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Rapid Communication

Opisthorchis viverrini antigen induces the expression of Toll-like receptor 2 in macrophage RAW cell line

Somchai Pinlaor[a,e], Saeko Tada-Oikawa[b], Yusuke Hiraku[b], Porntip Pinlaor[c,e], Ning Ma[d], Paiboon Sithithaworn[a,e], Shosuke Kawanishi[b,*]

[a]*Department of Parasitology, Khon Kaen University, Khon Kaen 40002, Thailand*
[b]*Department of Environmental and Molecular Medicine, Mie University School of Medicine, 2-174, Edobashi, Tsu, Mie 514-8507, Japan*
[c]*Department of Clinical Microbiology, Faculty of Associated Medical Science, Khon Kaen University, Khon Kaen 40002, Thailand*
[d]*Department of Anatomy, Mie University School of Medicine, Mie 514-8507, Japan*
[e]*Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand*

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Abstract

Opisthorchis viverrini infection induces inflammation in and around the bile duct, leading to cholangiocarcinoma in humans. To examine the mechanism of *O. viverrini*-induced inflammatory response, we assessed the expression of Toll-like receptors (TLRs) in RAW 264.7 macrophage cell line treated with an extract of *O. viverrini* antigen. Flow cytometry and immunocytochemistry showed that *O. viverrini* antigen induced the expression of TLR2 but not TLR4. Western blotting and immunocytochemistry revealed that nuclear factor-κB (NF-κB), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were expressed in RAW 264.7 cells treated with *O. viverrini* antigen in a dose-dependent manner. These results suggest that *O. viverrini* induces inflammatory response through TLR2-mediated pathway leading to NF-κB-mediated expression of iNOS and COX-2.

Keywords: *Opisthorchis viverrini*; Toll-like receptor 2; Inducible nitric oxide synthase; Nuclear factor-κB; Cyclooxygenase-2; RAW 264.7 macrophage cell line

Opisthorchis viverrini infection is a major risk factor of cholangiocarcinoma (CCA) development in humans (IARC, 1994). Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) has been implicated in inflammation-mediated carcinogenesis (Hussain et al., 2003) including *O. viverrini*-caused CCA (Haswell-Elkins et al., 1994). Recently, we have demonstrated that chronic inflammation triggered by repeated *O. viverrini* infection mediates iNOS-dependent DNA damage in intrahepatic bile duct epithelium and inflammatory cells of hamsters, which may play an important role in CCA development (Pinlaor et al., 2003, 2004a). However, the underlining mechanism of *O. viverrini*-induced inflammatory response remains to be clarified.

Sripa and Kaewkes (2000) observed *O. viverrini* antigen in bile ducts of the liver, liver cells, Kupffer cells, macrophages, epithelioid and giant cells in the egg granuloma. The presence of the antigens was associated with heavy inflammatory cell infiltration, particularly with mononuclear cells. Importantly, Akai et al. demonstrated that antibody level against *O. viverrini* antigen was positively associated with the severity of hepatobiliary disease and CCA (Akai et al., 1994). Thus, local parasite-specific immune responses induced by *O. viverrini* antigens would play a role in the pathogenesis of opisthorchiasis associated with CCA development.

Toll-like receptors (TLR) are an important membrane receptor family, which actively participates in the stimulation of the innate immune response. To date, 10 TLR homologs have been found in humans. It has been reported that TLR2 and TLR4 contribute to immune response to parasitic infection including protozoa (Kropf et al., 2004)

* Corresponding author. Tel./fax: +81 59 231 5011.
E-mail address: kawanisi@doc.medic.mie-u.ac.jp (S. Kawanishi).

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and helminth (van der Kleij et al., 2002). TLRs activate homologous signal transduction pathways leading to nuclear localization of NF-κB/Rel-type transcription factors. NF-κB is a key player in inflammation that regulates expression of various genes involved in controlling inflammatory response such as proinflammatory mediator, iNOS and cyclooxygenese-2 (COX-2) expression (Balkwill and Coussens, 2004; Surh et al., 2001). In addition, COX-2 is involved in carcinogenesis through the induction of inflammatory process (Surh et al., 2001). The expression of COX-2 is increased in tumor tissue of CCA patients (Endo et al., 2002). Relevantly, NF-κB functions as a tumor promoter in inflammation-associated cancer (Pikarsky et al., 2004).

In the present study, we investigated expression of TLRs, NF-κB, iNOS and COX-2 in RAW 264.7 macrophage cell line treated with *O. viverrini* antigen using flow cytometry and immunofluorescence techniques. To confirm the expression of these molecules, we also performed Western blotting.

O. viverrini crude antigen was prepared as described previously (Pinlaor et al., 2004b) with a minor modification. Four months after hamsters were infected with 50 *O. viverrini*, adult worms were collected. Following the addition of protease inhibitors (0.1 mM phenylmethyl sulphonyl fluoride, 0.1 mM L-1-tosylamine-2-phenylethyl chloromethyl ketone and 0.1 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64)), the worms were homogenized with 10 passes of a microhomogenizer with a Teflon-coated pestle. The homogenate was frozen in liquid nitrogen and then thawed at 37 °C. The homogenate was centrifuged at 12,000 *g* at 4 °C for 30 min. The supernatant containing crude somatic antigen was stored at −80 °C until use. The protein concentration in the supernatant was determined using the Coomassie® Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, USA).

RAW 264.7 mouse macrophage cell line was cultured in high glucose Dulbecco's modified Eagle's medium (Gibco/BRL, New York, NY, USA) containing 10% heat-inactivated fetal bovine serum and 100 mg/l kanamycin. The cells (1×10^6 cells) were detached from culture dishes by vigorous pipetting, and were centrifuged and resuspended in 1 ml fresh medium. Cells (1×10^6 cells/ml) were incubated with crude *O. viverrini* antigen at 37 °C for indicated durations.

For analysis of TLR expression, *O. viverrini* antigen-treated RAW 264.7 cells were treated with 1 μg/ml of rabbit polyclonal anti-TLR2 antibody or mouse monoclonal anti-TLR4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then incubated with 1 μg/ml of Alexa

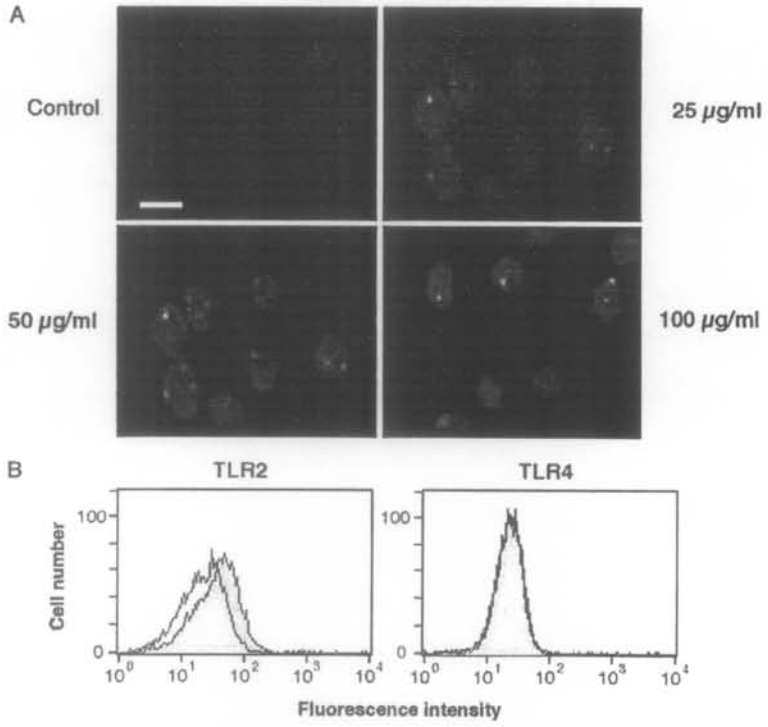

Fig. 1. TLR expression induced by *Opisthorchis viverrini* antigen. (A) Immunocytochemical analysis of TLR2 expression in RAW 264.7 cells treated with *O. viverrini* antigen. RAW 264.7 cells were treated with *O. viverrini* antigen for 12 h at 37 °C, and then treated with anti-TLR2 antibody overnight at room temperature. The cells were incubated with Alexa 594 anti-rabbit IgG antibody for 3 h and then viewed under a fluorescent microscope. Scale bar = 10 μm. (B) Flow cytometric fluorescence distribution of RAW 264.7 cells treated with *O. viverrini* antigen. RAW 264.7 cells were treated with 100 μg/ml of *O. viverrini* antigen for 12 h at 37 °C, and then incubated with anti-TLR2 or anti-TLR4 antibody for 30 min. The cells were then incubated with Alexa 488 anti-rabbit IgG or anti-mouse IgG antibody for 30 min and then analyzed with a flow cytometer. Open peaks, nontreated control cells; shaded peaks, *O. viverrini* antigen-treated cells.

488-labeled goat antibody against mouse IgG or against rabbit IgG (Molecular Probes, Eugene, OR, USA). The cells were resuspended in PBS, and then analyzed on a flow cytometer (FACScan; Becton Dickinson).

Localization of NF-κB and iNOS expression was assessed as described previously (Pinlaor et al., 2004a) with a minor modification. RAW 264.7 cells treated with *O. viverrini* antigen were smeared on a glass slide. After drying at 60 °C for 2 h, cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature. The cells were treated with 0.1% Triton X^{-1} 100 for 15 min and then incubated with 1% skim milk for 30 min.

For simultaneous analysis of iNOS and NF-κB expression, the cells were incubated with the primary antibodies, rabbit polyclonal anti-iNOS antibody (1:300, Calbiochem-Novabiochem Corporation, San Diego, USA) and mouse monoclonal anti-NF-κB antibody (1:300, Santa Cruz Biotechnology, Inc, USA) overnight at room temperature. Then, the cells were incubated with Alexa 594-labeled goat antibody against rabbit IgG and Alexa 488-labeled goat antibody against mouse IgG (1:400, Molecular Probes) for 3 h. The cells were examined under an inverted Laser Scan Microscope (LSM 410, Zeiss, Gottingen, Germany).

For analysis of the expression of TLR2, TLR4 and COX-2, the cells were incubated with the primary antibody [rabbit polyclonal anti-TLR2 antibody, mouse monoclonal anti-TLR4 antibody (1 μg/ml, Santa Cruz Biotechnology) or rabbit polyclonal anti-COX-2 antibody (1:400, Oxford Biomedical Research, Oxford, Michigan, USA)] overnight at room temperature. Subsequently, the cells were incubated with Alexa 594-labeled goat antibody against rabbit IgG or Alexa 488-labeled goat antibody against mouse IgG (1:400, Molecular Probes). The cells were examined under an inverted Laser Scan Microscope as described above.

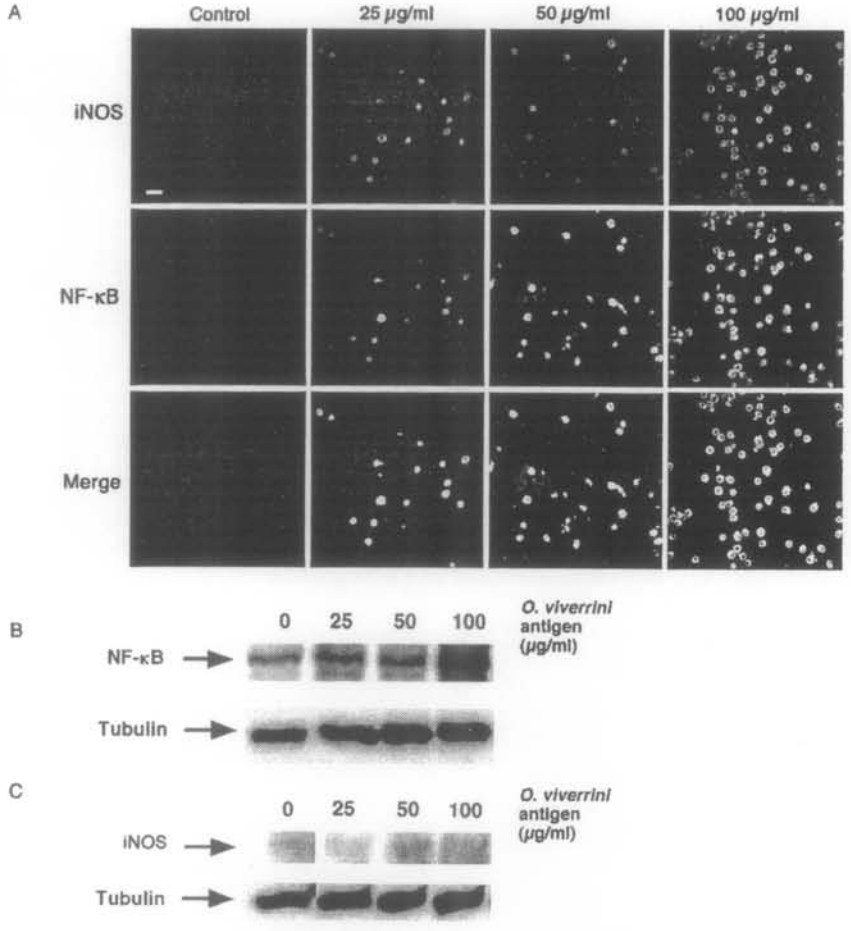

Fig. 2. Expression of NF-κB and iNOS induced by *Opisthorchis viverrini* antigen. (A) Immunocytochemical analysis of iNOS and NF-κB expression in RAW 264.7 cells treated with *O. viverrini* antigen. RAW 264.7 cells were treated with indicated concentration of *O. viverrini* antigen for 12 h, and then incubated with anti-iNOS and anti-NF-κB antibodies followed by Alexa 594 anti-rabbit IgG or Alexa 488 anti-mouse IgG antibodies for 3 h and then viewed under an inverted Laser Scan Microscope. iNOS expression (red color) is observed in the cytoplasm, whereas NF-κB (green color) is expressed in both nucleus and cytoplasm. Scale bar = 10 μm. (B, C) Western blotting analysis of NF-κB and iNOS expression in RAW 264.7 cells treated with *O. viverrini* antigen. Proteins extracted from *O. viverrini* antigen-treated RAW 264.7 cells were immunoblotted and probed with mouse monoclonal anti-NF-κB antibody (B) or rabbit polyclonal anti-iNOS antibody (C). (For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article.)

RAW 264.7 cells treated with crude *O. viverrini* antigen were solubilized in sample buffer and boiled for 5 min. Samples were separated by 4–20% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS, pH 7.5). The membranes were then incubated with the primary antibody [mouse monoclonal anti-NF-κB antibody (1:1000), rabbit polyclonal anti-iNOS antibody (1:2000) or rabbit polyclonal anti-COX-2 antibody (1:1000)]. The membranes were washed in TBS and then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:2000, Santa Cruz Biotechnology) or anti-rabbit IgG antibody (1:2000, New England Biolabs, Beverly, MA, USA). After extensive washings in TBS, enhanced chemiluminescence assay was performed using an ECL detection reagent (Amersham) and positive bands were detected on X-ray films.

Fig. 1 shows the expression of TLRs in RAW 264.7 cells treated with *O. viverrini* antigen. Immunocytochemistry revealed that *O. viverrini* antigen increased the expression of TLR2 dose-dependently (Fig. 1A), but not TLR4 (data not shown). TLR2 was not expressed in nontreated control cells (Fig. 1A). Flow cytometry confirmed that *O. viverrini* antigen increased the fluorescence intensity of TLR2, but not TLR4 (Fig. 1B).

Fig. 2 shows the expression of NF-κB and iNOS in RAW 264.7 cells treated with *O. viverrini* antigen. iNOS expression (red color) was observed in the cytoplasm, whereas NF-κB expression (green color) was observed in both nucleus and cytoplasm (Fig. 2A). *O. viverrini* antigen increased expression of NF-κB and iNOS dose-dependently. Western blotting showed that the expression of NF-κB (approximately 65 kDa, Fig. 2B) and iNOS (approximately 130 kDa, Fig. 2C) were increased dose-dependently in RAW 264.7 cells treated with *O. viverrini* antigen.

Fig. 3 shows COX-2 expression in RAW 264.7 cells treated with *O. viverrini* antigen. The immunoreactivity of COX-2 increased in the cytoplasm depending on the concentration of *O. viverrini* antigen (Fig. 3A). COX-2 expression was not observed in nontreated control cells (Fig. 3A). Western blotting showed that COX-2 protein (approximately 70 kDa) was increased dose-dependently in RAW 264.7 cells treated with *O. viverrini* antigen (Fig. 3B).

This is the first study showing that *O. viverrini* antigen induces the expression of TLR2, which may participate in NF-κB-mediated expression of iNOS and COX-2 in RAW 264.7 cells. The expression of these proteins was dependent on the concentration of *O. viverrini* antigen. TLR family recognizes conserved organism structures and activates signaling pathways (Barton and Medzhitov, 2003). Relevantly, it was reported that in schistosome infection, purified schistosomal antigen activates TLR2 in dendritic cells (van der Kleij et al., 2002). *O. viverrini* may mediate TLR2-dependent pathway through binding its lipoproteins as demonstrated using bacterial lipoproteins (Aliprantis et al., 1999). Our data can reasonably explain the clinical data showing that parasite-specific antibody levels in human hepatobiliary disease were associated with *O. viverrini* infection (Haswell-Elkins et al., 1991). On the other hand, TLR4

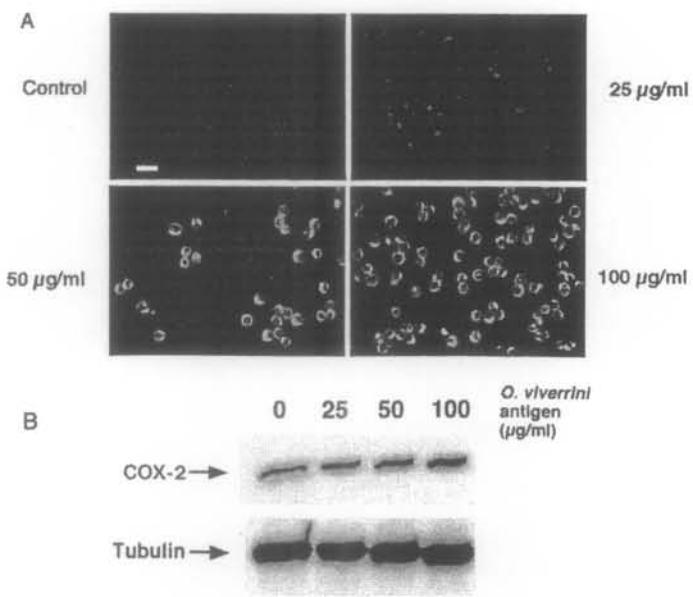

Fig. 3. COX-2 expression induced by *Opisthorchis viverrini* antigen. (A) Immunocytochemical analysis of COX-2 expression in RAW 264.7 cells. RAW 264.7 cells were treated with indicated concentration of *O. viverrini* antigen for 12 h, and then incubated with anti-COX-2 antibody followed by Alexa 594 anti-rabbit IgG antibody and then viewed under an inverted Laser Scan Microscope. COX-2 expression was observed in the cytoplasm dose-dependently, but not in untreated cells. Scale bar = 10 μm. (B) Western blotting analysis of COX-2 expression in RAW 264.7 cells treated with *O. viverrini* antigen. Proteins extracted from RAW 264.7 cells treated with *O. viverrini* antigen were immunoblotted and probed with anti-COX-2 antibody.

contributes to efficient control of infection with protozoa such as *Leishmania major* (Kropf et al., 2004), and lipopolysaccharide, a major component of the outer surface of Gram-negative bacteria (Andonegui et al., 2003). In the present study, TLR4 expression was not increased in *O. viverrini* antigen-treated cultured cells. It has been reported that constitutively active TLR4 induces NF-κB activation and subsequent production of inflammatory cytokines (Medzhitov et al., 1997). Thus, TLR4 may also participate in pathogenesis of *O. viverrini* infection.

Our results showed that NF-κB was expressed in both the nucleus and cytoplasm, suggesting that *O. viverrini* antigen induces not only NF-κB expression, but also its nuclear translocation and activation. NF-κB regulates the expression of iNOS and COX-2 (Surh et al., 2001; Balkwill and Coussens, 2004). We have previously reported that *O. viverrini* infection strongly induces iNOS in bile duct epithelial cells and inflammatory cells (Pinlaor et al., 2004a). This finding can be explained by assuming that *O. viverrini* antigen mediates NF-κB-mediated iNOS expression depending on TLR, since it has been reported that *O. viverrini* antigen is present in both epithelial bile duct and inflammatory cells (Sripa and Kaewkes, 2000). Relevantly, Harada et al. have reported that NF-κB is expressed through TLR and related molecules in cultured biliary epithelial cells treated with bacterial LPS (Harada et al., 2003). COX-2 expression induces inflammatory cell infiltration (Surh et al., 2001) and participates in carcinogenesis (Oshima et al., 2004). Moreover, COX-2 expression is increased in tumor tissue of cholangiocarcinoma patients (Endo et al., 2002). Thus, COX-2 may participate in the pathogenesis of *O. viverrini* infection through an additional inflammatory response.

We have recently demonstrated that *O. viverrini* infection induces inflammation-mediated DNA damage through NO production via iNOS expression (Pinlaor et al., 2004a). DNA damage was observed in bile duct epithelial cells and inflammatory cells in the liver of hamsters with repeated *O. viverrini* infection and was associated with the progression of pathological changes (Pinlaor et al., 2004a). In the present study, in vitro experiments have shown that *O. viverrini* antigen induces the expression of TLR2, NF-κB, iNOS and COX-2 in macrophage cell line. It is reasonably considered that TLR2 expression mediates NF-κB expression and its nuclear translocation, leading to expression of iNOS and COX-2. In conclusion, the pathogenesis of *O. viverrini* infection, including carcinogenesis, may be mediated by inflammatory response triggered by its antigen via TLR2-dependent pathway.

Acknowledgements

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References

Akai, P.S., Pungpak, S., Chaicumpa, W., Viroj, K., Bunnag, D., Befus, A.D., 1994. Serum antibody response to *Opisthorchis viverrini* antigen as a marker for opisthorchiasis-associated cholangiocarcinoma. Trans. R. Soc. Trop. Med. Hyg. 88, 471–474.

Aliprantis, A.O., Yang, R.B., Mark, M.R., Suggett, S., Devaux, B., Radolf, J.D., Klimpel, G.R., Godowski, P., Zychlinsky, A., 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 285, 736–739.

Andonegui, G., Bonder, C.S., Green, F., Mullaly, S.C., Zbytnuik, L., Raharjo, E., Kubes, P., 2003. Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. J. Clin. Invest. 111, 1011–1020.

Balkwill, F., Coussens, L.M., 2004. Cancer: an inflammatory link. Nature 431, 405–406.

Barton, G.M., Medzhitov, R., 2003. Toll-like receptor signaling pathways. Science 300, 1524–1525.

Endo, K., Yoon, B.I., Pairojkul, C., Demetris, A.J., Sirica, A.E., 2002. ERBB-2 overexpression and cyclooxygenase-2 up-regulation in human cholangiocarcinoma and risk conditions. Hepatology 36, 439–450.

Harada, K., Ohira, S., Isse, K., Ozaki, S., Zen, Y., Sato, Y., Nakanuma, Y., 2003. Lipopolysaccharide activates nuclear factor-κB through toll-like receptors and related molecules in cultured biliary epithelial cells. Lab. Invest. 83, 1657–1667.

Haswell-Elkins, M.R., Sithithaworn, P., Mairiang, E., Elkins, D.B., Wongratanacheewin, S., Kaewkes, S., Mairiang, P., 1991. Immune responsiveness and parasite-specific antibody levels in human hepatobiliary disease associated with *Opisthorchis viverrini* infection. Clin. Exp. Immunol. 84, 213–218.

Haswell-Elkins, M.R., Satarug, S., Tsuda, M., Mairiang, E., Esumi, H., Sithithaworn, P., Mairiang, P., Saitoh, M., Yongvanit, P., Elkins, D.B., 1994. Liver fluke infection and cholangiocarcinoma: model of endogenous nitric oxide and extragastric nitrosation in human carcinogenesis. Mutat. Res. 305, 241–252.

Hussain, S.P., Hofseth, L.J., Harris, C.C., 2003. Radical causes of cancer. Nat. Rev. Cancer 3, 276–285.

IARC Working Group, 1994. Infection with liver flukes (*Opisthorchis viverrini*, *Opisthorchis felineus* and *Clonorchis sinensis*), In: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 61. IARC Lyon pp. 121–175.

Kropf, P., Freudenberg, M.A., Modolell, M., Price, H.P., Herath, S., Antoniazi, S., Galanos, C., Smith, D.F., Muller, I., 2004. Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite *Leishmania major*. Infect. Immun. 72, 1920–1928.

Medzhitov, R., Preston-Hurlburt, P., Janeway Jr., C.A., 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388, 394–397.

Oshima, H., Oshima, M., Inaba, K., Taketo, M.M., 2004. Hyperplastic gastric tumors induced by activated macrophages in COX-2/mPGES-1 transgenic mice. EMBO J. 23, 1669–1678.

Pikarsky, E., Porat, R.M., Stein, I., Abramovitch, R., Amit, S., Kasem, S., Gutkovich-Pyest, E., Urieli-Shoval, S., Galun, E., Ben-Neriah, Y., 2004. NF-κB functions as a tumour promoter in inflammation-associated cancer. Nature 431, 461–466.

Pinlaor, S., Yongvanit, P., Hiraku, Y., Ma, N., Semba, R., Oikawa, S., Murata, M., Sripa, B., Sithithaworn, P., Kawanishi, S., 2003. 8-Nitroguanine formation in the liver of hamsters infected with *Opisthorchis viverrini*. Biochem. Biophys. Res. Commun. 309, 567–571.

Pinlaor, S., Ma, N., Hiraku, Y., Yongvanit, P., Semba, R., Oikawa, S., Murata, M., Sripa, B., Sithithaworn, P., Kawanishi, S., 2004a. Repeated

infection with *Opisthorchis viverrini* induces accumulation of 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. Carcinogenesis 25, 1535–1542.

Pinlaor, S., Sripa, B., Sithithaworn, P., Yongvanit, P., 2004b. Hepatobiliary changes, antibody response, and alteration of liver enzymes in hamsters re-infected with *Opisthorchis viverrini*. Exp. Parasitol. 108, 32–39.

Sripa, B., Kaewkes, S., 2000. Localisation of parasite antigens and inflammatory responses in experimental opisthorchiasis. Int. J. Parasitol. 30, 735–740.

Surh, Y.J., Chun, K.S., Cha, H.H., Han, S.S., Keum, Y.S., Park, K.K., Lee, S.S., 2001. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. Mutat. Res. 480-481, 243–268.

van der Kleij, D., Latz, E., Brouwers, J.F., Kruize, Y.C., Schmitz, M., Kurt-Jones, E.A., Espevik, T., de Jong, E.C., Kapsenberg, M.L., Golenbock, D.T., Tielens, A.G., Yazdanbakhsh, M., 2002. A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. J. Biol. Chem. 277, 48122–48129.

Synergistic effect of *Opisthorchis viverrini* infection and *N*-nitrosodimethylamine on the expression of iNOS, NF-kB and COX-2 in an animal model of cholangiocarcioma

Somchai Pinlaor[a,*], Puangrat Yongvanit[b,a], Somkid Dechakhamphu[b,a], Thidarut Boonmars[a,a], Paiboon Sithithaworna[a], Yusuke Hiraku[c], Shosuke Kawanishi [c,d]

[a]Department of Parasitology, [b]Department of Biochemistry, and [a]Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand,

[c]Department of Environmental and Molecular Medicine, Mie University Graduate School of Medicine,Japan, and [d]Faculty of Health Science, Suzuka University of Medical Science, Japan

Introduction

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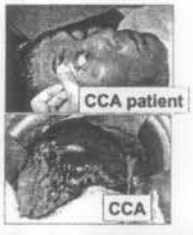

Infection with the liver fluke *Opisthorchis viverrini* (OV) and *N*-nitrosodimethylamine (NDMA) are the major risk factors of cholangiocarcinoma (CCA). OV infection is endemic in the Northeastern Thailand. Nitrate and nitrite are contained in fermented fish, the most favored food in this region. NDMA can be endogenously produced by the interaction of nitrite with secondary amines. The synergistic carcinogenic effect of OV plus NDMA is likely to occur in the environment.

We have recently reported OV infection mediates nitrative and oxidative DNA damage in an animal model through iNOS and NF-kappaB expression. The expression of Toll-like receptor 2, iNOS and NF-kappaB is a dose dependent with the induction by OV antigen in macrophage RAW cell line. Therefore, the study of these genes expression in cholangiocarcinogenesis may useful for chemoprevention and biomarker of CCA development.

Objective

To investigate the expression of iNOS, NF-kB, COX-2 and antioxidant enzymes in the liver of hamsters treated with OV and NDMA.

Methodology

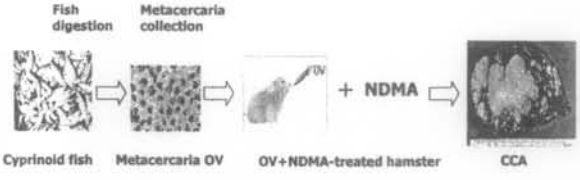

Fig.1 Methodological study in an animal model.

Experimental Design

Table.1 The experimental design of this study.

Groups	Time after treatment and the number of animals					
	21 D	1 M	2 M	3 M	4 M	6 M
OV	5	5	5	5	5	5
NDMA	5	5	5	5	5	5
OV + NDMA	5	5	5	5	5	5
Normal hamsters	5	5	5	5	5	5
Total number	20	20	20	20	20	20

Results

Real-time RT-PCR showed that OV plus NDMA induced synergistically increased the expression of iNOS, NF-kB and COX-2 in relation to CCA development. Expression of genes of antioxidant enzymes, such as Cu/Zn superoxide dismutase (SOD1), Mn SOD (SOD2), catalase (CAT) and glutathione peroxidase (GPx)-like genes, was decreased, while the expression of iNOS and COX-2 increased, suggesting that oxidative and nitrative stress occurred.

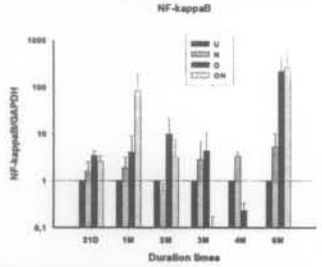

Fig2. Expression of NF-kappaB detected by SYBR Green real-time PCR. The expression of NF-kappaB synergistic increases in OV plus NDMA groups on 1M and the highest ratio in tumor group (n=3). The amount of mRNA was normalized to the internal control, GAPDH. U = normal, N = NDMA, O = OV, ON = OV plus NDMA.

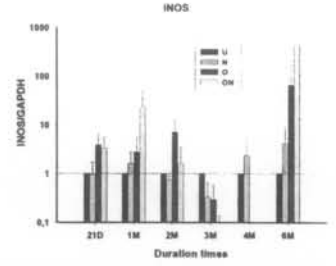

Fig3. Expression of iNOS detected by SYBR Green real-time PCR. The expression of iNOS synergistic increases in OV plus NDMA groups and the highest in tumor group (n=3). The amount of mRNA was normalized to the internal control, GAPDH. U = normal, N = NDMA, O = OV, ON = OV plus NDMA.

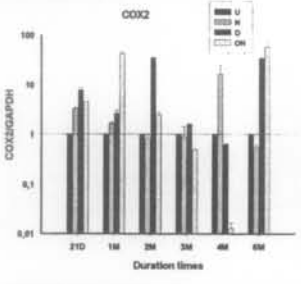

Fig4. Expression of COX2 detected by SYBR Green real-time PCR. The expression of COX2 synergistic increases in OV plus NDMA groups and the highest in tumor group (n=3). The amount of mRNA was normalized to the internal control, GAPDH. U = normal, N = NDMA, O = OV, ON = OV plus NDMA.

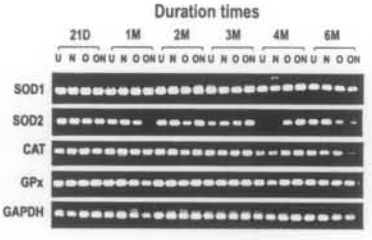

Fig5. Relative gel image expression of SOD1, SOD2, CAT and GPx detected by RT-PCR. The expression of SOD1, SOD2, CAT and GPx synergistic decrease in OV plus NDMA groups (n=3) compared with the internal control, GAPDH. U = normal, N = NDMA, O = OV, ON = OV plus NDMA.

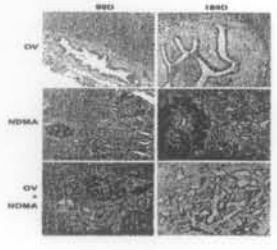

Fig.8 Histological feature of hamster liver. OV and NDMA synergistic induces CCA development on day 180.

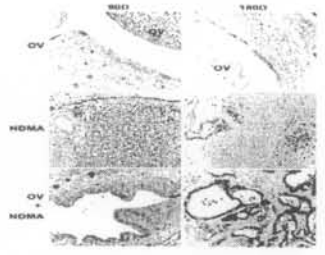

Fig.9 Localization of PCNA in hamster liver. OV and NDMA synergistic increases the expression of PCNA protein on epithelial bile ducts and inflammatory cells.

Conclusion

These results showed that the expression of iNOS, NF-kB and COX-2 was involved in the synergistic carcinogenic effect of NDMA and OV infection. Therefore, these genes and their products may be useful biomarkers to evaluate the potential risk of development of tumor and the candidate genes for cancer chemoprevention.

References

1.Pinlaor S, Ma N, Hiraku Y, Yongvanit P, Semba R, Oikawa S, Murata M, Sripa B, Sithithaworn P, Kawanishi S. Repeated infection with Opisthorchis viverrini induces accumulation of 5-nitrosouracil and 8-oxo-7,8-dihydro-2'-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. Carcinogenesis 2004;25(8):1535-42.

2.Pinlaor S, Sripa B, Ma N, Hiraku Y, Yongvanit P, Wongkham S, Pairojkul C, Bhudhisawasdi V, Oikawa S, Murata M, Semba R, Kawanishi S. Nitrative and oxidative DNA damage in intrahepatic cholangiocarcinoma patients in relation to tumor invasion. World J Gastroenterol 2005;11(30):4644-9.

3.Pinlaor S, Tada-Oikawa S, Hiraku Y, Pinlaor P, Ma N, Sithithaworn P, Kawanishi S. Opisthorchis viverrini antigen induces the expression of Toll-like receptor 2 in macrophage RAW cell line. Int J Parasitol 2005;35(6):691-6.

4.Pinlaor S, Hiraku Y, Yongvanit P, Tada-Oikawa S, Ma N, Pinlaor P, Sithithaworn P, Sripa B, Murata M, Oikawa S, Kawanishi S. iNOS-dependent DNA damage via NF-kappaB expression in hamsters infected with Opisthorchis viverrini and its suppression by the antihelminthic drug praziquantel. Int J Cancer 2006;119(5):1067-72.

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INDUCTION OF mRNA EXPRESSION INVOLVING IN OXIDATIVE AND NITRATIVE STRESS IN WHITE BLOOD CELLS CO-CULTURED WITH *Opisthorchis viverrini* CRUDE EXTRACT

Raynoo Thanan[1,2,4], Somchai Pinlaor[3,4], Watcharin Loilome[2,4], Anchalee Techasen[1,2,4], Nisana N Tepsiri[3,4], Paiboon Sithithaworn[3,4] and Puangrat Yongvanit[2,4]

[1]Graduate School, [2] Department of Biochemistry, [3] Department of Parasitology, [4] Liver fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

1. Abstract

We performed the experiments to prove that *Opisthorchis viverrini* infection induces oxidative and nitrative stress via inflammatory process in human. White blood cell (WBC) primary culture was exposed with OV crude antigens by the co-culture technique. Lipopolysaccharide (LPS) stimulation was used as the positive control. The amount of OV antigens and duration of co-culturing were optimized for the appropriate condition of the experiments. After co-culturing, WBC was collected for mRNA extraction to determine candidate gene expressions involving in reactive oxygen and reactive nitrogen species production, from which COX-2, TLR-2, NFκB expressions were primarily analyzed. Our preliminary data after 6, 24 and 72 hr induction of WBC by crude OV antigen showed that COX-2, iNOS, NFκB and TLR-2 mRNA were overexpressed.

2. Introduction

We had demonstrated that a single- and repeated-infections with *Opisthorchis viverrini* (OV) could mediate oxidative and nitrative DNA damage in hamster model. This may play role in initiation and/or promotion steps of CCA carcinogenesis through chronic inflammation as shown in Fig 1. Thus, we would further prove by a co-culture experiment whether oxidative and nitrosative stress in human is occurred via this mechanism.

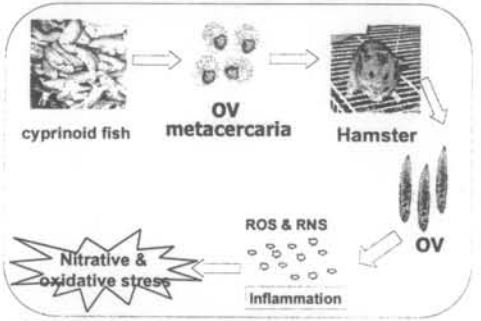

Fig 1. OV induced nitrative and oxidative stress in hamster model (Previous study by Pinlaor et al)

7. Financial Support

This work is supported by KKU-49 grant of Khon Kaen University and Thailand Research Fund through Royal Golden Jubilee Ph.D. Program to RT and PY.

3. Methodology

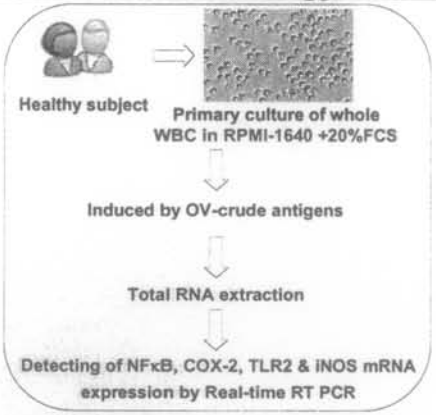

Fig 2. Experimental protocol of OV crude extract antigens inducing nitrative and oxidative stress in human WBC co-culture

4. Result

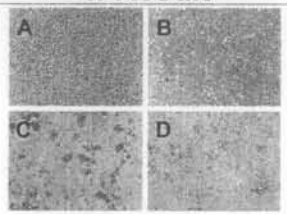

Fig 3. The morphology of WBC. Whole WBC was isolated from buffy coat then cultured in RPMI-1640+20%FCS (A). Twenty-four hours later the cells were co-cultured with OV crude extract for 24 h as well as control; no OV-antigens & LPS (B), induced by 100 ug/ml of OV-crude extract (C), and induced by 10 ug/ml of LPS (D).

5. Conclusion

Our study suggests that COX-2, iNOS, NFκB and TLR-2 genes could be induced in WBC at different time intervals as early and late response gene to OV crude antigens. The results indicated that OV infection may play roles in causing oxidative and nitrative stress in human. Fundamental knowledge of this study may be applied for chemoprevention study to inhibit cholangiocarcinogenesis in the future.

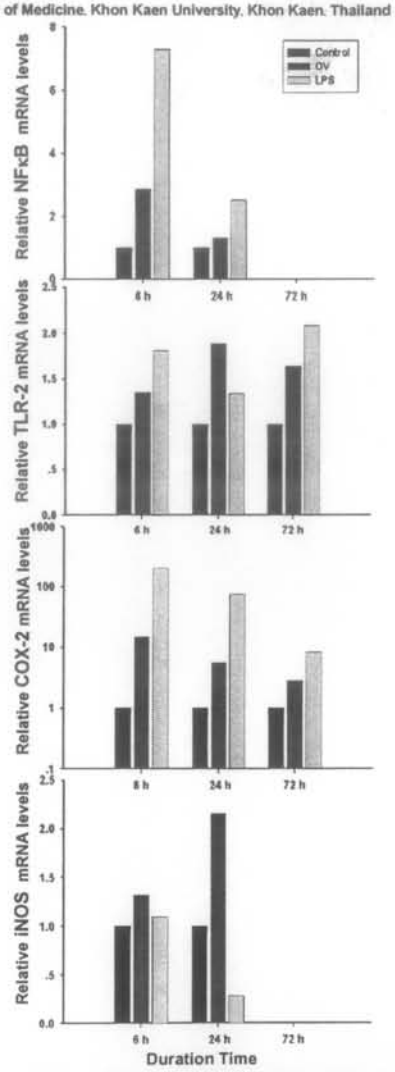

Fig 4. Relative mRNA expression of NFκB, TLR2, COX-2 and iNOS genes in whole WBC co-cultured with OV crude extract. After whole WBC was induced by OV crude antigens, whole WBC was collected and RNA was extracted. The levels of mRNA was analyzed by Real time RT PCR.