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BASIC RESEARCH

Inflammatory cytokines promote inducible nitric oxide synthase-mediated DNA damage in hamster gallbladder epithelial cells

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Abstract

AIM: To investigate the link between chronic biliary inflammation and carcinogenesis using hamster gallbladder epithelial cells.

METHODS: Gallbladder epithelial cells were isolated from hamsters and cultured with a mixture of inflammatory cytokines including interleukin-1β, interferon-γ, and tumor necrosis factor-α. Inducible nitric oxide synthase (iNOS) expression, nitric oxide (NO) generation, and DNA damage were evaluated.

RESULTS: NO generation was increased significantly following cytokine stimulation, and suppressed by an iNOS inhibitor. iNOS mRNA expression was demonstrated in the gallbladder epithelial cells during exposure to inflammatory cytokines. Furthermore, NO-dependent DNA damage, estimated by the comet assay, was significantly increased by cytokines, and decreased to control levels by an iNOS inhibitor.

CONCLUSION: Cytokine stimulation induced iNOS expression and NO generation in normal hamster gallbladder epithelial cells, which was sufficient to cause DNA damage. These results indicate that NO-mediated genotoxicity induced by inflammatory cytokines through activation of iNOS may be involved in the process of biliary carcinogenesis in response to chronic inflammation of the biliary tree.

Key words: Biliary carcinoma; Inflammation; Inflammatory cytokine; Nitric oxide; Inducible nitric oxide synthase; DNA damage; Gallbladder epithelial cell; Hamster

INTRODUCTION

It is well known that chronic local inflammation increases the risk for cancer development in several organs, such as colon, lung, pancreas, and esophagus[1,2]. Similarly, biliary carcinoma develops under chronic inflammatory conditions involving the biliary epithelium in the setting of gallstone disease, congenital choledochal cyst, pancreaticobiliary maljunction, or primary sclerosing cholangitis[3,4]. Moreover, recent reports have described the occurrence of secondary biliary carcinomas in patients with persistent reflux cholangitis after bilioenterostomy, transduodenal sphincteroplasty, or endoscopic sphincterotomy for both benign and malignant diseases of the liver, bile duct, and pancreas[5-7]. However, the molecular mechanisms of biliary carcinogenesis as a consequence of chronic biliary inflammation remain unclear. We have previously demonstrated that persistent reflux cholangitis after bilioenterostomy accelerates biliary carcinogenesis through activation of biliary epithelial cell kinetics in hamsters[8-10]. Furthermore, more severe cholangitis is associated with a high occurrence of biliary carcinoma. However, the molecular mechanisms by which chronic biliary inflammation increases the risk of biliary carcinogenesis are obscure, similar to the clinical occurrence in humans. Meanwhile, we have established a method for culturing biliary epithelial cells from the hamster using a collagen gel technique[11].

Chemically reactive oxidants, radicals, and electrophilic mediators, such as hydrogen peroxide and oxyradicals, nitric oxide, malondialdehyde, 4-hydroxynonenal, or eicosanoids, are produced during inflammation, and these chemical mediators are known to induce a variety of biological reactions[2]. Recently, attention has been focused on nitric oxide (NO) as an endogenous mutagen, an angiogenesis factor, and an inhibitor of apoptosis[12]. NO is a free radical that is synthesized from L-arginine by the family of nitric oxide synthases (NOSs). Three

isoforms of the NOSs have been isolated: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS)[13,14]. Although nNOS and eNOS are present constitutively, iNOS is induced in inflamed tissues and generates relatively large amounts of NO, compared with nNOS and eNOS[13-15]. Cytokine and/or lipopolysaccharide stimulation is known to induce iNOS expression in macrophages, hepatocytes, and many other cell types including certain epithelial cells[16-19]. Moreover, iNOS expression and generation of NO in inflamed tissues have been postulated to potentiate epithelial cells to malignant transformation through the ability of NO to promote mutagenic changes in DNA through DNA oxidization and protein nitrosylation[20,21].

In this study, we investigated the role of iNOS activation, NO generation, and DNA damage as the link between chronic inflammation and biliary carcinogenesis, by utilizing normal hamster gallbladder epithelial cells cultured with inflammatory cytokines.

MATERIALS AND METHODS

Animals

Five-week-old female Syrian golden hamsters (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were used. The animals were kept under standard laboratory conditions in the Laboratory Animal Center for Biochemical Research at Nagasaki University Graduate School of Biochemical Sciences. All experiments were performed following the Guidelines for Animal Experimentation of Nagasaki University.

Isolation and culture of biliary epithelial cells from hamsters

Biliary epithelial cells were isolated from the biliary tree of hamsters as previously described[11]. After laparotomy, inferior vena cava was paracentesed with a 22G needle, and the liver was perfused in situ with 100 mL Ca^{2+} - and Mg^{2+} - free phosphate-buffered saline (CMF-PBS) containing 10 mmol/L 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) at pH7.4, and 1 mmol/L ethylene glucol-bis (E-aminoethylether) N, N, N', N'-tetraacetic acid (EGTA) for 10 min at 37℃. The vena cava was clamped above the diaphragm, and the perfusing solution was drained *via* the incised portal vein, followed by perfusion with 100 mL Hanks' balanced salt solution (GIBCO, Grand Island, NY) containing 50 mmol/L HEPES and 0.04% collagenase (Nittazeratin, Osaka, Japan) for 10 min at 37℃. After perfusion with the collagenase solution, the liver, gall bladder, and extrahepatic bile duct were removed en bloc. The biliary tree was isolated and separated into the intrahepatic and extrahepatic bile ducts and the gall bladder in CMF-PBS containing 0.1 mmol/L EGTA. The biliary fragments were minced.

The biliary fragments were embedded on collagen gel plated (Collagen Gel Culture kit; Nittazeratin, Osaka, Japan) 60-mm petri dishes with 2 mL of an ice-cold mixture of collagen solution composed of 0.3% acid solution collagen (Cellmatrix Type I -A), 10 × HamF12, and 0.8 N NaOH at 8:1:1 dilution. After incubation at 37℃ with 5% CO_2 and 95% humidity for 20-30 min, collagen gels were overlaid with 5 mL of culture medium composed of Dulbecco's modified Eagle medium/HamF12 medium (DMEM/HamF12, GIBCO) and 10% fetal bovine serum (GIBCO). After incubation of biliary fragments for 7-10 d, the epithelial cells extended widely on the surface of the gel, while the mesenchymal cells progressed toward the inside of the gel. The biliary epithelial cells were isolated from the peripheral region of cellular sheets (Figure 1).

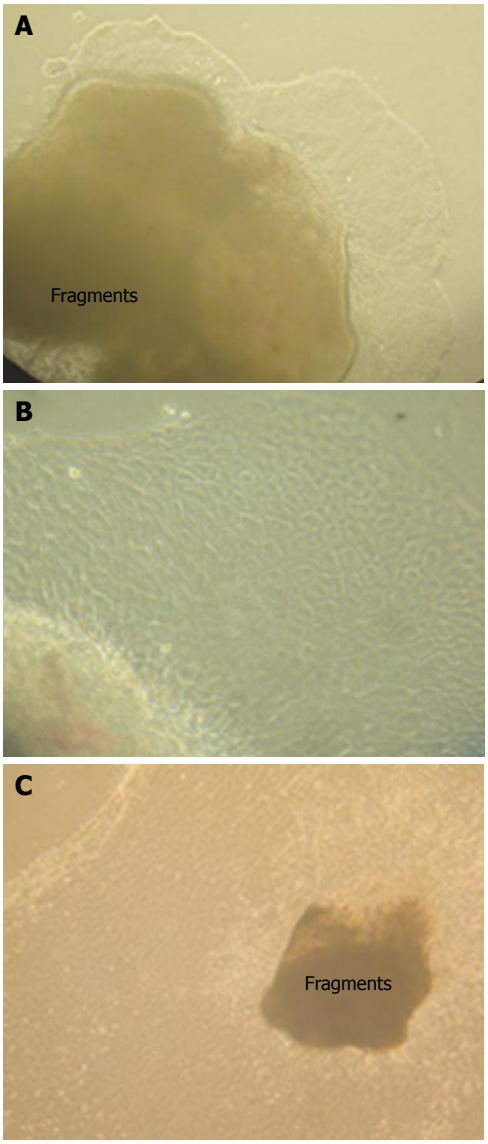

Figure 1 Isolation of hamster biliary epithelial cells. **A**: Phase contrast microscopy revealing a small amount of hamster gallbladder epithelial cells growing on the collagen gels 24 h after culture (× 100); **B**: High magnification of hamster gallbladder epithelial cells demonstrating cuboidal cells around the biliary fragments (× 400); **C**: Phase contrast microscopy showing widely extended gallbladder epithelial cells on the surface of the gel 7 d after culture (× 40).

Addition of inflammatory cytokines

Gallbladder epithelial cells isolated from hamsters were used in this study because of its higher cellular activity compared to other biliary epithelial cells. Resuspended gallbladder epithelial cells (1×10^5 cells/mL) were plated on collagen-coated plates. After incubation for 24 h, the epithelial cells were prepared for three different

Table 1 Oligonucleotide primers used for RT-PCR in this study

Primers	Sequences	References
NOS-590F	GGYTGGTACATGRGCACYGAGATYGG	Cox *et al*[25], 2001
NOS-893R	AAGGCRCARAASTGDGGRTA	Cox *et al*[25], 2001
NOS40F	GCAGGATGGGAAACTGAGGCCCAG	Ramirez-Emiliano *et al*[24], 2005
NOS40R	TGAACAAGGCAGCCAGGTCCCGG	Ramirez-Emiliano *et al*[24], 2005
GAPDHF	TCCCTCAAGATTGTCAGCAA	Liu *et al*[26], 1993
GAPDHR	AGATCCACAACGGATACATT	Liu *et al*[26], 1993

experimental protocols: incubation with culture medium alone (control group), incubation with a cytokine mixture known to increase iNOS expression in other cell types[18,22] consisting of human recombinant interleukin (IL) 1-β (0.5 ng/mL), interferon (IFN)-γ (5 ng/mL), and tumor necrosis factor (TNF)-α (250 ng/mL) (CM group), or incubation with the same cytokine mixture and an iNOS inhibitor L-N (G)-monomethyl arginine (L-NMMA, 0.03 mmol/L) (CM + L-NMMA group). These human recombinant cytokines and L-NMMA were obtained from the Sigma Chemical Co. (St. Louis, MO). Gallbladder epithelial cells in each group were incubated at 37℃ for 24 h, and then processed for the following analyses.

Measurement of NO_2^- and NO_3^- in the medium

To determine the amount of NO produced by gallbladder epithelial cells, nitrite (NO_2^-) and nitrate (NO_3^-) levels were measured in the culture media by high performance liquid chromatography with a NOx analyzer (ENO-10; Eicom, Kyoto)[23].

RT-PCR

iNOS mRNA was amplified using a nested RT-PCR method[24]. The sequences of primers (Invitrogen Life Technologies, Carlsbad, CA) used in this study are shown in Table 1[24-26].

Total RNA was extracted from gallbladder epithelial cells using a RNA extraction kit (ISOGEN; Nippon Gene, Tokyo). Reverse transcription and PCR amplification were performed with a RNA PCR kit Ver.3.0 (Takara Shuzo, Tokyo, Japan). After reverse transcription, the first amplification was performed using 1 μmol/L of the cDNA, together with PCR primers NOS-590F and NOS-893R. The PCR conditions were one cycle of denaturing at 95℃ for 2 min, followed by a touch-down protocol consisting of 18 cycles of 15 s at 95℃, 30 s at 60℃, and 1 min at 72℃, then 25 cycles of 15 s at 95℃, 30 s at 42℃ minus 1℃ per cycle, 1 min at 72℃, and a final extension at 72℃ for 10 min[24]. Next, 1 μL of the cDNA from this amplification was reamplified using the PCR primers specific for iNOS (NOS40F and NOS40R)[24]. The PCR conditions were an initial denaturation at 95℃ for 2 min, followed by a touch-down protocol consisting of 13 cycles of 15 s at 95℃, 30 s at 70℃ minus 1℃ per cycle, 1 min at 72℃, then 30 cycles of 15 s at 95℃, 30 s at 57℃, 1 min at 72℃, and a final extension at 72℃ for 10 min[24]. As a control, amplification of mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed[24,26], and the PCR conditions were the same as those described for the first nested PCR amplification. The amplification products were resolved by electrophoresis on 3% (w/v) agarose gels containing 0.1 μg/mL ethidium bromide and photographed under UV trans-illumination.

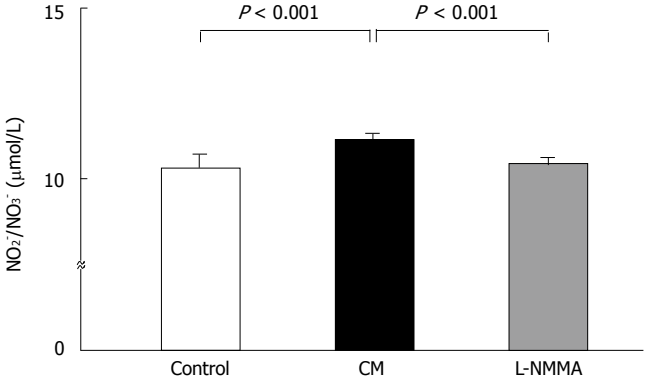

Figure 2 High performance liquid chromatography showing significantly elevated NO_2^- + NO_3^- levels in the CM group compared with the control group, similar NO generation in the CM + L-NMMA group and control group, being significantly lower than that in the CM group.

Comet assay (single cell gel electrophoresis assay)

Comet assay was performed as described previously[27,28]. The gallbladder epithelial cells in each group were resuspended at 1×10^5 cells/mL in ice cold CMF-PBS and combined with molten LMAgarose (Trevigen, Gaitherburg, MD) at a ratio of 1:10 (v/v). The sample was immediately pipetted onto a frosted microscope slide (CometSlide; Trevigen, Gaitherburg, MD). The slides were placed flat at 4℃ in the dark for 10 min, immersed in prechilled lysis solution (Trevigen, Gaitherburg, MD), and left at 4℃ for 30 min to remove cellular proteins, leaving DNA as nucleoids. The slides were then immersed in an alkaline solution (pH > 13, 0.3 mol/L NaOH and 0.001 mol/L EDTA) for 30 min to denature the DNA and hydrolyze sites of damage. The samples were electrophoresed for 10 min and stained with SYBR green I (Trevigen, Gaitherburg, MD) according to the manufacturers instructions. At least 75 cells on each slide, randomly selected by fluorescence microscopy, were analyzed using National Institutes of Health image (Netscape Navigator) with the comet analysis macro (comet 1.4 macro)[29].

Statistical analysis

Values for NO_2^- + NO_3^- levels and proportion of DNA damage were expressed as mean ± SE. For statistical analysis, ANOVA was used to compare non-repeated measurements between groups, followed by Bonferroni correction for paired comparison. $P < 0.05$ was considered statistically significant.

RESULTS

Generation of NO_2^- and NO_3^-

The amount of NO measured by high performance liquid chromatography in each group is shown in Figure 2. The concentration of NO_2^- + NO_3^- in the media was

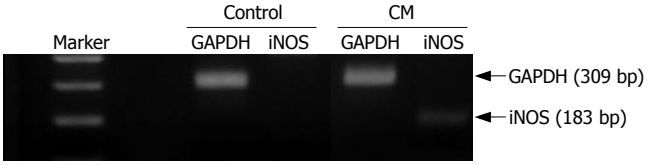

Figure 3 iNOS mRNA expression in gallbladder epithelial cells in the CM group and no iNOS mRNA expression in the control group.

10.35 ± 0.47 μmol/L in the control group (n = 26), 11.06 ± 0.18 μmol/L in the CM group (n = 26), and 10.46 ± 0.18 μmol/L in the CM + L-NMMA group (n = 27). NO generation was significantly higher in the CM group than in the control group ($P < 0.001$). Meanwhile, NO generation in the CM + L-NMMA group and control group was similar, and significantly lower than that in the CM group ($P < 0.001$).

RT-PCR

RT-PCR/touch down amplification of iNOS mRNA in gallbladder epithelial cells is shown in Figure 3. iNOS mRNA (183 bp) expression was demonstrated in the CM, while none of the control cells expressed iNOS mRNA.

Comet assay

Representative fluorescent micrograph images evaluated by single cell gel electrophoresis using comet assay are shown in Figure 4A. In contrast to the intact spherical nuclei observed in the control group, the cells treated with cytokine mixture demonstrated a comet tail indicative of DNA damage. In the CM + L-NMMA group, the cells also exhibited intact spherical nuclei. The ratio of DNA exhibiting comet tails evaluated by using NIH image was 2.14% ± 0.59% in the control group, 5.14% ± 0.69% in the CM group, and 1.70% ± 0.62% in the CM + L-NMMA group (Figure 4B). The level of DNA damage in the CM group was significantly higher than that in the control group ($P < 0.001$), and decreased to the control level after the addition of L-NMMA.

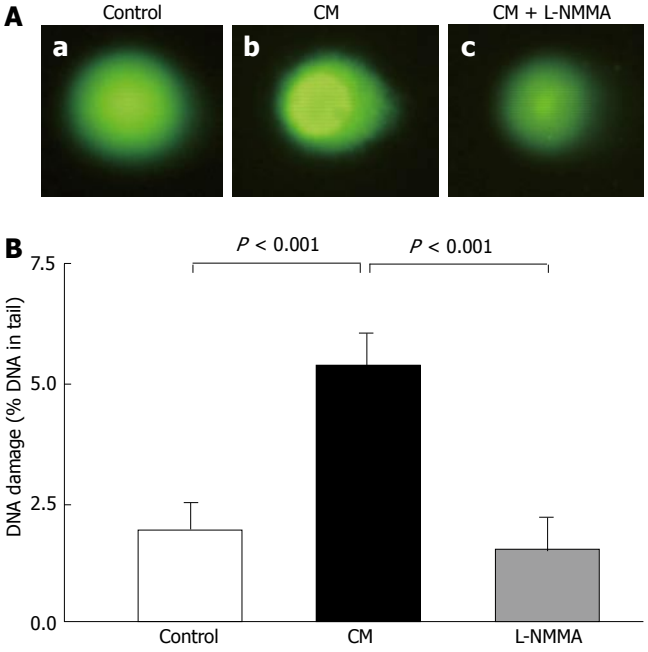

Figure 4 NO-dependent DNA damage evaluated by comet assay. **A**: Fluorescence microscopy showing the apparent comet tail in gallbladder cells stimulated by the cytokine mixture and intact spherical nuclei of the cells in the control group and CM + L-NMMA group; **B**: Analysis of the proportion of DNA migrated to comet tails using NIH image showing a significantly higher level of DNA damage in the CM group than in the control group, which decreased to the control level after the addition of L-NMMA.

DISCUSSION

The induction of iNOS expression and NO production has previously been described in a variety of precancerous or cancerous lesions, in which the NO level of production is in proportion to the degree of malignancy[30,31]. Recently, Jaiswal *et al*[27,32] reported that human cholangiocarcinomas exhibit intensive immunohistochemical staining for iNOS and that cholangiocarcinoma cell lines stimulated by inflammatory cytokines and cholangiocyte cell lines transfected with iNOS produce large amounts of NO due to iNOS expression, which result in both oxidative DNA damage and inhibition of the excision DNA repair process. In the present study, we used primary epithelial cells isolated from the gallbladder because these normal epithelial cells should be suitable for estimating the involvement of iNOS and NO in biliary carcinogenesis, especially in the initiation of biliary carcinoma in response to chronic inflammation.

Following stimulation with a mixture of inflammatory cytokines of IL-1β, IFN-γ, and TNF-α, NO generation was significantly increased in the gallbladder epithelial cells. Furthermore, the production of NO in the presence of inflammatory cytokines was completely suppressed by the addition of an iNOS inhibitor, L-NMMA. Moreover, the expression of iNOS mRNA in the gallbladder epithelial cells was clearly demonstrated in the presence of cytokine-stimulation using nested RT-PCR, but not in the absence of stimulatory cytokines. These findings indicate that inflammatory cytokines promote iNOS expression and NO generation in normal epithelial cells of hamster gallbladder.

NO has the ability to directly oxidize DNA, causing mutagenic changes[20,21]. Although NO also contributes to intracellular communication, inhibition of apoptosis, and enhancement of vascular dilatation, permeability, and neovascularization[33,34], DNA damage may be an essential and initial process involved in the malignant transformation of a wide variety of epithelial cells, and DNA damage in the individual cell can be detected using the highly sensitive comet assay[27,28,32]. In this assay, damaged single- and double-stranded DNA within the nucleus are allowed to migrate toward the anode, by an alkaline hydrolysis process during electrophoresis, resulting in the appearance of a "comet tail". In our study, the comet assay clearly demonstrated that the proportion of DNA moving to the comet tail, i.e., indicating DNA damage, was significantly higher in the cytokine mixture group than in the control group, and that the increased DNA damage was completely inhibited by L-NMMA. These data indicate that inflammatory cytokines stimulate iNOS-mediated DNA damage in normal gallbladder

epithelial cells. Although almost all DNA oxidative breaks can be excised by multiple excision DNA repair processes before mutations occur[35], DNA damage can lead to p53-mediated cell growth arrest and apoptosis, and the accumulation of p53 protein can repress the transcription of iNOS[36]. Furthermore, a recent report has shown that the tumor suppressant organization of normal p53 protein is inhibited in the presence of large amounts of NO in inflamed tissues[37]. In consideration of these facts, the results of our study suggest that NO-mediated oxidative DNA damage produced by inflammatory cytokines through iNOS expression is involved in an initiation process linking chronic biliary inflammation to malignant transformation.

Inflammatory cytokines released in the inflamed tissues initiate the induction of iNOS, resulting in increased NO production. As demonstrated in our study, iNOS-mediated NO production is sufficient to induce DNA damage in normal biliary epithelial cells. In association with persistency of biliary inflammation, the accumulation of NO-mediated genotoxicity may initiate the malignant transformation of epithelial cells lining the biliary tree. Our hamster models of both *in vivo* and *in vitro* tumorigenesis will enhance the understanding of the mechanisms of inflammation-related biliary carcinogenesis.

ACKNOWLEDGMENTS

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COMMENTS

Background

It is well known that biliary carcinoma develops under chronic inflammatory conditions involving the biliary epithelium. We have previously demonstrated that persistent reflux cholangitis after bilioenterostomy accelerates biliary carcinogenesis through activation of biliary epithelial cell kinetics in hamsters. However, the cellular mechanisms of biliary carcinogenesis in response to inflammation remain unclear.

Research frontiers

In the present study, we used primary epithelial cells isolated from the gallbladder of hamsters. These normal epithelial cells should be suitable for estimating the involvement of iNOS and NO in biliary carcinogenesis, especially in initiation of biliary carcinoma in response to chronic inflammation.

Innovations and breakthroughs

In normal hamster gallbladder epithelial cells, cytokine stimulation induced iNOS expression and NO generation which was sufficient to cause DNA damage. The results suggest that NO-mediated oxidative DNA damage produced by inflammatory cytokines through iNOS expression is involved in an initiation process linking chronic biliary inflammation to malignant transformation.

Applications

Our hamster models of both *in vivo* and *in vitro* tumorigenesis will enhance the understanding of the mechanisms of inflammation-related biliary carcinogenesis.

Peer review

The authors investigated the role of inducible nitric oxide (iNOS) in DNA damage using cultured gallbladder epithelial cells. They speculated that the DNA damage would be related to biliary carcinogenesis. The data were interesting.

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