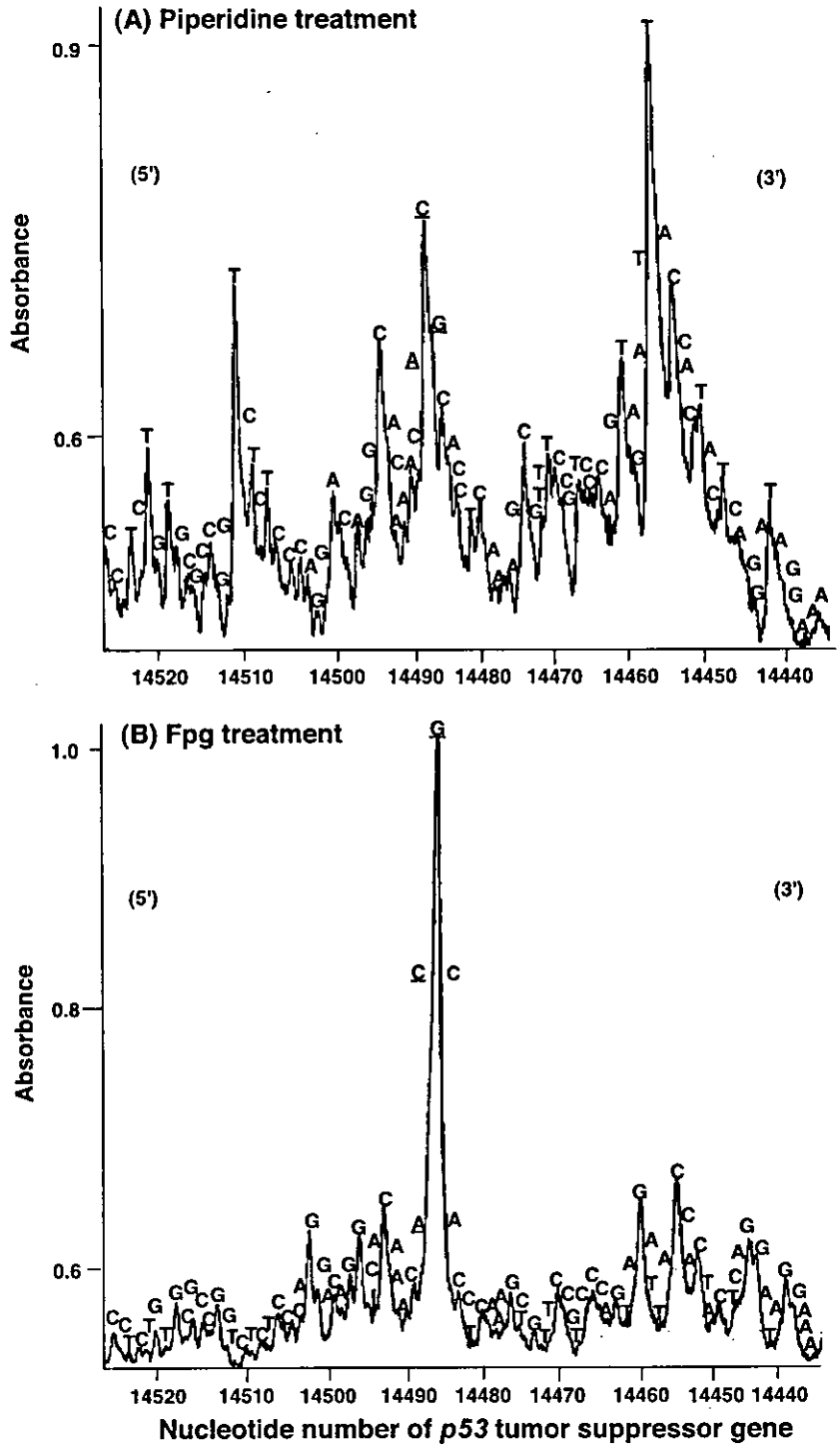

Fig. 6. Site specificity of Cu(II)-mediated DNA cleavage induced by 4-HC in the presence of NADH. Reaction mixtures contained ^{32}P-5'-end-labeled 443 bp fragment (*ApaI* 14179–*Eco*RI*14621) of the *p53* tumor suppressor gene, 5 μM per base calf thymus DNA, 10 μM 4-HC, 200 μM NADH, and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The reaction mixtures were incubated at 37°C for 1 h. The mixture was followed by (A) piperidine treatment or (B) Fpg treatment. After each treatment, the DNA fragments were analyzed as described in the legend to Fig. 4. The relative amounts of oligonucleotide were measured by scanning the autoradiogram with a laser densitometer. The horizontal axis shows the nucleotide number of the human *p53* tumor suppressor gene and underscoring shows complementary sequence to codon 273 (nucleotides 14486–14488).

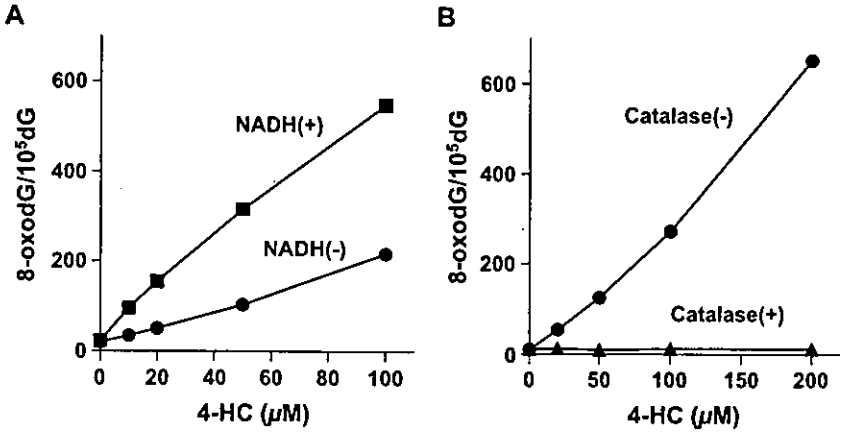

Fig. 7. Cu(II)-mediated formation of 8-oxodG in calf thymus DNA by 4-HC in the presence and absence of NADH and the effect of catalase. Calf thymus DNA (100 μM per base) was incubated with the indicated concentrations of 4-HC and 20 μM $CuCl_2$ in the presence and absence of (A) 200 μM NADH or (B) 30 units of catalase at 37°C for 30 min. After ethanol precipitation, DNA was enzymatically digested to the component nucleosides and analyzed with an HPLC-ECD. The symbols are as follows: ■, 4-HC + Cu(II) + NADH; ●, 4-HC + Cu(II); ▲, 4-HC + Cu(II) + catalase.

be explained by the fact that CP requires bioactivation through cytochromes P450-catalyzed metabolism to exhibit its cytotoxic activity [11,33].

To confirm the mechanism of DNA damage through H_2O_2 generation, we investigated the effects of various scavengers on DNA damage using ^{32}P-labeled DNA fragments obtained from the human *p53* tumor suppressor gene and the c-Ha-*ras*-1 proto-oncogene. The inhibitory effects of catalase and bathocuproine on DNA damage suggest that H_2O_2 and Cu(I) are required for the DNA damage. Typical free ·OH scavengers did not protect DNA but methional did, because methional can scavenge not only ·OH but also species like Cu(I)OOH that are less reactive than ·OH [35]. In the absence of Cu(II), DNA damage was not observed. These results suggest that reactive species is a complex of H_2O_2 and Cu(I) derived from Cu(II). The noninhibitory effect of ·OH scavengers can be explained by assuming that DNA damage is induced by ·OH generated by a metal ion bound in very close proximity to the nucleic acid. Furthermore, because of site-specific binding of copper ions to DNA, it might be expected that site-specific DNA damage is mediated by ·OH generated by bound copper.

In this study, we showed that 4-HC caused site-specific DNA damage in the presence of Cu(II). 4-HC induced piperidine-labile sites frequently at thymine and cytosine residues. When DNA fragments were treated with Fpg protein, 4-HC caused DNA damage mainly at guanine residues. Fpg protein mainly catalyzes the excision of piperidine-resistant 8-oxodG [21] and further oxidizes piperidine-labile guanine residues [22]. In addition, experiments with piperidine and Fpg treatment revealed

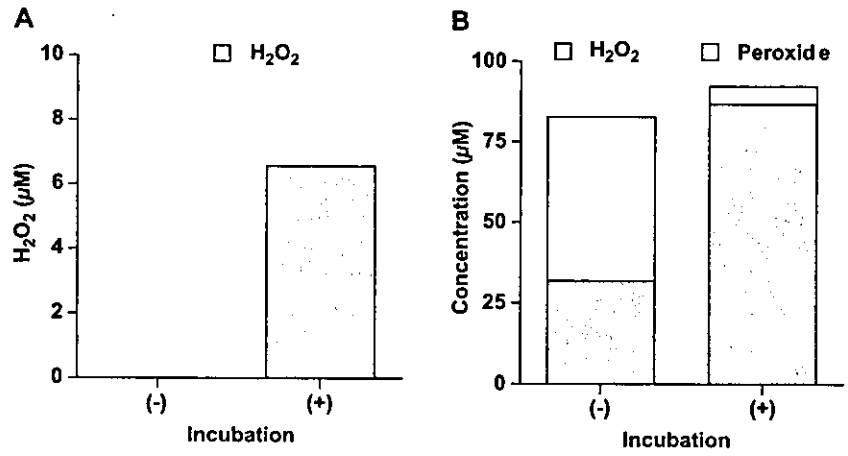

Fig. 8. The amounts of peroxide and H_2O_2 generation from 4-HC. The amounts of H_2O_2 and peroxide were measured by using (A) scopoletin and (B) iodometric procedure. The reaction mixture contained (A) 10 μM or (B) 100 μM 4-HC, in 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The effects of incubation at 37°C for 1 h on the amount of H_2O_2 and peroxide were investigated as described under Materials and Methods.

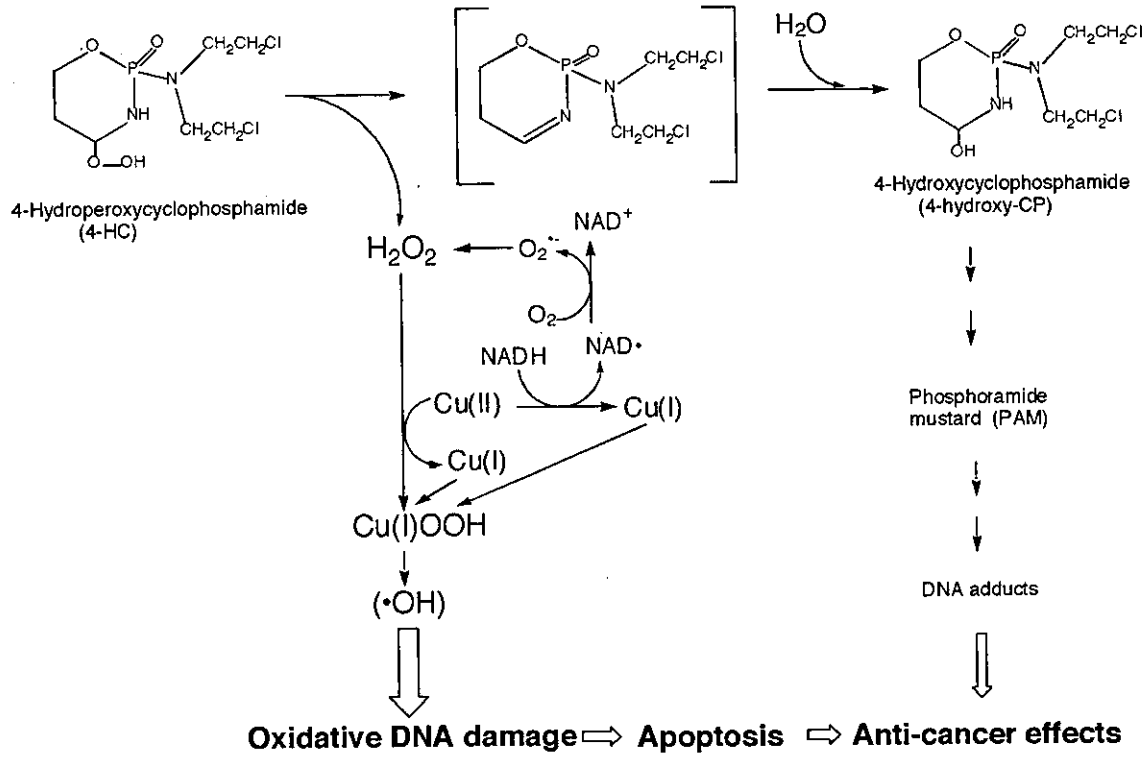

Fig. 9. A proposed mechanism for the generation of H_2O_2 and DNA damage by 4-HC.

that C and G of the 5′-ACG-3′ sequence, the complementary sequence to codon 273 (a known hot spot) in exon 8 of the *p53* gene [28,29], were significantly damaged.

A possibility of metal-mediated conversion of 4-HC to the corresponding peroxy radical causing DNA damage was denied, because DNA damage and 8-oxodG formation were notably suppressed in the presence of catalase. The degradation of 4-HC and H_2O_2 generation were ascertained in the present study. On the basis of our results and the literature [33], the possible mechanisms of H_2O_2 generation and DNA damage due to 4-HC have been proposed as follows (Fig. 9). We consider that 4-HC is converted to imino-CP as an intermediate via elimination of H_2O_2, as pointed out by Borch et al. [36]. We previously reported that H_2O_2 reacts with Cu(II) [20], then the reaction between generated Cu(I) and H_2O_2 yields Cu(I)OOH, which can damage DNA. The oxidative DNA damage induced by 4-HC was markedly enhanced by the addition of NADH. On the basis of our reports [37,38] and the literature [39–41], this enhancement can be explained as follows: NADH derives one-electron reduction of Cu(II) to give Cu(I) with concomitant formation of NAD$^\bullet$. NAD$^\bullet$ reacts with O_2 to form NAD^+ and superoxide ($O_2^{\bullet -}$) at almost diffusion-controlled rates [42]. Then, $O_2^{\bullet -}$ is dismutated to H_2O_2, resulting in enhancement of DNA damage. The biological importance of NADH as a nuclear reductant has been described [43]. The concentration of NADH in certain tissue was estimated to be as high as 100–200 μM [44]. Thus, the NADH-dependent H_2O_2 generation by 4-HC may enhance oxidative DNA damage in cells. In addition to H_2O_2 generation, 4-HC may yield PAM, which is known to adduct to DNA including the N7 position of guanine. Some of the adducts undergo ring opening, resulting in formation of phosphoramide mustard–imidazole ring-opened deoxyguanosine, formamidopyrimidine (Fapy) derivatives. The Fapy derivatives are also good substrates for Fpg protein [45]. However, we could not detect Fpg-sensitive sites when ^{32}P-labeled DNA was treated with 4-HC and Cu(II) in the presence of catalase under the conditions used (data not shown). Maccubbin et al. [46] reported that the adduct occurred at a level of $1/10^5$ nucleotides in calf thymus DNA treated with metabolically activated CP (200 μM). We showed Cu(II)-mediated 8-oxodG formation at the level of $200/10^5$ dG in calf thymus DNA treated with 100 μM 4-HC. The yield of oxidative DNA damage was much higher than that of DNA adduct formation. PAM-induced Fapy lesions may have been formed with a frequency not sufficient to be detected in our system. 4-HC can induce more efficiently oxidative DNA damage through H_2O_2 generation than DNA alkylation by PAM.

The concentration of 4-HC commonly used for purging is 60 μg/ml [47]. In this study, we used equal or lower concentrations of 4-HC. Therefore, in addition to the previously reported cross-linking, the oxidative DNA

damage may play a role in the anti-tumor effects of 4-HC. Greater effectiveness of 4-HC compared with CP has been thought to be due to the excretion of CP before metabolic activation [11]. In addition, we consider that oxidative DNA damage by 4-HC through H_2O_2 generation may contribute to the superiority of 4-HC's anti-tumor effect compared to CP.

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ABBREVIATIONS

CP — cyclophosphamide
DTPA — diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid
Fapy — formamidopyrimidine
Fpg — formamidopyrimidine-DNA glycosylase
4-HC — 4-hydroperoxycyclophosphamide
HPLC-ECD — high-pressure liquid chromatography coupled with an electrochemical detector
NADH — β-nicotinamide adenine dinucleotide (reduced form)
$O_2^{\bullet -}$ — superoxide anion radical
$^{\bullet}OH$ — hydroxyl radical
8-oxodG — 8-oxo-7,8-dihydro-2′-deoxyguanosine
PAM — phosphoramide mustard
SOD — superoxide dismutase

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Accumulation of 8-nitroguanine in human gastric epithelium induced by *Helicobacter pylori* infection

Ning Ma,[a] Yukihiko Adachi,[b] Yusuke Hiraku,[c] Noriyuki Horiki,[b] Shinichirou Horiike,[b] Ichiro Imoto,[d] Somchai Pinlaor,[c] Mariko Murata,[c] Reiji Semba,[a] and Shosuke Kawanishi[c,*]

[a] *Department of Anatomy, Mie University School of Medicine, Tsu, Mie 514-8507, Japan*
[b] *Department of Internal Medicine, Mie University School of Medicine, Tsu, Mie 514-8507, Japan*
[c] *Department of Environmental and Molecular Medicine, Mie University School of Medicine, Tsu, Mie 514-8507, Japan*
[d] *Department of Endoscopic Medicine, Mie University School of Medicine, Tsu, Mie 514-8507, Japan*

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Abstract

Helicobacter pylori infection causes chronic inflammation, which can lead to gastric carcinoma. A double immunofluorescence labeling study demonstrated that the level of 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) apparent in gastric gland epithelium was significantly higher in gastritis patients with *H. pylori* infection than in those without infection. A significant accumulation of proliferating cell nuclear antigen, a prognostic factor for gastric cancer, was observed in gastric gland epithelial cells in patients with *H. pylori* infection as compared to those without infection, and its accumulation was closely correlated with the formation of 8-nitroguanine and 8-oxodG. These results suggest that nitrosative and oxidative DNA damage in gastric epithelial cells and their proliferation by *H. pylori* infection may lead to gastric carcinoma. 8-Nitroguanine could be not only a promising biomarker for inflammation but also a useful indicator of the risk of gastric cancer development in response to chronic *H. pylori* infection.

Keywords: *Helicobacter pylori*; Gastric cancer; DNA damage; 8-Nitroguanine; 8-Oxo-7,8-dihydro-2′-deoxyguanosine; Proliferating cell nuclear antigen; Gastric gland epithelial cell; Infection; Inflammation; Double immunofluorescence labeling

Helicobacter pylori (*H. pylori*) infection, which is the major cause of atrophic gastritis, is a high risk factor for gastric carcinoma [1]. Recent studies have provided evidence that inflammation plays a role in the pathogenesis of cancer [2]. Reactive oxygen species (ROS) and reactive nitrogen species generated by inflammatory cells may contribute to carcinogenesis through the formation of DNA base lesions, such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which can lead to a G:C-to-T:A transversion [3]. 8-oxodG, a marker of oxidative DNA damage, is found at a significantly increased level in the gastric epithelium of *H. pylori*-infected patients [4,5]. Recently, we have reported that 8-nitroguanine is formed in hamster livers in response to an inflammatory reaction caused by a parasitic infection in association with cholangiocarcinoma development [6]. Therefore, in addition to 8-oxodG formation, the accumulation of 8-nitroguanine may play a key role in the initiation and/or promotion of inflammation-mediated carcinogenesis. However, whether 8-nitroguanine is formed in the gastric epithelium of patients with gastric carcinoma that are infected with *H. pylori* has yet to be determined.

To evaluate whether nitrosative DNA damage plays a role early in the carcinogenic process triggered by *H. pylori*, we used a double-immunofluorescent staining procedure to compare the formation of both 8-nitroguanine and 8-oxodG in the gastric epithelium of gastritis patients with and without *H. pylori* infection. Proliferating cell nuclear antigen (PCNA), which

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

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functions as a cofactor for DNA polymerase δ, is associated with DNA replication [7]. As Schipper et al. [7] reviewed, several studies have demonstrated that PCNA is an independent prognostic factor for gastric cancer in patients with *H. pylori* infection. To evaluate the proliferating activity of gastric epithelial cells in patients infected with *H. pylori*, we examined accumulation of PCNA by immunohistochemical technique.

Materials and methods

Patients. We tested eight gastritis patients with *H. pylori* infection that was confirmed by histology, bacterial culture, and a rapid urease test (five men and three women; mean age, 58.1 ± 15.1 years), and eight gastritis patients without *H. pylori* infection (five men and three women; mean age, 44.9 ± 15.7 years). There was no significant difference in the age or sex ratio of the patients with or without *H. pylori* infection. The permission No. 300 was obtained from the Ethics Committee of the Mie University School of Medicine (9 May, 2002). Informed consent was obtained from each patient. Four biopsy samples, consisting of two antrum and two corpus samples, were taken from each patient.

Production of anti-8-nitroguanine antibody. A polyclonal antibody raised against 8-nitroguanine was produced using a modified method [8]. 8-Nitroguanosine was incubated with sodium metaperiodate for 20 min at room temperature and was then allowed to conjugate to rabbit serum albumin (RSA) for 1 h, followed by a 1-h incubation with sodium borohydride. The conjugate was dialyzed overnight against 150 mM NaCl. The resulting product, 8-nitroguanine-aldehyde-RSA, along with Freund's complete adjuvant was injected in rabbit by intracutaneous administration. Four weeks after the initial immunization, the same antigen was again administered, followed by serum collection after subsequent ten days. The antibody was purified using an 8-nitroguanine conjugated column. Specificity of the purified antibody was evaluated using a dot immunobinding assay and an absorption test [9,10].

Immunohistochemical study. 8-Nitroguanine and 8-oxodG immunoreactivity in the gastric epithelium was assessed by a double immunofluorescence labeling study as previously described [11]. Briefly, paraffin sections (6-μm thickness) were incubated with a rabbit polyclonal anti-8-nitroguanine antibody (2 μg/ml) and a mouse monoclonal anti-8-oxodG antibody (5 μg/ml, Japan Institute for the Control of Aging, Fukuroi, Japan) overnight at room temperature. Next, the sections were incubated for 3 h with an Alexa 594-labeled goat antibody against rabbit IgG and an Alexa 488-labeled goat antibody against mouse IgG (1:400) (Molecular Probes, Eugene, Oregon, USA). The stained sections were examined under an inverted Laser Scan Microscope (LSM 410, Zeiss, Gottingen, Germany).

The accumulation of PCNA was also assessed by immunohistochemistry. Briefly, paraffin sections (6-μm thickness) were incubated overnight at room temperature with a mouse monoclonal anti-PCNA antibody (1:100; Novocastra Laboratories, Newcastle, United Kingdom). Then, the sections were incubated with a goat anti-mouse IgG conjugate to HRP (1:200). Sections were visualized with 3,3-diaminobenzidine tetrahydrochloride as the chromogen.

Sections were blindly evaluated by two anatomists (M.D., Ph.D.) who were unaware of the clinical status of each patient.

Statistical analysis. Comparisons between the data sets were made using the χ^2 test. Spearman's rank correlation coefficient was used to analyze the relative correlation of the qualitative data. Quantitative data from the two groups were compared using the Student's t test. $P < 0.05$ was considered to be statistically significant.

Results

Formation of 8-nitroguanine and 8-oxodG in gastric epithelium

The formation of 8-nitroguanine and 8-oxodG in gastric epithelium in gastritis patients with *H. pylori* infection is shown in Fig. 1. Notably, intense immunoreactivity of both compounds was observed to colocalize in gastric gland epithelial cells in patients with *H. pylori* infection (Fig. 1, *HP*(+)). On the other hand, in gastritis patients without *H. pylori* infection, little or no immunoreactivity was observed in gastric gland epithelial cells (Fig. 1, *HP*(−)). 8-Nitroguanine formation was observed in both the nuclei and the cytoplasm of the labeled epithelial cells, suggesting that it can form in both DNA and RNA. The 8-oxodG immunoreactivity was coincident with that of 8-nitroguanine within the nuclei of gastric gland cells and surface epithelial cells in *H. pylori*-infected patients (Fig. 1, merged labeling in yellow). Regardless of the *H. pylori* infection status, immunoreactivity of 8-oxodG and 8-nitroguanine was observed in inflammatory cells.

Accumulation of PCNA in gastric epithelium

PCNA accumulation in gastric gland epithelial cells is shown in Fig. 2. PCNA immunoreactivity was not observed in the gastric epithelium of patients without *H. pylori* infection. In the *H. pylori*-infected patients, intense PCNA immunoreactivity was observed in the nucleus of gastric gland epithelial cells.

Relationship between the accumulation of 8-nitroguanine, 8-oxodG, and PCNA in patients with H. pylori infection

We tabulated the number of patients with and without *H. pylori* infection that exhibited immunoreactivity to 8-nitroguanine, 8-oxodG, and PCNA (Table 1). The level of immunoreactivity of 8-nitroguanine, 8-oxodG, and PCNA in patients with *H. pylori* infection was significantly ($P < 0.01$) higher than that observed in patients without infection. The formation of 8-nitroguanine ($R = 0.848$, $P < 0.001$ in corpus and $R = 0.893$, $P < 0.001$ in antrum) and 8-oxodG ($R = 0.882$, $P < 0.001$ in corpus and $R = 0.907$, $P < 0.001$ in antrum) was significantly correlated with PCNA accumulation in the epithelium. 8-Nitroguanine formation was significantly correlated with 8-oxodG formation ($R = 0.976$, $P < 0.001$ in corpus and $R = 0.958$, $P < 0.001$ in antrum).

Discussion

In this study, we have first demonstrated that *H. pylori* infection can promote 8-nitroguanine formation

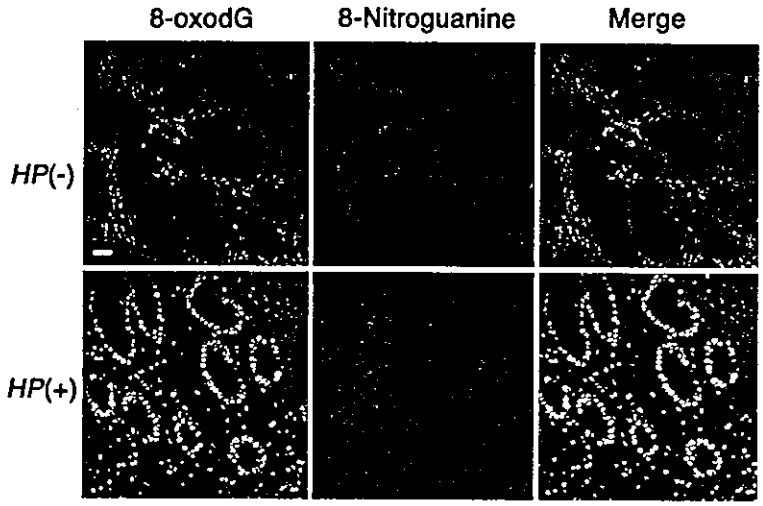

Fig. 1. 8-oxodG and 8-nitroguanine formation in gastritis patients with and without *H. pylori* infection. Double immunofluorescence staining of paraffin sections shows the localization of 8-oxodG and 8-nitroguanine in the gastric epithelium. In *H. pylori*-infected patients (*HP*(+)), the immunoreactivity of 8-oxodG and 8-nitroguanine colocalizes primarily in the nuclei of gastric gland epithelial cells and in some inflammatory cells in the corpus (Merge). In chronic gastritis patients without *H. pylori*-infection (*HP*(−)), the immunoreactivity of 8-oxodG and 8-nitroguanine is observed mainly in the inflammatory cells, while the gastric gland epithelial cells displayed little or no immunoreactivity. Scale bar represents 50 μm.

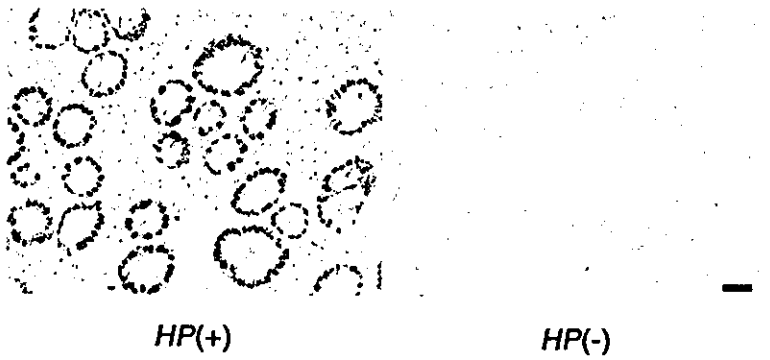

Fig. 2. PCNA accumulation in gastric gland epithelial cells of patients with and without *H. pylori* infection. PCNA accumulation was analyzed by immunohistochemistry with an HRP-conjugated antibody. Intense immunoreactivity of PCNA is observed in the gastric epithelium in the corpus of patients with *H. pylori* infection. Scale bar represents 50 μm.

in human gastric epithelium in addition to 8-oxodG formation. A high level of 8-oxodG and 8-nitroguanine formation was observed, particularly in the gastric gland epithelial cells of patients with *H. pylori* infection; this phenomenon was associated with the persistence of inflammatory cell infiltration. The accumulation of DNA damage in human gastric epithelium induced by *H. pylori* infection can be reasonably explained by the following mechanisms. Neutrophils, the first line of defense against *H. pylori* infection, are not able to kill the bacteria living in the gastric mucus, and persistent *H. pylori* infection will then promote additional infiltration by other inflammatory cells, including macrophages. In patients with *H. pylori*-induced gastritis or gastric ulcers, inducible nitric oxide synthase (iNOS) is expressed in the infiltrating inflammatory cells [12]. Sustained generation of ROS and NO derived from these inflammatory cells due to iNOS expression may contribute to 8-oxodG and 8-nitroguanine formation in the surrounding gastric gland epithelial cells [13]. However, inflammatory cells can also infiltrate into mucosa even in *H. pylori*-negative gastritis. Therefore, *H. pylori* infection must have some additional effects on the host to induce the observed higher levels of 8-oxodG and 8-nitroguanine formation. Fu et al. [14] demonstrated that the expression of iNOS mRNA was significantly increased in *H. pylori*-positive compared to *H. pylori*-negative gastritis, and that the iNOS protein could be detected at a higher level in the epithelial cells of *H. pylori*-positive gastritis patients. We have previously demonstrated that repeated parasitic infections can induce stronger iNOS expression in the epithelium of a target organ in an animal model, probably through cytokine production [9]. There are some reports suggesting that specific cytokines, such as TNF-α [15] and IL-8 [16], induced iNOS. The host immune response to *H. pylori* mediated by cytokines, and the

Table 1
Relationship of 8-nitroguanine, 8-oxodG, and PCNA in gastric epithelium of patients with and without *H. pylori* infection

Area	Immunoreactivity	8-Nitroguanine		8-oxodG		PCNA	
		H. pylori		*H. pylori*		*H. pylori*	
		(−)	(+)	(−)	(+)	(−)	(+)
Corpus	−	7	0	8	0	6	0
	+	1	0	0	0	2	0
	++	0	5	0	5	0	6
	+++	0	3	0	3	0	2
		$P = 0.00113$		$P = 0.00034$		$P = 0.00113$	
Antrum	−	8	0	8	0	4	0
	+	0	0	0	0	4	0
	++	0	2	0	4	0	2
	+++	0	6	0	4	0	6
		$P = 0.00034$		$P = 0.00034$		$P = 0.00113$	
Correlation with PCNA							
Corpus		$R = 0.848$		$R = 0.882$			
		$P = 0.00003$		$P = 0.00001$			
Antrum		$R = 0.893$		$R = 0.907$			
		$P = 0.00000$		$P = 0.00000$			

subsequent iNOS expression, may lead to an increase in the accumulation of 8-nitroguanine and 8-oxodG in epithelium.

The formation of 8-oxodG is known to cause a G → T transversion, which may promote carcinogenesis [17,18]. 8-Nitroguanine undergoes spontaneous depurination, which leads to apurinic sites in DNA [19]. The resulting apurinic sites in DNA can also lead to G → T transversions [20]. The mutation frequency in the gastric gland was 4-fold higher in mice infected with *H. pylori* than in uninfected mice, and it was associated with a high frequency of G → T transversions [21]. Because 8-nitroguanine can also cause G → T transversions, it may contribute to *H. pylori*-induced carcinogenesis in addition to 8-oxodG. This study also showed that PCNA accumulates in gastric epithelial cells in patients with *H. pylori* infection, suggesting an increased proliferation rate of these cells [7]. This observation accords with a previous study which highlighted the importance of an increased proliferation rate in gastric carcinogenesis in response to *H. pylori* infection [22]. Ongoing tissue repair in response to tissue damage, as well as the secretion of cytokines such as growth factors, both stimulate epithelial cell proliferation. Since rapid DNA replication allows the accumulation of potential oncogenic mutations [23], *H. pylori* infection-mediated DNA damage and epithelial cell proliferation may promote gastric carcinogenesis.

3-Nitrotyrosine is a well-known marker of inflammation [24]. 8-oxodG is a representative marker of oxidative DNA damage caused by oxidative stress, including inflammation, mitochondrial respiratory chain reactions, and several $O_2^{\cdot-}$-generating enzymes. We have shown that 8-nitroguanine formation is significantly correlated with PCNA expression. In conclusion, 8-nitroguanine could be not only a promising biomarker for inflammation but also a useful indicator of the risk of gastric cancer development induced by chronic *H. pylori* infection.

Acknowledgments

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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

Somchai Pinlaor[1,3,6], Ning Ma[2], Yusuke Hiraku[1], Puangrat Yongvanit[3,6], Reiji Semba[2], Shinji Oikawa[1], Mariko Murata[1], Banchob Sripa[4,6], Paiboon Sithithaworn[5,6] and Shosuke Kawanishi[1,7]

[1]Department of Environmental and Molecular Medicine and [2]Department of Anatomy, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan and [3]Department of Biochemistry, [4]Department of Pathology, [5]Department of Parasitology and [6]Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

[7]To whom correspondence should be addressed. Tel: +81 59 231 5011; Fax: +81 59 231 5011;
Email address: kawanisi@doc.medic.mie-u.ac.jp

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

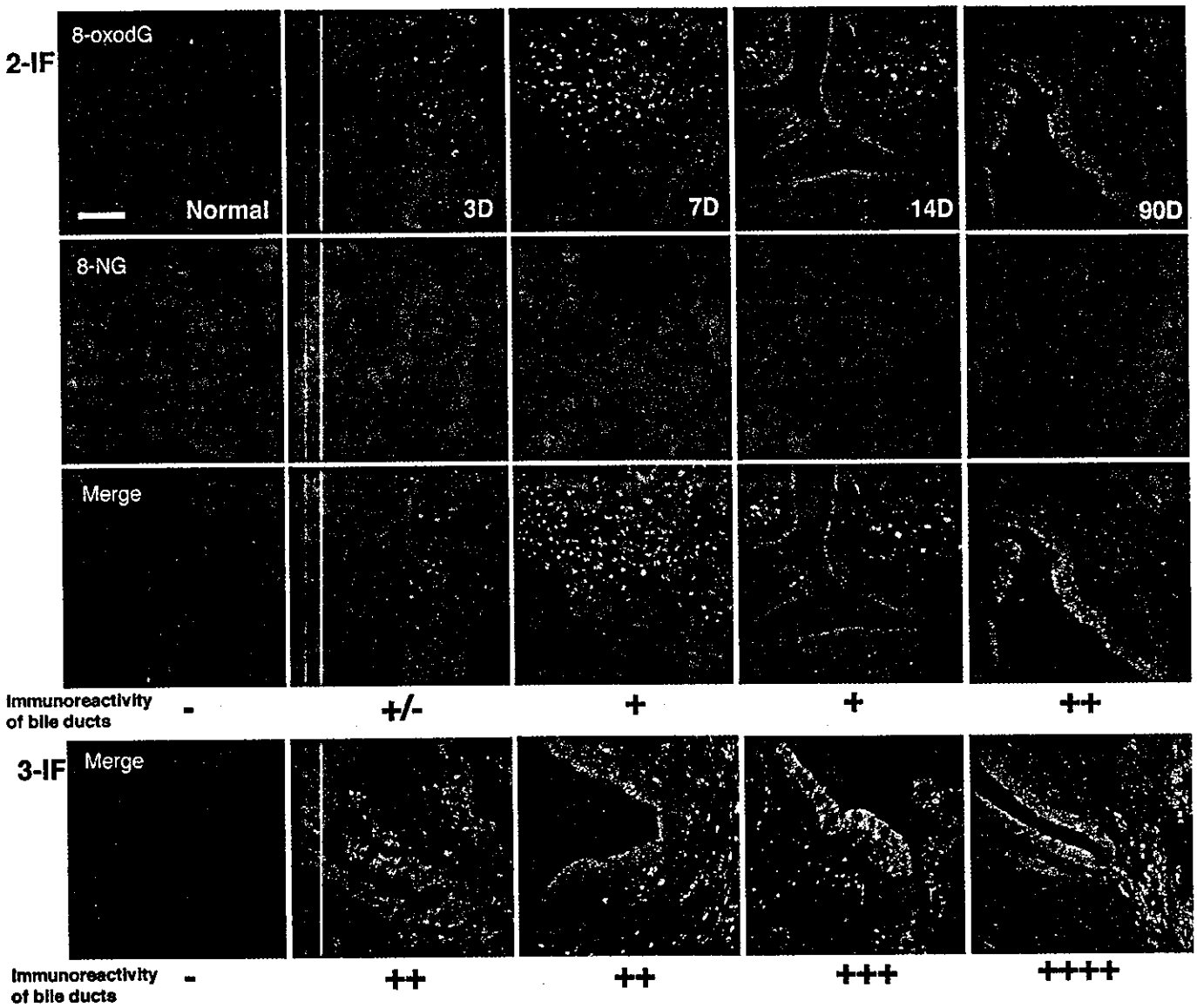

Fig. 1. A time profile of 8-oxodG and 8-nitroguanine formation in the liver of hamsters reinfected with *O.viverrini*. Localization of 8-oxodG and 8-nitroguanine in paraffin liver sections was assessed by double immunofluorescence staining. Immunoreactivity of 8-oxodG and 8-nitroguanine was observed 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

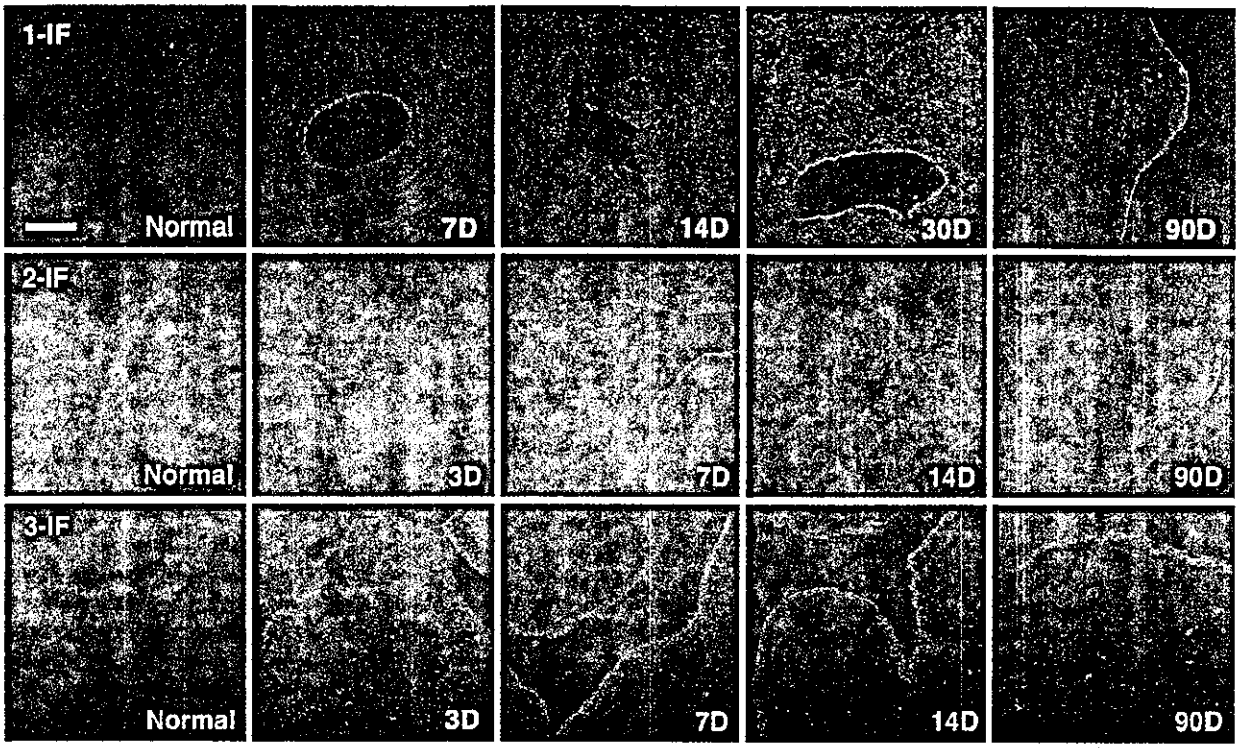

Fig. 2. Alteration of iNOS expression in the liver of hamsters infected with *O.viverrini*. iNOS expression by reinfection with *O.viverrini* was analyzed and compared with single infection (1-IF) using an immunohistochemical technique as described in Materials and methods. Immunoreactivity in inflammatory 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

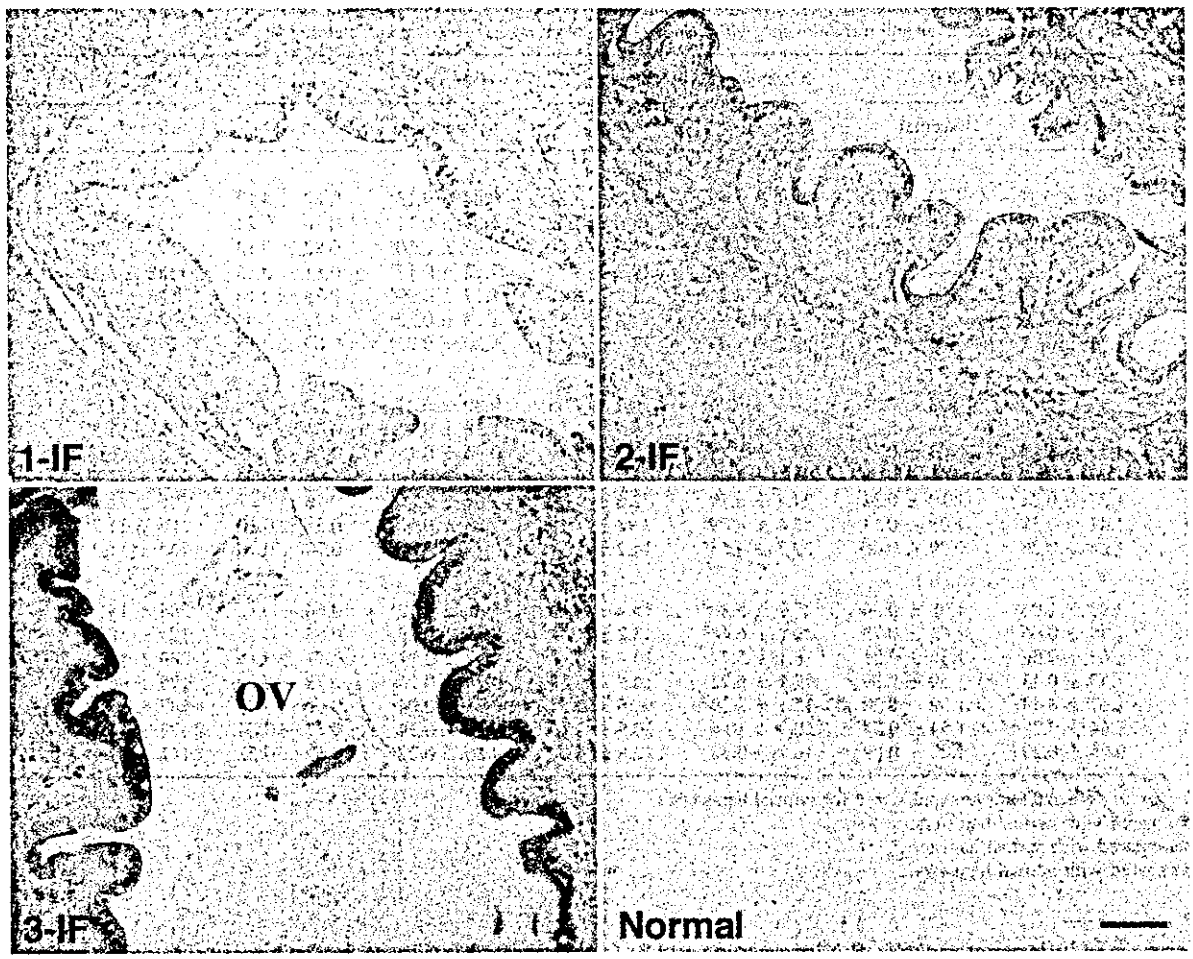

Fig. 3. PCNA accumulation in the liver of hamsters infected with *O.viverrini*. PCNA accumulation due to reinfection with *O.viverrini* was analyzed on day 90 using an immunohistochemical technique as described in Materials and methods. Immunoreactivity of PCNA in the epithelium of bile ducts increased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

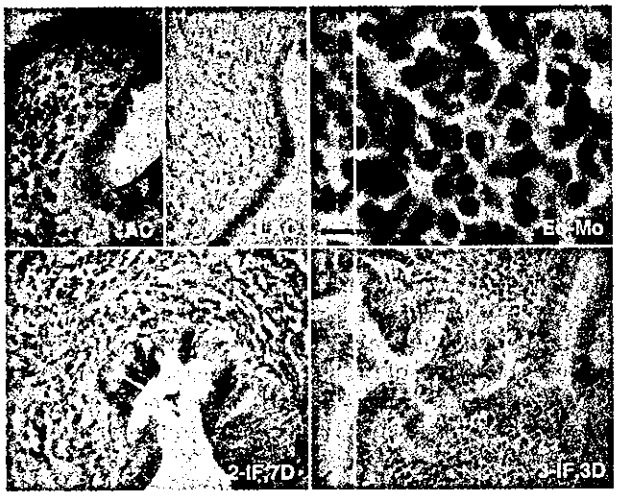

Fig. 4. Histological changes in the liver of hamsters reinfected with *O.viverrini*. In short-term infection, hyperplasia of the epithelium of bile ducts and inflammatory cell infiltration were frequently observed (AC, day 7 in 2-IF). In long-term infection, a decrease in the number of inflammatory cells 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.

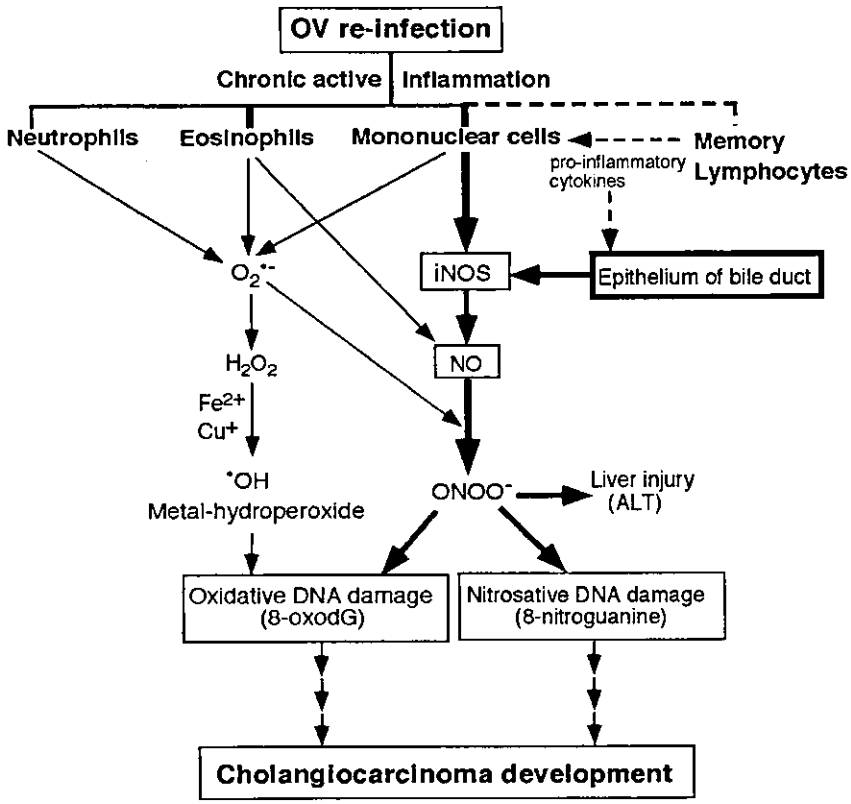

Fig. 5. The potential mechanisms of CHCA development due to reinfection with *O.viverrini* through oxidative and nitrosative DNA damage.

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 •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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Copper-mediated oxidative DNA damage induced by eugenol: possible involvement of *O*-demethylation

Katsuhisa Sakano, Yuji Inagaki, Shinji Oikawa, Yusuke Hiraku, Shosuke Kawanishi*

Department of Environmental and Molecular Medicine, Mie University School of Medicine, Mie 514-8507, Japan

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Abstract

Eugenol used as a flavor has potential carcinogenicity. DNA adduct formation via 2,3-epoxidation pathway has been thought to be a major mechanism of DNA damage by carcinogenic allylbenzene analogs including eugenol. We examined whether eugenol can induce oxidative DNA damage in the presence of cytochrome P450 using [^{32}P]-5′-end-labeled DNA fragments obtained from human genes relevant to cancer. Eugenol induced Cu(II)-mediated DNA damage in the presence of cytochrome P450 (CYP)1A1, 1A2, 2C9, 2D6, or 2E1. CYP2D6 mediated eugenol-dependent DNA damage most efficiently. Piperidine and formamidopyrimidine-DNA glycosylase treatment induced cleavage sites mainly at T and G residues of the 5′-TG-3′ sequence, respectively. Interestingly, CYP2D6-treated eugenol strongly damaged C and G of the 5′-ACG-3′ sequence complementary to codon 273 of the *p53* gene. These results suggest that CYP2D6-treated eugenol can cause double base lesions. DNA damage was inhibited by both catalase and bathocuproine, suggesting that H_2O_2 and Cu(I) are involved. These results suggest that Cu(I)–hydroperoxo complex is primary reactive species causing DNA damage. Formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine was significantly increased by CYP2D6-treated eugenol in the presence of Cu(II). Time-of-flight-mass spectrometry demonstrated that CYP2D6 catalyzed *O*-demethylation of eugenol to produce hydroxychavicol, capable of causing DNA damage. Therefore, it is concluded that eugenol may express carcinogenicity through oxidative DNA damage by its metabolite.

Keywords: DNA damage; Eugenol; Cytochrome P450; 8-OxodG; Copper; Hydrogen peroxide

Abbreviations: 8-OxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine (and also known as 8-hydroxy-2′-deoxyguanosine); HPLC–ECD, an electrochemical detector coupled to a high-performance liquid chromatography; Fpg, *E. coli* formamidopyrimidine-DNA glycosylase; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; O_2^-, superoxide anion radical; H_2O_2, hydrogen peroxide; DMSO, dimethyl sulfoxide; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate (oxidized form); CIP, calf intestine phosphatase; SOD, superoxide dismutase; CYP-cytochrome P450; TOF-MS, time-of-flight-mass spectrometry; G-6-PDH, glucose 6-phosphate dehydrogenase; G-6-P, glucose 6-phosphate

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

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1. Introduction

Eugenol (4-allyl-2-methoxy phenol) is a naturally occurring compound that has been used extensively as a flavoring agent and fragrance. Human exposure to eugenol also occurs through its use as an analgesic and from clove cigarettes [1,2]. Eugenol has anti-inflammation activity and might be a plausible lead candidate for further development of the COX-2 inhibitor [3]. In addition, eugenol has antimutagenic and anticarcinogenic potential [3–6]. Thus, eugenol is expected to act as a potential chemopreventive agent.

On the other hand, the US National Toxicology Program (NTP) showed that animals had an increased incidence of hepatocelluar carcinomas in male mice at low dose of eugenol. Eugenol is believed to have some mutagenic capacity in mice and should be evaluated for further toxicological effects [7]. Eugenol induced chromosomal aberrations in Chinese hamster ovary cells [8]. In animal studies, methyleugenol, a natural constituent of many plant essential oils, with structure similar to eugenol given orally to rats induced liver and stomach tumors in both sexs and kidney, mammary gland, and skin tumors in males [9]. DNA adduct formation via 2,3-epoxidation pathway is thought to be a major cause of DNA damage by carcinogenic allylbenzene analogs including eugenol and methyleugenol [10]. In addition to DNA adduct formation, eugenol also forms of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a DNA lesion characteristic of oxidative damage, through oxidation mechanism [11]. However, the mechanism for oxidative DNA damage by eugenol remains to be clarified.

In this study, to clarify a mechanism other than DNA adduct formation, we have investigated whether oxidative DNA damage is induced by eugenol in the presence of cytochrome P450 (CYP) using [^{32}P]-5′-end-labeled DNA fragments obtained from the human *p16* and *p53* tumor suppressor genes and the c-Ha-*ras*-1 protooncogene. These genes are suitable for studying the mechanisms of chemical carcinogenesis because they are known to be targets for chemical carcinogens [12,13]. We also analyzed the formation of 8-oxodG using an electrochemical detector coupled to a high-performance liquid chromatography (HPLC–ECD). To clarify the ultimate carcinogen causing DNA damage, we utilized time-of-flight-mass spectrometry (TOF-MS) to identify the *O*-demethylated metabolite generated by the treatment with CYP.

2. Materials and methods

2.1. Materials

The restriction enzymes (*Sma*I, *Bss*HII, *Eco*RI, *Apa*I and *Sty*I) and glucose 6-phosphate dehydrogenase (G-6-PDH) were purchased from Boehringer Mannheim GmbH (Germany). The restriction enzymes (*Hind*III and *Xba*I) and T_4 polynucleotide kinase were obtained from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP (222 TBq/mmol) was acquired from New England Nuclear (Boston, MA). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproine disulfonic acid were procured from Dojin Chemical Co. (Kumamoto, Japan). Acrylamide, piperidine, dimethyl sulfoxide (DMSO), bisacrylamide, β-nicotinamide adenine dinucleotide phosphate (oxidized form) ($NADP^+$) and glucose 6-phosphate monosodium salt (G-6-P) were purchased from Wako (Osaka, Japan). CYP isozymes from human microsomes (1A1, 1A2, 2C9, 2D6 and 2E1) and CYP reductase (10.0 mg/ml protein from human microsomes) were purchased from Gentest Corporation (Woburn, MA). $CuCl_2$, ethanol, D-mannitol and sodium formate were acquired from Nacalai Tesque (Kyoto, Japan). Calf thymus DNA, calf intestine phosphatase (CIP), superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes), α-cyano-4-hydroxycinnamic acid, eugenol and catalase (45,000 units/mg from bovine liver) were obtained from Sigma Chemical Co. (St. Louis, MO). Nuclease P_1 (400 units/mg) was purchased from Yamasa Shoyu Co. (Chiba, Japan). *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) was obtained from Trevigen Inc. (Gaithersburg, MD).

2.2. Preparation of [^{32}P]-5′-end-labeled DNA fragments

Exon-containing DNA fragments were obtained from the human *p53* [14] and *p16* [15] tumor suppressor genes and the c-Ha-*ras*-1 protooncogene [16]. DNA fragment of the *p53* tumor suppressor gene was prepared from pUC18 plasmid, ligated fragments