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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

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Chronic inflammation induced by repeated infection with *Opisthorchis viverrini* has been postulated to be a risk factor for cholangiocarcinoma. To clarify the mechanism of carcinogenesis induced by repeated *O.viverrini* infection, we investigated the timecourse of 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation, inducible nitric oxide synthase (iNOS) expression, nitric oxide production and pathological features in hamsters with two (2-IF) or three (3-IF) *O.viverrini* infections. Inflammatory cell infiltration triggered by repeated infection (3-IF > 2-IF > 1-IF) was earlier than by single infection (1-IF). HPLC coupled with an electrochemical detector revealed that 8-oxodG level in the liver was the highest on day 3 in 3-IF and day 7 in 2-IF, earlier than that on day 21 in 1-IF. Notably, a double immunofluorescence study revealed that formation of 8-nitroguanine and 8-oxodG appeared to increase in the epithelium of bile ducts in the order 3-IF > 2-IF > 1-IF after the decrease in inflammatory cells. This may be explained by the fact that repeated infection increased iNOS expression in the epithelium of bile ducts in the order 3-IF > 2-IF > 1-IF on day 90. Proliferating cell nuclear antigen accumulated in the epithelium of bile ducts on day 90 after repeated *O.viverrini* infection, supporting the hypothesis that cell proliferation was promoted by inflammation-mediated DNA damage. In conclusion, more frequent *O.viverrini* infection can induce the expression of iNOS not only in inflammatory cells but also in the epithelium of bile ducts and subsequently cause nitrosative and oxidative damage to nucleic acids, which may participate in the initiation and/or promotion steps of cholangiocarcinoma development.

Abbreviations: ALT, alanine aminotransferase; CHCA, cholangiocarcinoma; GSH, reduced glutathione; HPLC-ECD, electrochemical detector coupled to HPLC; 1-IF, single infection; 2-IF, two infections; 3-IF, three infections; iNOS, inducible nitric oxide synthase; LPO, lipid peroxidation; MDA, malondialdehyde; $ONOO^-$, peroxynitrite; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; RSA, rabbit serum albumin; TBARS, thiobarbituric acid-reactive substances.

Introduction

Chronic inflammation induced by infection has been postulated to be a risk factor of various cancers (1–3), especially during chronic active inflammation (4,5). Reinfection with *Opisthorchis viverrini* is a major risk factor of cholangiocarcinoma (CHCA) in north-east Thailand, probably through inflammation (1,6). In endemic communities, several investigators reported that reinfection with *O.viverrini* occurs frequently after treatment (7,8). The majority (70%) of *O.viverrini*-induced CHCA occur in the intrahepatic bile ducts, with the remainder occurring in the extrahepatic duct (30%) (9), although generally the incidence of intrahepatic CHCA is relatively low. Upatham *et al*. have shown that the rate of reinfection is markedly higher than infection of those who are negative or have only a light infection (7). The severity of the disease and CHCA development are associated with duration and frequency of reinfection (1,10). *Opisthorchis viverrini* infection is known to cause persistent infection and several histopathological changes, such as hyperplasia, adenomatous hyperplasia and periductal fibrosis. However, the mechanism by which *O.viverrini* induces CHCA is not well understood. Therefore, investigations of reinfection in hamsters as a model of humans may provide an insight into the mechanism linking it to CHCA carcinogenesis.

Overproduction of NO by inflammatory cells plays a crucial role in multistage carcinogenesis (3,11). Higher levels of nitrate and nitrite, which reflect endogenous generation of NO due to *O.viverrini* infection, have been previously reported in humans (12) and animals (3). In particular, oxidative DNA damage induced by NO or NO-derived reactive nitrogen species such as peroxynitrite ($ONOO^-$) could be significant sources of the genomic instability characteristic of human cancers (13). 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a major indicator of oxidative DNA damage, has been implicated in cancer triggered by infection (14,15). In addition, a close association of 8-nitroguanosine formation with NO production in mice with viral pneumonia has been established (16). We have also reported 8-nitroguanine and 8-oxodG formation in hamsters on single infection with *O.viverrini*. 8-Nitroguanine and 8-oxodG formation in inflammatory cells reached its highest intensity on days 30 and 21 in the acute phase, respectively, and remained high in the chronic phase (17). 8-Nitroguanine and 8-oxodG are known to cause G:C → T:A transversions, which are frequently found in various tumor-relevant genes (3,13,18). In the present study we have investigated 8-nitroguanine and 8-oxodG formation in the liver of hamsters triggered by reinfection with *O.viverrini* and compared it with single infection (1-IF).

In order to clarify the mechanism of carcinogenesis induced by repeated *O.viverrini* infection, we used hamsters as a model of reinfection in humans. We produced a specific anti-8-nitroguanine antibody using 8-nitroguanine aldehyde–rabbit serum albumin (RSA) conjugate. 8-Nitroguanine and 8-oxodG

formation were assessed by double immunofluorescence using this antibody. We also measured the amount of 8-oxodG in the liver using an electrochemical detector coupled to HPLC (HPLC-ECD). Inducible nitric oxide synthase (iNOS) was assessed in the liver of hamsters by an immunohistochemical technique. Plasma nitrate/nitrite, end products of NO generated by inflammatory cells, was analyzed. Furthermore, we measured malondialdehyde (MDA), a marker of lipid peroxidation (LPO), in plasma. To evaluate liver tissue damage, we measured plasma alanine aminotransferase (ALT) activity and examined histopathological changes by hematoxylin and eosin staining. Liver glutathione (GSH) level, which appears to be most sensitive to oxidative stress and plays a key role in antioxidative defence, was determined by a sensitive and specific method using a HPLC-ECD method with a gold electrode, which we recently developed (19). Proliferating cell nuclear antigen (PCNA) functions as a cofactor of DNA polymerase δ associated with repair of DNA damage (20). We examined accumulation of PCNA in the liver of *O.viverrini* -infected hamsters by an immunohistochemical technique.

Materials and methods

Chemicals

8-Nitroguanine was purchased from Biolog Life Science Institute (Bremen, Germany). Mouse monoclonal anti-8-oxodG antibody was purchased from the Japan Institute for the Control of Aging (Fukuroi, Japan). Rabbit polyclonal anti-iNOS antibody was purchased from Calbiochem-Novabiochem Corp. (Darmstadt, Germany). Mouse monoclonal anti-PCNA antibody was obtained from Novocastra Laboratories (Newcastle, UK). Alexa 594-labeled goat antibody against rabbit IgG and Alexa 488-labeled goat antibody against mouse IgG were obtained from Molecular Probes Inc. (Eugene, OR).

Parasites

Metacercariae of *O.viverrini* were obtained from naturally infected cyprinoid fish from an endemic area in Khon Kaen Province, Thailand. Cysts of *O.viverrini* were collected and identified under a dissecting microscope as described previously (21).

Animals and experimental design

The animal experiments were conducted according to the guidelines of the National Committee of Animal Ethics. Permission no. AE003/2002 was obtained from the Animal Ethic Committee of the Faculty of Medicine, Khon Kaen University, Thailand. 286 male Syrian golden hamsters (Animal Unit, Faculty of Medicine, Khon Kaen University) ranging in age from 6 to 8 weeks were housed under conventional conditions and fed with stock diet and water *ad libitum*.

Animals were fed with 50 metacercariae of *O.viverrini* by intragastric intubation for single infections (1-IF). For repeated infections, animals were given 30 metacercariae and, after 3 months, they were then reinfected with 20 metacercariae (2-IF). After a further 3 months, some hamsters were again fed with 20 metacercariae (3-IF). In addition, normal hamsters were treated with saline solution by the same route. Seven groups of seven *O.viverrini*-infected hamsters and six non-infected hamsters were used for each time point (days 3, 7, 14, 21, 30, 60 and 90 of infection). Finally, hamsters were anesthetized and blood was taken from the heart with an EDTA tube. Days 3, 7, 14 and 21 were defined as short-term infection with immature new *O.viverrini* and days 30, 60 and 90 were defined as long-term infection with mature *O.viverrini*.

Production of anti-8-nitroguanine antibody

Anti-8-nitroguanine polyclonal antibody was produced by a modified method (22). 8-Nitroguanosine was incubated with sodium metaperiodate for 20 min at room temperature and was conjugated with RSA for 1 h, followed by incubation with sodium borohydride for 1 h. The conjugate was dialyzed against 150 mM NaCl overnight. 8-Nitroguanine aldehyde–RSA conjugate was injected into a rabbit with Freund's complete adjuvant by intracutaneous administration. After 4 weeks, the same antigen was again given and blood was taken 10 days later. The antibody was purified using an 8-nitroguanine-conjugate column. Specificity of the purified antibody was examined by a dot immunobinding assay and absorption test (23).

Immunofluorescence staining and histopathological study

8-Nitroguanine and 8-oxodG immunoreactivity in hamster livers were assessed by double immunofluorescence labeling studies as described previously (24). Briefly, paraffin sections (6 μm thickness) were incubated with rabbit polyclonal anti-8-nitroguanine antibody (2 μg/ml) and mouse monoclonal anti-8-oxodG antibody (5 μg/ml) overnight at room temperature. Then the sections were incubated with Alexa 594-labeled goat antibody against rabbit IgG and Alexa 488-labeled goat antibody against mouse IgG (1:400) for 3 h. The immunostained sections were examined under an invert Laser Scan Microscope (LSM 410; Zeiss, Gottingen, Germany).

iNOS expression in the liver of hamsters infected with *O.viverrini* was performed by an indirect immunofluorescence technique as described previously (17). Briefly, paraffin sections were incubated with rabbit polyclonal anti-iNOS antibody (1:400) overnight at room temperature. Subsequently, the sections were incubated with Alexa 594-labeled goat antibody against rabbit IgG (1:400) for 3 h and were analyzed using a Laser Scan Microscope.

Accumulation of PCNA in the hamster liver was assessed by immunohistochemistry. Briefly, paraffin sections were incubated with mouse monoclonal anti-PCNA antibody (1:100) overnight at room temperature. Then the sections were incubated with goat anti-mouse IgG–horseradish peroxidase (1:200). Sections were visualized with 3,3-diaminobenzidine tetrahydrochloride as chromogen.

A histopathological study was also performed with hematoxylin and eosin staining in paraffin sections as described previously (21).

HPLC analysis of 8-oxodG and GSH in the liver

The amount of 8-oxodG in liver DNA was measured by HPLC-ECD with a minor modification (25). Briefly, 200 mg hamster liver were homogenized in 0.25 M saccharose solution and the pellet was then washed five times with cold phosphate-buffered saline (PBS), pH 7.4. DNA was extracted from the homogenate under anerobic conditions. The 8-oxodG content was measured as described previously (17).

Liver GSH content was determined by a minor modification of HPLC-ECD equipped with a gold electrode (19). Approximately 100 mg liver was scissored into small pieces, followed by homogenization in 4 vol PBS with a microhomogenizer with a Teflon-coated pestle and protein was precipitated with trichloroacetate. The supernatant was diluted with 0.1 N HCl and the amount of GSH was measured as described previously (19). The amount of protein in the supernatant from the homogenate was measured by a modification of a method described previously (26).

Determination of nitrate/nitrite, MDA and ALT activity in plasma

Plasma nitrate/nitrite was determined by Griess reaction as described previously (17). Nitrate concentration in plasma was measured after reduction to nitrite using as the catalyst copper-coated cadmium. The absorbance at 545 nm was measured using a spectrophotometer and sodium nitrite as the standard.

Thiobarbituric acid-reactive substances (TBARS) reaction equivalent to MDA was measured with a slightly modified method (27). TBARS were determined with a fluorescence spectrofluorometer (Hitachi 650-40; Hitachi, Japan) with 520 nm excitation and 550 nm emission, using 1,1,3,3-tetramethoxypropane as the standard.

Plasma ALT activity was analyzed using a spectrophotometer (automate RA100) and a commercial kit (Thermo Trace Ltd., Melbourne, Australia). Conformance® biochemistry control was used as the standard for this enzyme.

Statistical analysis

Student's *t*-test was used to compare infected and non-infected samples. Results were considered significant at $P < 0.05$.

Results

Timecourse of 8-nitroguanine and 8-oxodG formation and expression of iNOS and PCNA in the liver after repeated infection with O.viverrini

We produced specific anti-8-nitroguanine antibody using 8-nitroguanine–RSA conjugate. A dot immunobinding assay and absorption test showed that purified antibody gave a strong immunoreactivity only with 8-nitroguanine conjugate without cross-reaction with 3-nitrotyrosine, guanosine, 8-oxodG or deoxyguanosine (data not shown).

Figure 1 shows the time profile of 8-oxodG and 8-nitroguanine formation in the liver of hamsters reinfected with *O.viverrini*. 8-OxodG and 8-nitroguanine immunoreactivity

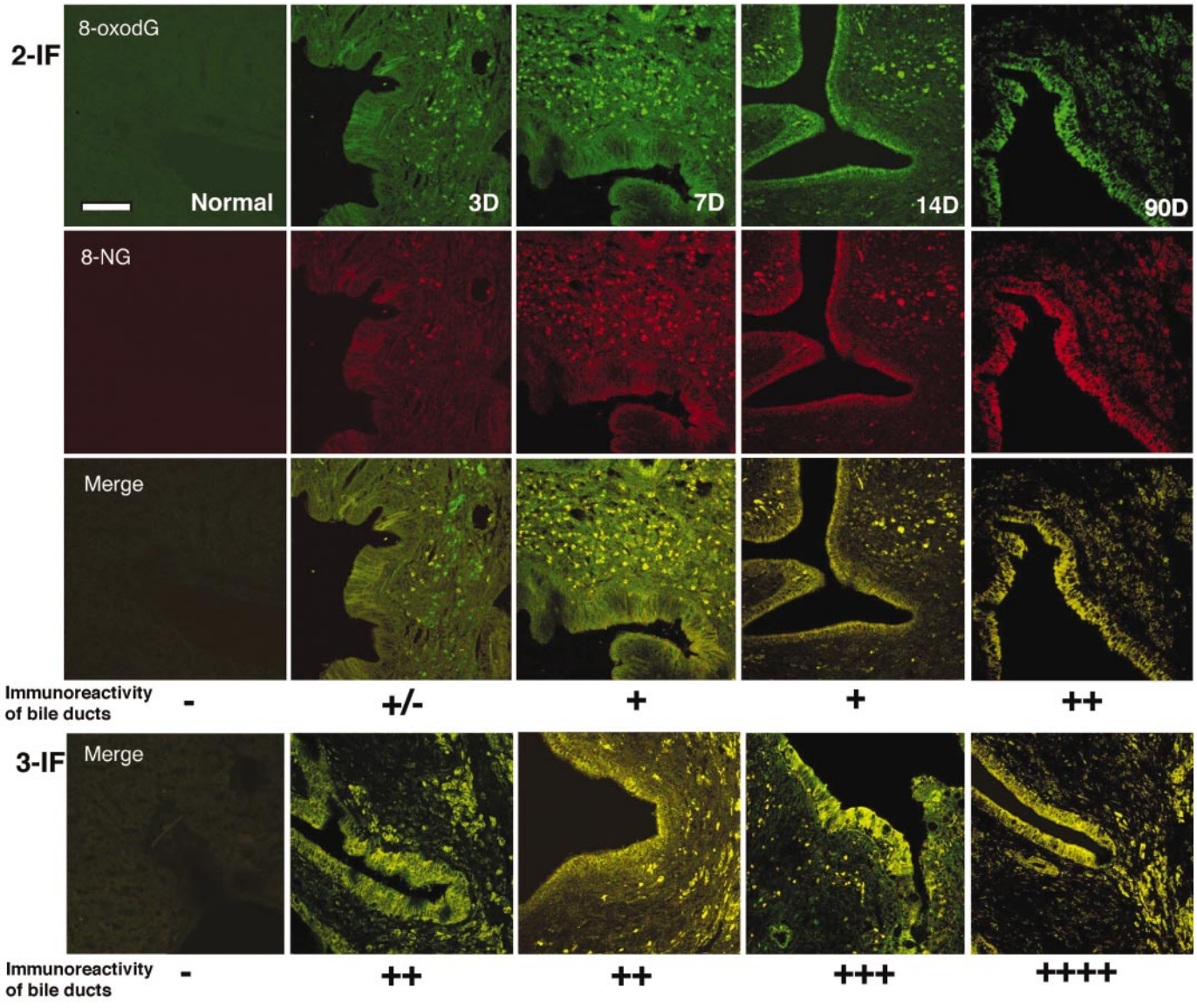

Fig. 1. A time profile of 8-oxodG and 8-nitroguanine formation in the liver of hamsters reinfected with *O.viverrini*. Localization of 8-oxodG and 8-nitroguanine in paraffin liver sections was assessed by double immunofluorescence staining. Immunoreactivity of 8-oxodG and 8-nitroguanine was observed mostly in the same inflammatory cells and the staining intensity was greatest on day 7 in 2-IF and day 3 in 3-IF. 8-OxodG and 8-nitroguanine immunoreactivity remained in the epithelium of the bile duct on day 90. 8-NG, 8-nitroguanine. Bar = 50 μm.

was not observed in the liver of normal hamsters. In the 2-IF and 3-IF groups, both 8-oxodG and 8-nitroguanine formation were mainly observed in the nucleus of the same inflammatory cells and in the cytoplasm of the epithelium of bile ducts. The number of intensely immunoreactive inflammatory cells was highest on day 7 in 2-IF and on day 3 in 3-IF, compared with days 21–30 in 1-IF. 8-OxodG and 8-nitroguanine formation increased time-dependently in the epithelium of bile ducts by day 90 in the 2-IF and 3-IF groups, although the number of inflammatory cells decreased. Immunoreactivity of 8-oxodG and 8-nitroguanine appeared to increase in the epithelium of bile ducts in the order 3-IF > 2-IF > 1-IF.

Figure 2 shows alterations in iNOS expression in the liver of hamsters reinfected with *O.viverrini*. iNOS immunoreactivity was not observed in the liver of normal hamsters. iNOS immunoreactivity in the cytoplasm of inflammatory cells and the epithelium of bile ducts reached its highest intensity on days 30, 7 and 3 in 1-IF, 2-IF and 3-IF, respectively. iNOS immunoreactivity in the epithelium of the bile duct persisted whereas that in inflammatory cells decreased. The intensity of iNOS immunoreactivity increased in the epithelium of bile ducts in the order 3-IF > 2-IF > 1-IF on day 90. Weak iNOS expression was found in hepatocytes (data not shown). PCNA immunoreactivity was not observed in the liver of normal hamsters (Figure 3). The intensity of PCNA immunoreactivity increased in the epithelium of bile ducts in the order 3-IF > 2-IF > 1-IF on day 90 (Figure 3).

Histopathological changes in liver of hamsters reinfected with O.viverrini

Histopathological changes induced by *O.viverrini* reinfection are shown in Figure 4. Hyperplasia of the epithelium of bile ducts and accumulation of inflammatory cells, characteristics of chronic active inflammation, were most frequently observed on days 3–14 in 2-IF and days 3–7 in 3-IF (Figure 4, AC). Inflammatory cells consisted of eosinophils, mononuclear

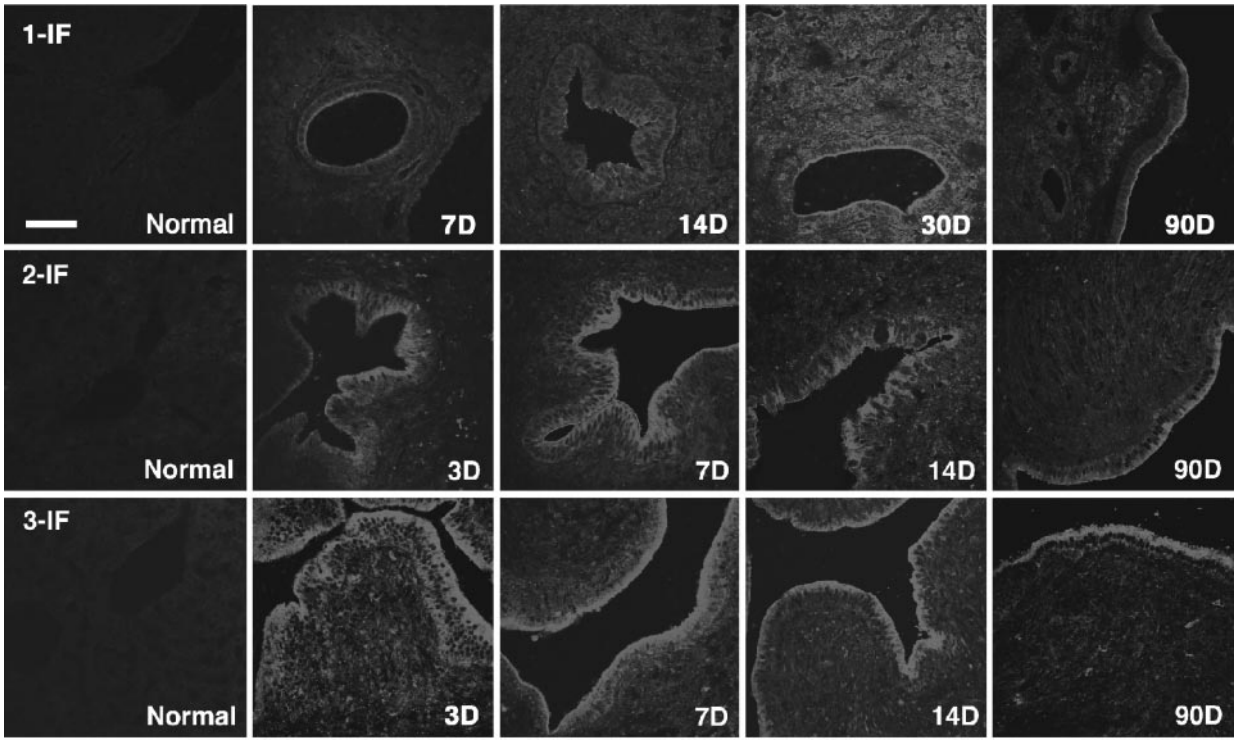

Fig. 2. Alteration of iNOS expression in the liver of hamsters infected with *O.viverrini*. iNOS expression by reinfection with *O.viverrini* was analyzed and compared with single infection (1-IF) using an immunohistochemical technique as described in Materials and methods. Immunoreactivity in inflammatory cells and the epithelium of bile ducts was highest on day 7 in 2-IF and day 3 in 3-IF compared with day 30 in 1-IF. iNOS expression in the epithelium of bile ducts continued until day 90. Bar = 50 μm.

cells (macrophages, lymphocytes and plasma cells) and a few neutrophils (Figure 4, Eo-Mo). These changes were most frequently observed on day 7 in 2-IF (Figure 4, 2-IF, 7D) and day 3 in 3-IF (Figure 4, 3-IF, 3D). In contrast, the decrease in the number of inflammatory cells and less active epithelium with short columnar cells were seen from day 21 in 2-IF and day 14 in 3-IF. In long-term infections (days 30–90) in 2-IF and 3-IF, the decrease in the number of inflammatory cells and periductal fibrosis were observed (Figure 4, LAC). Adenomatous hyperplasia and ductal dilation were also found (data not shown).

The effect of repeated infection with O.viverrini on 8-oxodG formation in the liver

8-OxodG content in the liver of hamsters infected with *O.viverrini* was determined by HPLC-ECD, and the time course is shown in Table I. In 1-IF, the peak 8-oxodG level was observed on day 21. In 2-IF, a significant difference in 8-oxodG formation was found on day 3 between infected and non-infected hamsters ($P < 0.05$). Its peak level was reached on day 7 ($P < 0.01$). 8-OxodG content remained at a higher level than in normal cells but the difference was not significant. In 3-IF, the highest level of 8-oxodG formation was found on day 3 compared with non-infected hamsters ($P < 0.05$). Interestingly, repeated infections induced faster 8-oxodG formation compared with 1-IF.

Alteration of plasma nitrate/nitrite concentration by O.viverrini infection

Table I shows the change in plasma nitrate/nitrite concentration. In 1-IF, the level of nitrate/nitrite peaked on day 30. In 2-IF, plasma nitrate/nitrite level rapidly reached a peak on day 7 ($P < 0.001$). This concentration was sustained at a higher level than that in non-infected animals on days 60–90 ($P < 0.05$). In 3-IF, nitrate/nitrite level was maximal and significantly higher than that in normal hamsters on day 3 ($P < 0.001$). Then the concentration gradually decreased, but was still significantly higher on days 7 and 30 ($P < 0.05$ and 0.001, respectively). The timecourse of plasma nitrate/nitrite level was closely related with that of iNOS immunoreactivity in inflammatory cells.

Change in plasma MDA concentration in hamsters infected with O.viverrini

Table I shows the alterations in plasma MDA concentration. In 1-IF, plasma MDA level was significantly higher than that in normal hamsters from day 3 ($P < 0.05$) and gradually increased. In 2-IF, plasma MDA level was significantly higher than that in normal hamsters and reached a peak on day 7 ($P < 0.01$). This concentration remained significantly higher until day 60 ($P < 0.01$). In 3-IF, plasma MDA level was significantly higher than that in normal hamsters on day 3 ($P < 0.05$). This concentration remained at a higher level than in non-infected hamsters until day 90, although a statistically significant difference was found only on day 30 ($P < 0.01$).

The effect of repeated infection with O.viverrini on ALT activity

Changes in ALT activity in hamsters reinfected with *O.viverrini* are shown in Table I. The level of ALT reached a peak on day 21 in 1-IF. In 2-IF, ALT activity was significantly higher than that in control hamsters from day 3 ($P < 0.05$) and reached a peak on day 14 ($P < 0.001$). This activity remained at a significantly higher level than that in normal hamsters until

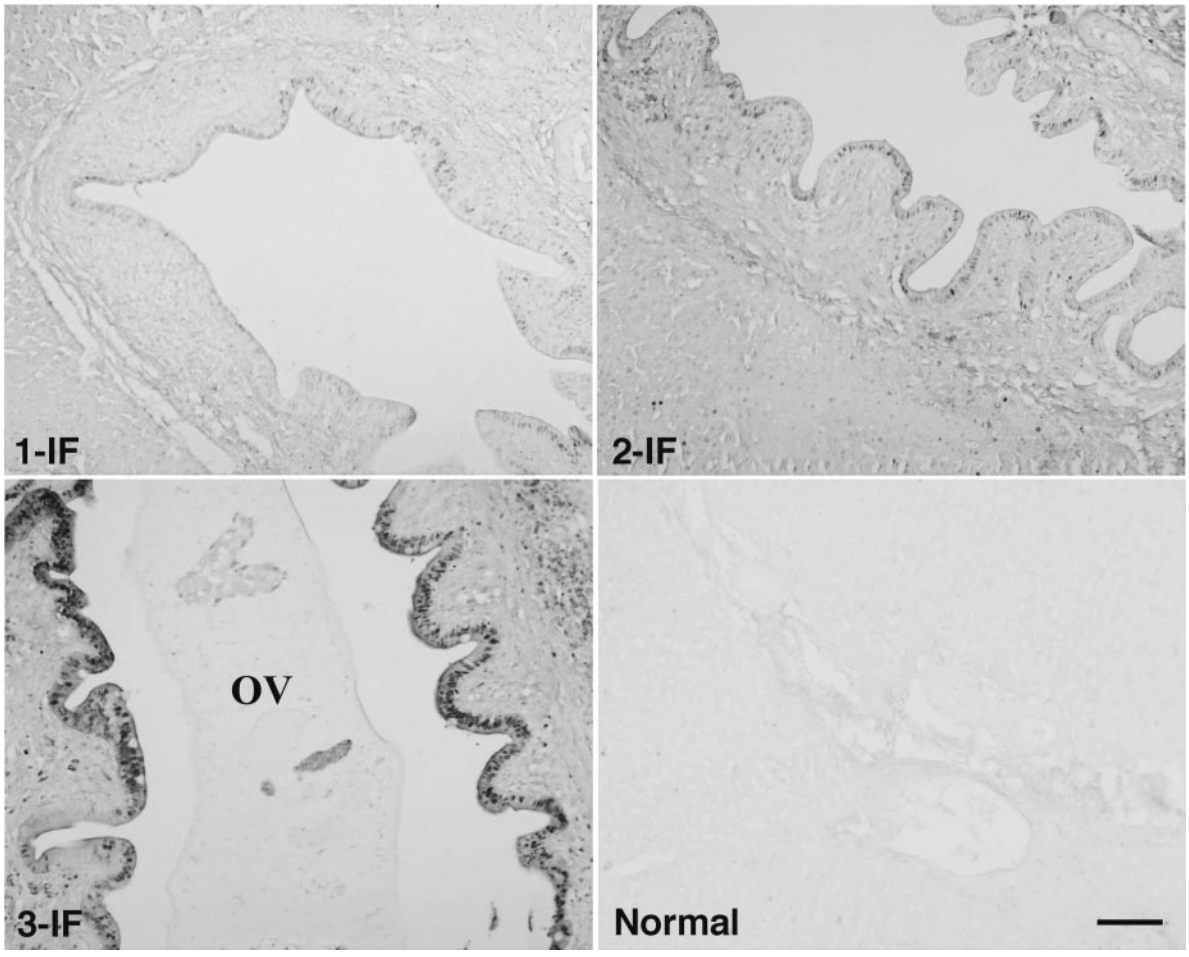

Fig. 3. PCNA accumulation in the liver of hamsters infected with *O.viverrini*. PCNA accumulation due to reinfection with *O.viverrini* was analyzed on day 90 using an immunohistochemical technique as described in Materials and methods. Immunoreactivity of PCNA in the epithelium of bile ducts increased in the order 3-IF > 2-IF > 1-IF. Normal, corresponding control hamsters of 3-IF group. Bar = 100 μm. Original magnification 100×.

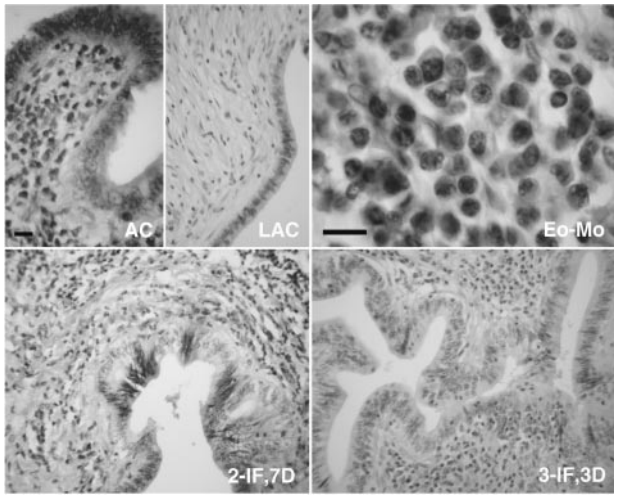

Fig. 4. Histological changes in the liver of hamsters reinfected with *O.viverrini*. In short-term infection, hyperplasia of the epithelium of bile ducts and inflammatory cell infiltration were frequently observed (AC, day 7 in 2-IF). In long-term infection, a decrease in the number of inflammatory cells and periductal fibrosis were observed (LAC, day 90 in 2-IF). Marked accumulation of eosinophils, mononuclear cells and a few neutrophils (Eo-Mo) was observed on day 7 in 2-IF (2-IF, 7D) and day 3 in 3-IF (3-IF, 3D). Short bar = 10 μm for AC, LAC, 2-IF, 7D and 3-IF, 3D; long bar = 10 μm for Eo-Mo.

day 90 ($P < 0.01$). In 3-IF, ALT activity was significantly higher than that in control hamsters from day 3 ($P < 0.01$) and was sustained at a higher level than that of normal hamsters until day 60 ($P < 0.01$).

Alterations in GSH content in liver of hamsters infected with O.viverrini

We examined the changes in GSH content in the liver of hamsters infected with *O.viverrini* by HPLC-ECD. GSH level was significantly increased on day 14 in 2-IF ($P < 0.05$) and 3-IF ($P < 0.01$), whereas in 1-IF it was not. On the other hand, GSH level was significantly decreased on day 21 in 1-IF ($P < 0.05$), although not in 2-IF and 3-IF (data not shown).

Discussion

The present study has clearly demonstrated that repeated infection with *O.viverrini* induced an early inflammatory response in association with expression of iNOS and increased NO production. An earlier response of inflammatory cells due to repeated infection produced a faster increase in oxidative and nitrosative DNA damage and the activity of ALT, which was associated with liver tissue injury. Notably, formation of 8-nitroguanine and 8-oxodG appeared to increase in the epithelium of bile ducts in the order 3-IF > 2-IF > 1-IF

Table I. Amounts of 8-oxodG in the liver and nitrate/nitrite, MDA and ALT in plasma of *O.viverrini*-infected hamsters

Post-infection duration	8-OxodG ($\times 10^{-5}$ dG)		Nitrate/nitrite (μM)		MDA (μM)		ALT (U/l)	
	Infected	Normal	Infected	Normal	Infected	Normal	Infected	Normal
1-IF								
3D	1.65 ± 0.27	1.43 ± 0.15	20.1 ± 7.92	17.8 ± 3.21	0.84 ± 0.13[a]	0.62 ± 0.17	198 ± 22.9	92.8 ± 44.1
7D	1.76 ± 0.62	1.36 ± 0.38	21.3 ± 6.22	18.6 ± 5.02	1.01 ± 0.11[a]	0.70 ± 0.24	132 ± 94.4	87.1 ± 10.8
14D	1.98 ± 0.32[b]	1.33 ± 0.16	28.3 ± 7.94	19.7 ± 6.65	0.97 ± 0.10[a]	0.82 ± 0.13	527 ± 287[c]	97.7 ± 21.5
21D	3.17 ± 1.28[c]	1.44 ± 0.34	26.3 ± 6.88[a]	14.9 ± 8.71	1.12 ± 0.17[a]	0.81 ± 0.15	1188 ± 332[b]	101 ± 27.4
30D	2.31 ± 0.18[b]	1.32 ± 0.34	35.8 ± 3.24[c]	21.2 ± 4.80	1.17 ± 0.09[a]	0.75 ± 0.15	573 ± 161[b]	120 ± 58.1
60D	2.03 ± 0.30[c]	1.57 ± 0.25	22.2 ± 8.41	14.8 ± 3.11	1.18 ± 0.32[a]	0.76 ± 0.04	270 ± 110[b]	52.8 ± 16.1
90D	1.65 ± 0.38	1.26 ± 0.35	28.2 ± 3.09	23.1 ± 9.88	1.20 ± 0.22[a]	0.69 ± 0.15	227 ± 88.2[b]	86.7 ± 21.6
2-IF								
3D	1.20 ± 0.22[a]	0.88 ± 0.21	18.3 ± 9.76	16.3 ± 4.08	0.89 ± 0.27	0.80 ± 0.10	259 ± 215[a]	109 ± 38.9
7D	1.78 ± 0.50[c]	1.03 ± 0.22	30.8 ± 5.72[b]	18.5 ± 4.13	1.18 ± 0.18[c]	0.81 ± 0.15	178 ± 51.3[c]	88.1 ± 37.9
14D	1.51 ± 1.27	1.03 ± 0.18	22.3 ± 5.19	17.7 ± 4.39	0.94 ± 0.15[a]	0.74 ± 0.08	458 ± 161[b]	126 ± 57.8
21D	1.13 ± 0.61	1.06 ± 0.29	25.1 ± 7.22	21.4 ± 3.97	0.99 ± 0.16[c]	0.66 ± 0.14	243 ± 72.1[a]	118 ± 107
30D	1.37 ± 0.26	1.10 ± 0.31	24.4 ± 6.79	22.6 ± 4.88	1.04 ± 0.26[a]	0.78 ± 0.11	197 ± 48.1[b]	66.6 ± 14.9
60D	1.41 ± 0.38	1.08 ± 0.11	16.4 ± 4.09[a]	7.98 ± 1.73	0.83 ± 0.12[c]	0.58 ± 0.10	186 ± 51.2[a]	111 ± 11.4
90D	1.04 ± 0.26	0.99 ± 0.40	20.7 ± 4.65[a]	14.2 ± 1.47	0.94 ± 0.24	0.74 ± 0.16	144 ± 51.2 [c]	81.8 ± 17.7
3-IF								
3D	2.93 ± 1.06[a]	1.68 ± 0.36	27.7 ± 5.80[b]	16.4 ± 3.44	0.80 ± 0.12[a]	0.64 ± 0.12	284 ± 131[c]	77.1 ± 8.81
7D	1.99 ± 0.66	1.63 ± 0.73	20.2 ± 6.48[a]	13.2 ± 3.34	0.75 ± 0.08	0.64 ± 0.16	129 ± 50.2[c]	79.7 ± 6.98
14D	1.63 ± 0.56	1.32 ± 0.21	18.1 ± 5.72	16.4 ± 5.13	1.07 ± 0.37	0.71 ± 0.15	233 ± 129[c]	67.2 ± 23.2
21D	1.53 ± 0.43	1.50 ± 0.39	17.1 ± 6.22	16.9 ± 6.59	0.76 ± 0.15	0.72 ± 0.10	175 ± 96.1[c]	65.5 ± 27.4
30D	1.47 ± 0.43	1.39 ± 0.20	15.1 ± 2.39[b]	9.56 ± 1.32	0.76 ± 0.04[c]	0.60 ± 0.05	147 ± 39.1	115 ± 44.3
60D	1.64 ± 0.39	1.54 ± 0.34	20.9 ± 10.4	20.9 ± 4.72	1.00 ± 0.34	0.77 ± 0.04	173 ± 48.1[c]	96.5 ± 29.8
90D	0.96 ± 0.29	1.22 ± 0.09	16.7 ± 3.58	12.8 ± 6.27	0.86 ± 0.22	0.69 ± 0.22	141 ± 49.6	103 ± 64.4

$n = 7$ for *O.viverrini*-infected hamsters and $n = 6$ for normal hamsters.
[a]$P < 0.05$ compared with normal hamsters.
[b]$P < 0.001$ compared with normal hamsters.
[c]$P < 0.01$ compared with normal hamsters.

after the decrease in inflammatory cells. 8-Nitroguanine and 8-oxodG formation in the epithelium of bile ducts was increased depending on the time and frequency of *O.viverrini* infection (3-IF > 2-IF > 1-IF). On the other hand, HPLC-ECD analysis revealed that 8-oxodG levels in liver did not significantly increase on long-term infection. This difference means that although 8-oxodG formation in bile ducts contributes only a small part to its level in whole liver, oxidative and nitrosative DNA damage in bile duct would contribute to CHCA development. DNA damage may persist during long-term reinfection. This may be explained by the fact that repeated infection increased iNOS expression in the epithelium of bile ducts in the same order on day 90. Repeated infection increased accumulation of oxidative and nitrosative DNA damage via NO production by inflammatory cells and the epithelium of bile ducts.

Experiments using HPLC revealed that the amount of 8-oxodG in the liver was at its highest on day 7 in 2-IF and day 3 in 3-IF, earlier than day 21 in 1-IF. Double immunofluorescence showed that 8-oxodG and 8-nitroguanine formation in inflammatory cells was highest on day 7 in 2-IF and day 3 in 3-IF. In 1-IF, eosinophils predominantly appeared on day 21, followed by accumulation of mononuclear cells on day 30. In 2-IF and 3-IF, these cells appeared on day 7 and day 3, respectively. Faster oxidative and nitrosative DNA damage due to *O.viverrini* reinfection may be explained by the immune response of memory cells via cytokine production. Our result is supported by a recent study that memory cells provide a faster and more effective secondary response against the same pathogen (28).

On the basis of the present results, the potential mechanisms of carcinogenesis induced by reinfection with *O.viverrini* are illustrated in Figure 5. The superoxide anion radical ($O_2^{\bullet -}$) is derived from eosinophils (29) and macrophages (30) and is dismutated to H_2O_2, which induces metal-dependent 8-oxodG formation (31). In addition, the timecourse of iNOS expression in inflammatory cells was closely related to the plasma nitrate/nitrite level. NO reacts with $O_2^{\bullet -}$ to produce $ONOO^-$, which can induce 8-oxodG (32,33) and 8-nitroguanine formation (34,35). The present study has shown that 8-oxodG and 8-nitroguanine were formed mainly in the nucleus, probably in nuclear DNA, in inflammatory cells. This observation is supported by reports that 8-oxoguanine (36) and 8-nitroguanine (35,37) are formed in cellular DNA. 8-OxodG and 8-nitroguanine were also formed in the cytoplasm of the epithelium of bile ducts. This result is supported by the finding that 8-nitroguanine formation could be observed in RNA (36,38) and 8-oxodG was found in mitochondrial DNA (36). It is relevant that formation of 8-oxodG and 8-nitroguanine continued in the epithelium of bile ducts on day 90. iNOS expression in the epithelium of the bile duct remained whereas that in inflammatory cells decreased. Notably, expression of iNOS increased in epithelial cells of bile ducts in the order 3-IF > 2-IF > 1-IF. This result suggests that NO may be mainly derived from iNOS in the epithelium of bile ducts (39), particularly with repeated infections. Moreover, PCNA accumulated in the epithelium of bile ducts, especially in hamsters with repeated *O.viverrini* infections, supporting the hypothesis that *O.viverrini* infection promotes cell proliferation via inflammation-mediated DNA damage. Therefore, reinfection promotes 8-oxodG and 8-nitroguanine formation in the epithelium of bile ducts, which may play a key role in *O.viverrini*-induced CHCA development.

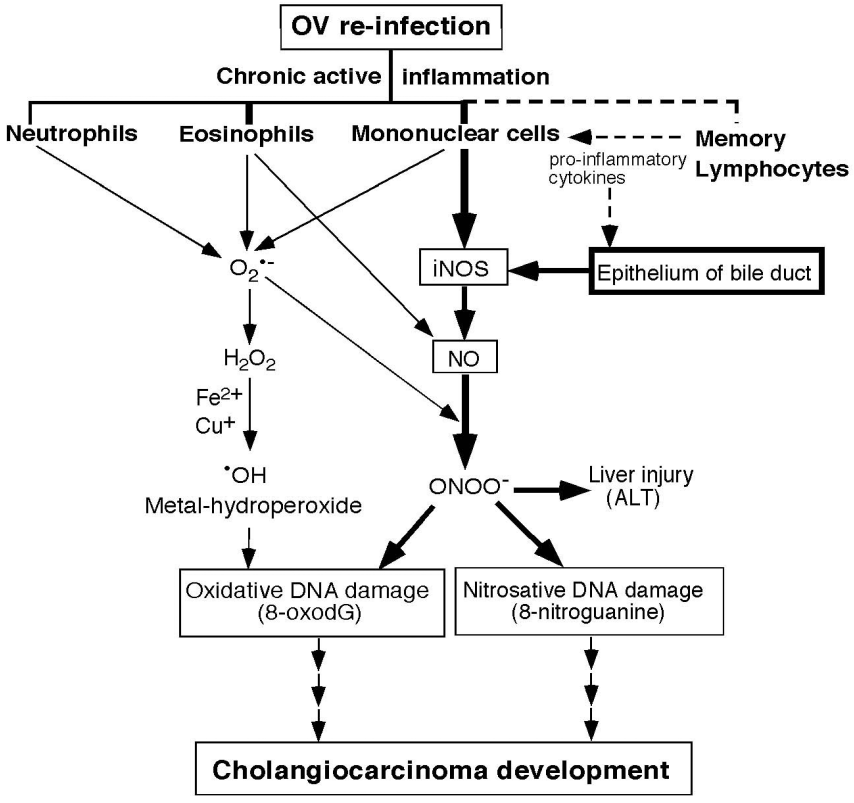

Fig. 5. The potential mechanisms of CHCA development due to reinfection with *O.viverrini* through oxidative and nitrosative DNA damage.

Plasma MDA concentration increased significantly in the 1-IF, 2-IF and 3-IF groups and LPO persisted during long-term infection. It is well known that ROS such as $^{\bullet}OH$ can mediate LPO. In addition, LPO may be induced by NO and its products, such as $ONOO^-$ and nitrogen dioxide (40). Increased LPO can also induce 8-oxodG formation via reaction of LPO products such as lipid hydroperoxides with ferric ion (41). Besides direct oxidative DNA damage, LPO could lead to the formation of etheno DNA adducts via aldehyde (42). Therefore, LPO may contribute to the initiation of carcinogenesis in liver fluke-associated cancer.

Interestingly, the amount of GSH increased on day 14 in 2-IF and 3-IF, although it decreased on day 21 in 1-IF. This result leads to the idea that repeated infection induces GSH synthesis, a member of an antioxidant defence system. This idea is supported by a report showing increased GSH level and γ-glutamyl cysteine synthetase expression on NO induction in rat fibroblasts (43). Of relevance, an antigen of a certain parasite can both induce NO production and increase GSH level (44). Our hypothesis is supported by several papers showing that an increase in GSH content occurs in tumor cells (45).

The present study has demonstrated that in repeated infections, oxidative and nitrosative DNA damage occurred faster than in a single infection. Furthermore, infection-associated carcinogenesis through chronic inflammation has been observed in hepatocytes of transgenic mice (4) and humans (46) with chronic active hepatitis destined to develop hepatocellular carcinoma. Our experimental conditions are considered to be similar to humans in north-east Thailand. The *O.viverrini*-infected hamsters used in this study are suitable models for humans experiencing repeated *O.viverrini* infection. We observed marked periductal fibrosis in the long-term reinfection. Similar pathological features were observed in a human study in endemic areas where reinfection is common (6). Repeated infection may be a risk factor for CHCA development. A majority (70%) of CHCA occur in intrahepatic bile ducts in *O.viverrini*-infected patients (9). This can be explained by our findings that 8-oxodG and 8-nitroguanine were formed in intrahepatic bile ducts in *O.viverrini*-infected hamsters. In conclusion, oxidative and nitrosative DNA damage induced by repeated infection through chronic active inflammation may play an important role in the initiation and promotion steps of CHCA in communities where *O.viverrini* reinfection is frequent.

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