cytokines such as IL-1, IL-6 and TNF α and alpha- and oxidative stress response enzymes such as COX-2 and free radical molecules, may disturb the balance of antioxidant–anti-oxidant molecules and DNA repair mechanisms leading to enhanced survival. Moreover, fibrosis caused by dysregulation of tissue repair in chronic inflammation may affect nodal cell function leading to enhanced survival.

In conclusion, we identified the oxygenates Triol and 3K4 as potential mediators of cholangiocarcinogenesis. Triol and 3K4 induced cell apoptosis via mitochondrial-dependent mechanisms. Triol and 3K4 induced DNA damage in MMWK-1 cells. We propose that chronic liver fibrotic infection increases the production of ROS/RNS in the setting of chronic inflammation leading to the formation of oxygenates via oxidation of hiliar cholesterol. Oxygenates and free radicals can induce DNA damage. Damaged hiliar epithelial cells are eliminated through induction of apoptosis. Ineffective DNA repair and ongoing exposure to DNA damaging agents select for resistant cells that clonally expand to become malignant (-).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

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Increased expression of TLR-2, COX-2, and SOD-2 genes in the peripheral blood leukocytes of opisthorchiasis patients induced by *Opisthorchis viverrini* antigen

Puangrat Yongvanit · Raynoo Thanan · Somchai Pinlaor · Pulboon Sithithaworn · Watcharin Lollome · Nisann Namwat · Anchalee Techan · Somkid Dechakhamphu

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Abstract Re-infection with liver fluke, *Opisthorchis viverrini*, increases proinflammatory molecules involved in inflammation-mediated disease and carcinogenesis in an animal model. To clarify whether these genes respond to parasite antigen in peripheral blood leukocytes (PBL) of opisthorchiasis patients, we examined the transcriptional level of oxidant-generating (toll-like receptor 2 (TLR-2), nuclear factor-kappa B (NF-KB), and cyclooxygenase 2 (COX-2)), anti-oxidant-generating (manganese superoxide dismutase 2 (SOD-2) and catalase (CAT)), proinflammatory cytokine (interleukin (IL)-1β), and anti-inflammatory cytokine (IL-10), in PBL exposed to parasite antigen in *O. viverrini*-infected patients compared with healthy individuals in an *in vitro* experiment. After *O. viverrini* antigen-treated PBL, quantitative RT-PCR analysis revealed that increased expression of cytokines and oxidant-generating genes in PBL was similar between *O. viverrini*-infected and healthy groups. Interestingly, compared with healthy subjects, increase of TLR-2, COX-2, and SOD-2 and decreased CAT mRNA expression levels were observed in *O. viverrini*-infected group. The results indicate that *O. viverrini* antigen induces upregulation of TLR-2, COX-2, and SOD-2 and downregulation of CAT genes in opisthorchiasis patients, suggesting that imbalance of oxidant/anti-oxidant transcripts during re-infection may be involved in the inflammatory-driven carcinogenesis. These molecules may be used as the chemopreventive target for intervention of opisthorchiasis patients in an endemic area.

Introduction

The liver fluke, *Opisthorchis viverrini*, is classified as a group I carcinogen by the IARC, which is a relative risk factor for cholangiocarcinoma (CCA), in humans in Southeast Asia (IARC 1994). *O. viverrini* infection is endemic in northeastern Thailand, Khon Kaen Province, where the highest incidence of CCA in the world has been reported (Sriamporn et al. 2004). In Thailand, the estimated number of people infected with *O. viverrini* is eight million with an average of 9.6% of the population to be infected (Jongsukuntigul and Imsonboon 2003). Mass treatment programs seem to decrease the burden of infection; however, re-infection more likely occurs after praziquantel treatment in endemic areas (Sripa et al. 2007; Saowakontha et al. 2000). Humans become infected with *O. viverrini* by consuming raw or undercooked fish that contain metacercaria, the infective stage. Adult worm lives in the biliary tract and produces eggs, which is excreted in the feces after 1 month post-infection. Most of the infection-induced chronic disease remains clinically silent unless actively detected by
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Increased expression of TLR-2, COX-2, and SOD-2 genes in the peripheral blood leukocytes of opisthorchiasis patients induced by Opisthorchis viverrini antigen

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ultrasound (Mairiang et al. 2006). Antigens released by adult worms stimulate inflammation around biliary ducts not only in the large bile duct but also in the small bile duct where flukes are not resident (Sripa and Kaewkes 2000).

Several studies report that *O. viverrini* infection is a relative risk to CCA via chronic inflammation (Yongvanit et al. 2012; Pinlaor et al. 2004a; Sattrung et al. 1998). Host immune responses can mediate further consequent pathogenesis such as releasing of growth factors, proteolytic enzymes, and fibrogenic cytokines in response to local mechanical and immune irritation to parasite infection, resulting in a persistent inflammatory condition which contributes to the disease and carcinogenesis (Prakobwong et al. 2010; Prakobwong et al. 2009; Sripa et al. 2007). This immunologic reaction also leads to excessive production of reactive oxygen species (ROS) and reactive nitrogen species by inflammatory cells and epithelial bile duct lead to induce oxidative and nitrosative DNA damage which may be involved in the initiation and promotion step of experimental cholangiocarcinogenesis (Prakobwong et al. 2010; Pinlaor et al. 2004a). The regulation of inflammatory mediators, particularly cytokines and oxidative stress-related pathways, is believed to mediate antioxidant/pro-oxidant mechanisms (Haddad and Harb 2005; Strober and James 1986). We have also demonstrated a model of inflammation-mediated carcinogenesis via nitric oxide (NO)-mediated oxidative and nitrosative DNA damage in hamsters infected with *O. viverrini* (Pinlaor et al. 2004b). Subsequently, we have shown that hamsters infected with *O. viverrini* more than one time (re-infection) induced greater expression of apoptosis-related genes (Boonmars et al. 2007) and accumulation of DNA damage than a single dose through chronic inflammation (Pinlaor et al. 2004a). More recently, we also reported that urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), an oxidative DNA damage marker, level was observed in the order of CCA patients > *O. viverrini*-infected patients > healthy subjects and urinary 8-oxodG level was significantly correlated with its levels in leukocytes (Thanan et al. 2008). In addition, we detected in opisthochiriosis subject the etheno-DNA base adducts, 1,N²-etheno-2'-deoxyadenosine (edeA) and 3,N⁴-etheno-2'-deoxyctydine (edc) in white blood cell DNA and also as excretion products in urine, probably as a consequence of DNA repair (Dechakhampu et al. 2010; Dechakhampu et al. 2008). These findings demonstrated that inflammation-mediated DNA damage does not occur only at the site of inflammation but also in peripheral blood leukocytes (PBL) of *O. viverrini* infection. However, the mechanism of cellular immune and oxidative stress response to *O. viverrini* infection leading to CCA genesis remains unclear. Therefore, we hypothesized that *O. viverrini* antigen would induce cellular immune response via inflammatory/anti-inflammatory cytokines resulting in induction of oxidant related gene and adaptive anti-oxidant defense mechanism.

To clarify this hypothesis, the PBL taken from *O. viverrini*-infected patients was treated with *O. viverrini* antigen in vitro. Expression of genes involved in inflammation including oxidant-generating (toll-like receptor 2 (TLR-2), nuclear factor-kappa B (NF-κB)), and cyclooxygenase 2 (COX-2)), anti-oxidant-generating (manganese superoxide dismutase 2 (SOD-2) and catalase (CAT)), inflammatory cytokine (interleukin (IL)-1β) and anti-inflammatory cytokine (IL-10) against *O. viverrini* antigen in PBL was examined in *O. viverrini*-infected patients compared with age and sex-matched healthy individuals using quantitative RT-PCR analysis in vitro co-culture. In addition, lipopolysaccharide (LPS)-treated cells were used as a positive control.

Materials and methods

Subjects

The protocol for this study was approved by the Ethics Group of the Human Research Committee (HE480316), Khon Kaen University, Thailand. The subjects were divided into two pair-matched groups (matched for age and sex), namely, *O. viverrini*-infected patient and healthy individual groups. All participants volunteered for this study gave informed consent. Healthy individuals (n=4; three males and one female; age, 27–48 years) were defined as persons who are living in Khon Kaen Province and never had a history of *O. viverrini* infection as indicated by negative results for *O. viverrini* antibody and the absence of parasite egg in feces evaluated by modified formalin ether concentration technique. *O. viverrini*-infected patients (n=8; five males and one female; age, 35–50 years) who lived in an endemic area in Khon Kaen Province, Thailand and were positive for only *O. viverrini*-egg count and its antibody. Obese, diabetic patients, and patients with chronic inflammation diseases such as hepatitis virus and tuberculosis infections were excluded from the study. In addition, to exclude urinary tract infection and/or other related inflammatory disease, we examined the morning urine of all subjects by using urinary test kits. Positive urinary nitrate/nitrite in urine was also exclusion criteria.

Preparation of metacercariae and adult *O. viverrini*

*O. viverrini* metacercariae were obtained from naturally infected cyprinid fish and were digested by artificial pepsin digestion as previously described (Pinlaor et al. 2004b). Cyprinid fish was purchased from the endemic area in Khon Kaen Province. Fish was chopped into small pieces and minced using an electrical blender with freshly prepared
solution containing 0.25% artificial pepsin and incubated at 37°C in a shaking water bath for 1 h. After digestion, the digested content was filtered and washed several times with normal saline. Metacercariae of *O. viverrini* were collected and identified under a dissecting microscope. Fifty actively visible cysts were fed to Syrian golden hamsters. Adult worms were collected after 1–4 months post-infection. The Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (AEKIU012/48) approved this study.

Preparation of *O. viverrini* antigen

*O. viverrini* adult were used for crude extract preparation (Pinlaor et al. 2005). After hamsters were infected with *O. viverrini* for 1–4 months, animals were killed, and adult worms were obtained from livers and bile ducts. Complete and fresh parasites were washed several times in a cold normal saline solution containing penicillin (200 U/ml) and streptomycin (200 U/ml). To remove any debris and residual blood, parasites were washed twice with sterile phosphate-buffered saline (PBS) containing penicillin (100 U/ml) and streptomycin (100 U/ml). Subsequently, they were crushed and ground on ice in PBS containing protease inhibitors (Protease inhibitor cocktail, BioRad, CA, USA). The suspension was sonicated, centrifuged at 10,000×g for 30 min at 4°C, and the supernatant was filtered with 0.22-µm filter with sterile technique. Protein concentration of *O. viverrini* antigen was determined by Coomassie Plus™ Protein Assay Reagent (Rockford, IL, USA) following the manufacturer’s protocol. The solution was aliquoted with sterile technique and stored at −80°C until used.

White blood cell isolation and *in vitro* co-culture

To clarify the inflammation-mediated pathogenesis, we isolated all population of PBL and treated with parasite antigen. Twelve-hour fasting peripheral blood was obtained by sterilized venipuncture, collected in tubes (10 ml per tube) with heparin, centrifuged at 1,500 rpm at 25°C for 15 min, then the buffy coat and plasma were isolated with sterile technique. To obtain PBL pellets and to remove other contaminated cells, the buffy coat was treated with red blood cell lysis buffer and washed twice with sterile RPMI-1640 medium supplemented with 1-glutamine without Phenol Red (PAA Laboratories, Austria), 2% fetal calf serum gold (FCSg; Laboratories, Austria), and 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen, CA, USA). Then, the pellet was washed with RPMI-1640 medium containing 20% FCSg and 1% penicillin/streptomycin. PBL pellet was re-suspended in RPMI-1640 medium containing with 20% FCSg and 1% penicillin/streptomycin. Cell was cultured overnight in a 24-well plate (Corning Incorporated, NY, USA) with 10⁵ cells/ml/well at 37°C with 5% CO₂ incubator for 24 h. The primary PBL culture was treated with 100 µg/ml of *O. viverrini* worm antigen or 1 µg/ml of LPS (Sigma, MO, USA) for 3, 6, 12, 24, and 72 h. PBS-treated cell or untreated cell was used as a negative control and LPS-treated cell was used as a positive control. After stimulation, cells were collected and washed twice with cold PBS. Cell pellets were stored at −80°C until gene expression analysis.

Measurement of mRNA expression by quantitative RT-PCR

Total RNA extraction was isolated from PBL pellets by Trizol® reagent (Invitrogen, CA, USA) following the manufacturer’s protocol. A reverse transcription reaction consisted of 0.2 µg total RNA and random hexamer (2.5 mM), which was heated at 70°C for 10 min. The reaction mixture included the first-stranded cDNA synthesis buffer (75 mM KCl; 50 mM Tris-Cl, pH 8.3; 3 mM MgCl₂), 10 mM DTT, 0.5 mM each dNTP and 200 units reverse transcriptase (Promea, WI, USA). Reverse transcription was carried out using a DNA thermocycler (GeneAmp PCR system 2400, Applied Biosystems, CA, USA) at 25°C for 10 min, 37°C for 1 h, and 95°C for 5 min.

Quantitative PCR was performed using TaqMan® Gene Expression assay kits and the ABI 7500 real-time PCR system (Applied Biosystems, CA, USA). TaqMan® PCR primers and probes for TLR-2 (HS00152932_m1), COX-2 (HS00153133_m1), NFKB (HS00231653_m1), SOD-2 (HS00167309_m1), CAT (HS00156308_m1), IL-1β (HS99999029_m1), IL-10 (HS00961622_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs00414290_m1) were used in this experiment (Applied Biosystems, CA, USA). The reporter dyes of all targeted TaqMan® probes in this experiment were labeled with FAM dye whereas GADPH probe was labeled with VIC dye. PCR reaction was performed using TaqMan® Gene Expression Master Mix (Applied Biosystems, CA, USA) following the manufacturer’s protocol with duplicated sets. The data cycle threshold (ΔCt) method was performed to determine relative concentration, using GAPDH as the normalizing value. The mRNA expression level of targeted genes compared with the housekeeping gene was calculated as 2−ΔCt. A relative mRNA expression level of targeted genes was calculated from the mRNA expression level in treated PBL (*O. viverrini* antigen or LPS) normalized with the mRNA expression level of control PBL (2−ΔΔCt experiment/2−ΔCt control).

Statistical analysis

In order to normalize the normal distribution of the data, a Log transformation was performed before statistical analysis. Student’s *t* test was used to determine the significance in a normal distribution of independent data between
O. viverrini-infected patient and healthy individual groups. Pearson's correlation coefficient was used to analyze correlations for parametric data. Statistical analyses were performed by SPSS 16.0 for Windows software. P values of less than 0.05 were considered statistically significant.

Results

O. viverrini antigen mediates mRNA expression of inflammatory/anti-inflammatory cytokines

To clarify whether O. viverrini antigen induces cytokines associated with inflammation-related genes, we examined pro-inflammatory (IL-1β) and anti-inflammatory (IL-10) cytokine genes in PBL of O. viverrini-infected patients and healthy individuals. According to the time profile experiment at 6, 12, 24, and 72 h, both the pro-inflammatory and anti-inflammatory cytokines were detected as the upregulated genes in PBL from O. viverrini-infected patient and healthy individual groups after being induced by O. viverrini antigen and LPS and had the highest expression level at 6 and 12 h, respectively. IL-1β and IL-10 gene expression levels were not significantly different between PBL form O. viverrini-infected patients and healthy individual groups as shown in Fig. 1. O. viverrini antigen and LPS-treated PBL showed the same trend of induction.

O. viverrini antigen induces the expression of oxidant-generating genes

Since oxidant generates gene expression through TLR-dependent pathway and those that is involved in oxidative stress, we examined O. viverrini antigen-driven TLR-2, NF-κB, and COX-2 mRNA expression in PBL of O. viverrini-infected patient and healthy individual groups. According to the time profile experiment, TLR-2, NF-κB, and COX-2 gene expressions were detected as upregulated genes after the induction of O. viverrini antigen and LPS when compared with untreated and have the highest expression level at 6, 12, and 6 h, respectively. Interestingly, the expression levels of TLR-2 and COX-2 in O. viverrini antigen-treated PBL in O. viverrini-infected patient group were significantly higher than in healthy individual group. Moreover, the expression level of NF-κB expression level in O. viverrini antigen-treated PBL from O. viverrini-infected group was higher than healthy individual group as shown in Fig. 2. O. viverrini antigen- and LPS-treated PBL showed the same trend of induction.

Fig. 1 The relative mRNA expression of pro-inflammatory (IL-1β) and anti-inflammatory (IL-10) genes in PBL treated with O. viverrini antigen and LPS of O. viverrini-infected patients (OIP, gray bar) and in healthy individuals (HI, white bar). a The relative expression of IL-1β in O. viverrini antigen and LPS-treated PBL from OIP and HI groups at 6 h. b The relative expression of IL-10 in O. viverrini antigen and LPS-treated PBL from OIP and HI groups at 12 h. The data are shown as mean±SE of four healthy and six O. viverrini-infected subjects (Log scale). Transformed Log scale data were used for the statistical analysis between HI and OIP group by using Student's t test. The relative mRNA expression levels were adjusted by each control experiment (untreated) for individual samples.

O. viverrini antigen upregulates expression of SOD-2 but downregulates CAT anti-oxidant genes

To clarify whether O. viverrini antigen may induce anti-oxidant gene association with inflammation-related genes expression, we examined SOD-2 and CAT genes in PBL of O. viverrini-infected patient and healthy individual groups. The time profile alteration of the expression levels of SOD-2 and CAT genes had the highest expression at 12 h.
Fig. 2. The relative mRNA expression of oxidant-generating genes in PBL-treated with O. viverrini antigen and LPS of O. viverrini-infected patients (OIP; gray bar) and in healthy individuals (HI; white bar). a The relative expression of TLR-2 in O. viverrini antigen and LPS-treated PBL from OIP and HI groups at 6 h. b The relative expression of NF-κB in O. viverrini antigen and LPS-treated PBL from OIP and HI groups at 12 h. c The relative expression of COX-2 in O. viverrini antigen and LPS-treated PBL from OIP and HI groups at 6 h. The data are shown as mean±SE of four healthy and six O. viverrini-infected subjects (Log scale). Transformed Log scale data were used for the statistical analysis between HI and OIP group by using Student's t test. The relative mRNA expression levels were adjusted by each control experiment (untreated) for individual samples.

SOD-2 was detected as upregulated gene whereas CAT was detected as downregulated gene in PBL treated with O. viverrini antigen and LPS from both O. viverrini-infected patient and healthy individual groups. Interestingly, relative SOD-2 expression level in O. viverrini antigen-induced PBL from O. viverrini-infected patient group were significantly higher than healthy individual group, whereas relative CAT gene expression levels showed similar expressed level between the O. viverrini antigen-treated PBL from O. viverrini-infected patient and healthy individual groups as shown in Fig. 3. O. viverrini antigen and LPS-treated PBL showed the same trend of induction.

O. viverrini antigen induces ratio of oxidant/anti-oxidant genes

The ratio of TLR-2, NF-κB, and COX-2 by CAT expression levels are shown in Fig. 4. O. viverrini antigen significantly increased the ratio of TLR-2/CAT at 6-h post-treatment in O. viverrini-infected patient group, which was higher than that in the healthy individual group. A similar result was obtained for the LPS treated. O. viverrini antigen and LPS also increased the ratio of NF-κB/CAT and COX-2/CAT expression levels in O. viverrini-infected patient group higher than that in the healthy individual group, but this was not statistically significantly different probably due to a low sample size.

Correlations of inflammation-related genes expression level

A correlation of inflammation-related gene expression level induced by O. viverrini antigen was performed by linear regression analysis, and the results are shown in Table 1. Pearson’s correlation test of the data in Log scale (Log 2-transformed) obtained from all subjects (healthy and O. viverrini infection subjects) and experimental tests (untreated control and treated with O. viverrini antigen or LPS) were included. The expression of oxidant-generating genes (TLR-2, NF-κB, and COX-2) and anti-oxidant gene (SOD-2) levels were positively significantly correlated with pro-inflammatory (IL-1β) and anti-inflammatory (IL-10) cytokines. In contrast, anti-oxidant gene (CAT) was positively significantly correlated with only anti-inflammatory cytokine (IL-10). The positive correlations were shown among oxidant-generating genes expression (TLR-2, NF-κB, and COX-2) and the anti-
infected and uninfected healthy control was treated with parasite antigen. This study reports for the first time an association between transcript expression of cytokines and inflammatory related genes including pro- and anti-inflammatory cytokines, oxidant-generating, and antioxidant genes to *O. viverrini* infection. Our results showed that *O. viverrini* antigen increased IL-1β, IL-10, TLR-2, NF-κB, COX-2, and SOD-2 gene expression levels but do not alter the anti-oxidant gene expression, such as CAT in PBL obtained from *O. viverrini*-infected patients and in healthy individuals. Moreover, the alteration of these genes was rapid and highly responsive in PBL isolated from *O. viverrini*-infected patients at 6 h. The expression level ratio of oxidant-generating/CAT expressions gene levels were higher in infected patients than in healthy individuals. In addition, expression of oxidant-generated genes was found to be positively correlated with the expression of IL-1β and IL-10 cytokine genes, but the ratio of IL-1β/IL-10 was not different between *O. viverrini*-infected and uninfected groups. These results indicate that re-exposure to *O. viverrini* rapidly induces regulation of inflammatory mediators and SOD-2 while CAT expression remained unchanged. Therefore, this may lead to imbalanced expression of adaptive response genes in cellular antioxidant system.

Expression of oxidant-generating genes (TLR-2, NF-κB, and COX-2) leads to oxidative stress (Dai et al. 2007; Al-Ashy et al. 2006; Li and Vera 2002; Gino-Prase and Whisler 1998), and we found that these genes are responsible to *O. viverrini* antigen in *O. viverrini*-infected patients being at a higher level than in healthy individuals (Fig. 1). This observation can be explained by the expression of their mediators, IL-1β and IL-10, to *O. viverrini* antigen which showed a positive correlation with these oxidant-generating genes (Table 1). Expression of TLR-2 responsible to *O. viverrini* antigen significantly increased at the same time point for PBL of both groups, suggesting that *O. viverrini* antigen may increase activation of an inflammation-related pathway faster than 6 h. The higher responses to *O. viverrini* antigen in infected patients than in healthy individuals suggest that memory T cell responses are faster and more robust than those of their naive counterparts (Dutton et al. 1998).

Expression of these molecules may involve in the inflammatory-mediated pathogenesis and carcinogenesis (Pinnix et al. 2005). IL-1β is known to induce the synthesis of the enzyme COX-2 through the activation and translocation of p65 subunit of NF-κB (Al-Ashy et al. 2006). IL-1β and IL-10 cytokines are regulated by NF-κB (Dai et al. 2007). Alternatively, *O. viverrini* antigen may induce an inflammatory response through a TLR-2-mediated pathway leading to NF-κB-mediated expression of iNOS and COX-2 (Karim and Groten 2005). These results are in agreement with results in a mouse macrophage cell line which showed an increase of TLR-2, iNOS, and COX-2 at the protein level

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**Fig. 3** The relative mRNA expression of anti-oxidant genes in PBL-treated with *O. viverrini* antigen and LPS of *O. viverrini*-infected patients (OIP; gray bar) and in healthy individuals (HI; white bar). The relative expression of SOD-2 (a) and CAT (b) in *O. viverrini* antigen and LPS-treated PBL from OIP and HI groups at 12 h. The data are shown as mean±SE of four healthy and six *O. viverrini*-infected subjects (Log scale). Transformed Log scale data were used for the statistical analysis between HI and OIP group by using Student’s t test. The relative mRNA expression levels were adjusted by each control experiment (untreated) for individual samples.

oxidant enzyme (SOD-2). In addition, CAT mRNA expression level was positively significantly correlated with SOD-2 gene expression in which both gene products function as anti-oxidant enzymes.

**Discussion**

In order to test whether those who have been exposed to *O. viverrini* or the healthy control have different inflammatory response to parasite antigen, PBL isolated from *O. viverrini*...
Fig. 4. *O. viverrini* antigen induces the imbalance of oxidant/anti-oxidant ratio. Ratio of mRNA expression level of oxidant-generating genes (TLR-2, NF-κB, and COX-2) was divided by anti-oxidant (CAT) gene level and compared with *O. viverrini*-infected (OIP) and healthy (HI) subjects. A Student's *t*-test was used to test for significant differences.  

![Graph showing the comparison between TLR-2, NF-κB, COX-2, and CAT expression levels in HI and OIP subjects.](image)

Table 1. Pearson's correlation among gene expression levels (Log $2^{−\Delta C_{T}}$)

<table>
<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>IL-10</th>
<th>TLR-2</th>
<th>NF-κB</th>
<th>COX-2</th>
<th>SOD-2</th>
<th>CAT</th>
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<td></td>
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<tr>
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<td>0.561**</td>
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<tr>
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<td>0.460**</td>
<td>0.549**</td>
<td>1</td>
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</tr>
<tr>
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<td>0.475**</td>
<td>0.450**</td>
<td>0.772**</td>
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<tr>
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<td>0.609**</td>
<td>0.494**</td>
<td>0.708**</td>
<td>0.623**</td>
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<td>0.219*</td>
<td>0.368**</td>
<td>0.290**</td>
<td>0.100</td>
<td>0.449**</td>
<td>1</td>
</tr>
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*P*<0.05; **P**<0.01

to *O. viverrini* antigen (Pilaor et al. 2005). TLR signals stimulate not only pro-inflammatory cytokines such as IL-1β but also anti-inflammatory cytokines such as IL-10 and IL-6 (Ozato et al. 2002). Thus, we could not exclude IL-6 cytokine which is considered to be associated with cholangiocarcinogenesis (Sripa et al. 2009). In our model as shown in Table 1, infection with *O. viverrini* may enhance both IL-1β and IL-10 expression, and additionally activate TLR-2 leading to NF-κB-mediated pathway (Dai et al. 2007; Al-Ashy et al. 2006; Li and Verma 2002; Gunn-Pease and Whisler 1998).

Decreasing potential of antioxidant system is well recognized due to oxidative stress. Several antioxidant genes including CAT showed downregulation in animal model of
opisthorchiasis-associated cholangiocarcinoma detected by a kinetic analysis of cDNA microarray technique (Wu et al. 2011). In agreement with other liver fluke, Fasciola hepatica, antioxidant potential was decreased in rat livers after post-infection (Kołodziejczyk et al. 2005). Here, we demonstrated that the expression levels of SOD-2 and CAT genes had a similar trend in healthy individuals and O. viverrini-infected groups. SOD-2 gene expression increased when treated in PBL with O. viverrini antigen whereas CAT seems to be suppressed in both groups. Our result showed that SOD-2 was positively correlated with oxidant-generating genes, suggesting its expression is the first line to catalyze ROS generation (Feng et al. 1995). On the other hand, CAT expression was suppressed in both groups but more significantly lowered in the infected group, suggesting an increased oxidative stress by a decrease in anti-oxidant of CAT during the inflammatory response due to the detoxification function of hydrogen peroxide (H$_2$O$_2$) (Lupertz et al. 2008; Canonico et al. 1977). An adaptive imbalance of anti-oxidant enzymes to genotoxic stress can be the pro-carcinogenic effect which has been proposed by Hofseth et al. (2003). Our result showed that IL-10 was positively correlated with CAT expression, suggesting that the expression of IL-10 is also involved in the reduction of oxidative stress.

All population of white blood cell counts in uninfected and O. viverrini-infected groups were in the normal range. It was previously demonstrated that the eosinophil counts between these two groups also showed no significant change (Punppak et al. 1994); here, we, therefore, include all populations of leukocytes, including mononuclear cells, neutrophils, and eosinophils; infection by O. viverrini induces a cellular immune response by the induction of oxidant-generating genes but downregulation of anti-oxidant gene (CAT), which may be involved in oxidative stress. A previous human study in an endemic area showed that host cellular immune responses to chronic infection increased endogenous NO production which contributed to form a carcinogen, N-nitrosodiethylaniline (Satarug et al. 1998). It has also been demonstrated that T cells play an important role in O. viverrini infection-mediated disease in an animal model (Flavell and Flavell 1986). We have successively reported in an animal and in vitro model that O. viverrini induces inflammatory responses through a TLR-2-mediated pathway leading to NF-kB-mediated expression of iNOS and COX-2, resulting in oxidative and nitroative DNA damage (Pinlaor et al. 2004b).

Taken together, O. viverrini infection induced oxidative stress via disturbance of oxidant/anti-oxidant balancing system but does not alter the expression of pro-inflammation/anti-inflammation system, which may be one of the key-driven forces for cholangiocarcinogenesis. The present study provides insight into the selection of appropriate chemopreventive agents against opisthorchiasis in endemic areas for the reduction of the disease and CCA, and in the particular area where re-infection occurs more frequently.

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References


Haddad JJ, Harb HL (2005) L-gamma-glutamyl-L-cysteinyl-glycine (glutathione; GSH) and GSH-related enzymes in the regulation of pro- and anti-inflammatory cytokines: a signaling transcriptional scenario for redox(y) immunologic sensor(s)? Mol Immo- nol 42(9):987–1014