Short-term detection of gastric genotoxicity using the DNA double-strand break marker γ-H2AX

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Abstract: DNA damage caused by Helicobacter pylori infection and chronic inflammation or exposure to genotoxic agents is considered an important risk factor of gastric carcinogenesis. In this study, we have evaluated a short-term technique to detect DNA damage response to various chemical carcinogens; it involves visualization of Ser 139-phosphorylated histone H2AX (γ-H2AX) foci by immunohistochemistry and expression analysis of other genes by quantitative RT-PCR. Six-week-old male rats were intragastrically administered N-methyl-N-nitrosourea (MNU), 3,2′-dimethyl-4-aminobiphenyl (DMAB), dimethylnitrosamine (DMN), and 1,2-dimethylhydrazine (DMH) for 5 days/week for 4 weeks, using corn oil as a vehicle. Animals were sacrificed at day 28, and their stomachs were excised. γ-H2AX foci formation, indicating DNA double-strand breaks, was observed in the proliferative zone of both fundic and pyloric glands. The number of positive cells per gland was significantly high in pyloric glands in the MNU group and in fundic glands in the MNU and DMAB groups. A significant increase in p21waf1 mRNA level was observed in the DMN group compared with the control, which was in contrast to the decreasing tendency of the h2afx mRNA level in the MNU and DMN groups. Apoptotic cells positive for γ-H2AX pan or peripheral nuclear staining were observed on the surface layer of the fundic mucosa in the MNU group. The fundic pepsinogen a5 (pga5) mRNA level showed a significant decrease, indicating gland damage. The pyloric pepsinogen c mRNA level showed no change. In conclusion, γ-H2AX in combination with other gene expression analyses could be a useful biomarker in a short-term experiment on gastric chemical genotoxicity. (DOI: 10.1293/tox.2019-0007; J Toxicol Pathol 2019; 32: 91–99)

Key words: stomach, gastric cancer, DNA double-strand break, γ-H2AX, p21, pepsinogen

Introduction

Gastric cancer is the third leading cause of cancer-related death worldwide¹. Various genetic changes are closely related to gastric carcinogenesis. As summarized in our previous review², it is widely known that Helicobacter pylori (H. pylori) infection is correlated with gastric carcinogenesis. H. pylori infection triggers gastric inflammation and activates inflammation-related factors such as nuclear factor κB (NF-κB) in gastric epithelial cells⁴. Matsumoto and colleagues⁴ reported that H. pylori-induced NF-κB activation increases the expression of activation-induced cytidine deaminase (AID). Overexpression of AID alters the nucleotides of various genes, such as the tumor suppressor gene TP53³, indicating that critical genomic damage and mutations might occur at the early stage of gastric carcinogenesis. Therefore, a technique for detecting genetic changes in gastric epithelial cells would lead to early diagnosis of gastric cancer risk and enable large-scale drug screening⁶.

During cancer initiation and progression, DNA damages is either fixed by DNA repair mechanisms or becomes irreversible and is inherited by daughter cells after cell division⁷. The mammalian DNA double-strand break (DSB), which is one of the most lethal forms of DNA damage, can
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be induced by various genotoxic carcinogens. As DSBs at multiple sites are related to genomic instability and cancer initiation, they should be promptly repaired by either non-homologous end-joining (NHEJ) or homologous recombination (HR). Both NHEJ and HR are complex DSB repair systems involving a series of protein recruitments and accumulations. Hallmarks, such as DSB site-specific proteins, could be useful to indirectly detect DSBs in a given cell.

In a nucleosome, double-stranded linear DNA is wrapped around histone family proteins. Each nucleosome contains two molecules each of the core histone proteins H2A, H2B, H3, and H4, constituting an octamer. Specific amino acid residues of each histone are posttranscriptionally modified, producing biological varieties of histone proteins. In addition to core histones, several histone variants exist in mammals. Among them, H2AX, a variant of H2A, is rapidly phosphorylated at Ser 139 by ataxia telangiectasia mutated (ATM) protein and accumulated at DSB sites with other various proteins such as 53BP1 (p53 binding protein 1). Although γ-H2AX detection has been widely used for evaluation of DSB and other biological effects in some cancers, little has been reported on its role in gastric cancer.

In response to DNA-damaging agents, mammalian cells have evolved an elaborate system to control cell cycle transition and progression besides sensing and repairing DNA damage. According to some studies using animal models, p21 is induced at transcriptional and translational levels upon exposure to chemical genotoxic agents or X-rays in vitro and in vivo.

Identification of preneoplastic changes is an important way to determine the pathogenesis of gastric cancers. Pepsinogen enzyme 1 (Pg1, pyloric pepsinogen) has received attention as a marker of preneoplasia in the stomach. Focal reduction or disappearance of Pg1 expression in the pyloric mucosa during the early stages of gastric carcinogenesis before distinct morphological alterations has been reported.

In this study, we performed immunohistochemical analysis of γ-H2AX and evaluated quantitative alterations in transcriptional products of the H2AX gene (h2afx), p21 gene, and pyloric and fundic pepsinogen genes using carcinogen-administered rats to establish a rapid evaluation scheme for gastric genotoxicity.

Materials and Methods

Chemicals

N-methyl-N-nitrosourea (MNU) was obtained from Sigma-Aldrich (St. Louis, MO, USA). 3,2′-dimethyl-4-aminobiphenyl (DMAB) was purchased from Matsugaki Pharmaceutical (Osaka, Japan). Dimethylnitrosamine (DMN) and 1,2-dimethylhydrazine (DMH) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Corn oil was used as a vehicle.

Experimental design

This project was planned as a short-term, collaborative, multi-organ detection of carcinogenicity project and was supported by the Ministry of Health, Labour and Welfare (chief researcher, Prof. N. Yoshimi, Ryukyu University). Experiments were performed in the SPF facility at Nagoya City University with the approval by the Institutional Animal Care and Use Committee of Nagoya City University School of Medical Sciences. Five-week-old male F344 rats were purchased from Charles River Laboratories Japan (Kanagawa, Japan) and maintained at the Laboratory Animal Facility of Nagoya City University. Animals were housed in plastic cages on hardwood-chip bedding in an air-conditioned room with a 12-h light/12-h dark cycle and allowed free access to a basal diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. After a one-week acclimation period, the experiment was initiated. The animals were divided into 5 groups (corresponding to 5 chemicals) of 5 animals each, and 6 animals were assigned as controls. Chemicals were administered intragastrically 5 times per week for 4 weeks as shown in Table 1. Corn oil was used as a vehicle control. MNU has been reported to act as a direct carcinogen, inducing gastric cancer in mice and Mongolian gerbils. Other chemicals act as indirect carcinogens, inducing tumors not in the stomach but in other organs; DMAB, DMN, and DMH have been reported to induce prostate, liver, and colorectal tumors, respectively. The dose of each chemical was defined as approximately 1/20 of LD50 of each chemical as previously described. The rats were sacrificed by exsanguination under deep isoflurane anesthesia at experimental day 28. Several organs were excised for subsequent analyses. Small pieces of stomach at the fundic and pyloric border, approximately 4 × 2 mm in size, were cut and used for total RNA isolation. The rest of the stomach was fixed in 10% formalin for 24 h.

Histological analysis

Stomach tissue samples were sliced along the longitudinal axis into strips of equal width, dehydrated, and embedded in paraffin. Sections with a thickness of 3 µm were prepared, deparaffinized, and stained with hematoxylin (Merck KGaA, Darmstadt, Germany) and eosin (Muto Pure Chemicals, Co., Ltd., Tokyo, Japan) (H&E). For immunohistochemical detection of DSBs and apoptotic reaction, an antibody against phospho-Ser139-histone H2AX (γ-H2AX) (Cat. No. #2577S, Cell Signaling Technology, Danvers, MA, USA) was used. Immunohistochemical procedures were performed using an ultraView Universal DAB Detection Kit with a Ventana Benchmark Ultra apparatus according to the instructions from the manufacturer (Roche Diagnostics, Tokyo, Japan). Briefly, the sections were deparaffinized, treated with CC1 antigen retrieval buffer (5 mM ethylenediaminetetraacetic acid, pH 8.0), and incubated with the primary antibody described above. Then, the sections were treated with a universal secondary antibody (a mixture of anti-mouse and anti-rabbit antibodies).
visualized with 3,3′-diaminobenzidine, and counterstained with hematoxylin as described elsewhere. The number of γ-H2AX-positive cells per ten glands were counted in each animal and were expressed as an average number of cells per gland. Intranuclear focus formation was considered as indicative of DSBs, whereas pan/peripheral nuclear staining was regarded as indicating apoptosis.

RNA isolation and quantitative RT-PCR
Total RNA was extracted from the border of the antrum and corpus regions in the glandular stomach of rats using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). After DNase treatment, first-strand cDNAs were synthesized using a SuperScript VILO cDNA Synthesis kit for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Quantitative PCR of H2A histone family member X (H2afx), p21 (waf1), progastricsin (pepsinogen c; Pgc), considered as specifically expressed in pyloric gland and fundic mucous neck cells), and pepsinogen 5, group I (Pga5, originated from fundic chief cells), was performed using the Eco Real-Time PCR System (Illumina, San Diego, CA, USA) and a QuantiTect SYBR Green PCR kit (QIAGEN) according to the manufacturer’s instructions. The rat-specific glyceraldehyde-3-phosphate dehydrogenase (Gapdh) gene was used as an internal control. The Rattus norvegicus primer sequences for each marker are listed in Table 2. Specificity of the PCR reaction was confirmed using the High Resolution Melt (HRM) program provided with the Illumina software. To further confirm that there was no obvious primer dimer formation or nonspecific amplification, after the PCR reaction, the samples were electrophoresed in 2.5% agarose gels and visualized with GR Green Loading Buffer (GRG-1000, Bio Craft, Tokyo, Japan). Total RNA samples without RT were used as a control for PCR amplification (data not shown). Relative quantification was performed with the previously established ΔΔCt method using the internal control without the necessity for external standards. The relative expression levels of mRNAs were calculated by comparing them with those of the control group (set at 1.00) for each gene.

Statistical analysis
Quantitative values were expressed as means ± SE, and differences between means were statistically analyzed using ANOVA (analysis of variance), followed by Dunnett’s multiple comparisons test using the Prism 7 software (GraphPad Software, La Jolla, CA, USA). Values with P<0.05 were considered statistically significant.

Results
Animal experiments
There were no significant abnormal macroscopic findings in stomachs and other organs among experimental groups at sacrifice. There were no significant differences in major organ weights, including those of the liver and kidneys, although reduction of body weight was observed in the MNU group.

γ-H2AX as a histological marker for DSBs in the gastric mucosa
Histological evaluation of H&E-stained specimens revealed no obvious findings in any groups. Representative results are shown in Fig. 1a and 1d (pyloric gland) and 2a and 2c (fundic gland).

Immunoeexpression of γ-H2AX was evaluated in the pyloric mucosa (Fig. 1b,1c, 1e, 1f, and 3a). Although several small γ-H2AX-positive foci were observed in the nuclei of

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Table 1. List of Chemicals

<table>
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<tr>
<th>Groups</th>
<th>Chemicals</th>
<th>Dose</th>
<th>Mode of action</th>
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<td>1 Control (corn oil)</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2 N-methyl-N-nitrosourea (MNU)</td>
<td>5 mg/kg BW</td>
<td>Direct carcinogen</td>
<td>Stomach</td>
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<tr>
<td>3 3,2′-dimethyl-4-aminobiphenyl (DMAB)</td>
<td>5 mg/kg BW</td>
<td>Indirect carcinogen</td>
<td>Prostate gland</td>
<td></td>
</tr>
<tr>
<td>4 Dimethylnitrosamine (DMN)</td>
<td>2 mg/kg BW</td>
<td>Indirect carcinogen</td>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>5 1,2-dimethylhydrazine (DMH)</td>
<td>5 mg/kg BW</td>
<td>Indirect carcinogen</td>
<td>Colorectum</td>
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Table 2. Rattus norvegicus Primer Sequences for Relative Quantitative RT-PCR

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<td>pga5</td>
<td>ATCAAACCGTGTTAGCCTCGG</td>
<td>150</td>
<td>NM_021753</td>
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Fig. 1. Immunohistochemical detection of γ-H2AX-positive cells showing foci formation in the nuclei in the proliferating region of pyloric glands. (a–c) Control and (d–f) MNU groups. (a and d) Hematoxylin and eosin (H&E) staining; original magnification, 200×. (b, c, e, and f) γ-H2AX immunostaining; original magnification 630× (b and e); digitally zoomed to 6,300× (c and f).

Fig. 2. Immunohistochemical detection of γ-H2AX-positive cells in the proliferating region of fundic glands. (a and b) Control and (c and d) MNU groups. (a and c) H&E staining; original magnification, 200×. (b and d) γ-H2AX immunostaining; original magnification, 630×.
some epithelial cells in the proliferative region of the pyloric mucosa, which were also present in the control group, the number of γ-H2AX-positive cells was higher in the MNU group. These intranuclear focus formations were considered to be DSBs. The numbers of γ-H2AX-positive cells per pyloric gland were 1.10 ± 0.21, 2.54 ± 0.15, 1.66 ± 0.24, 1.42 ± 0.15, and 1.26 ± 0.16 (mean ± SE) in the control, MNU, DMAB, DMN, and DMH groups, respectively. Statistical significance was observed in the MNU group compared with the control (P<0.0005, ANOVA; P=0.0001, Dunnett’s multiple comparisons test). The pattern of foci formation did not show any difference among the control and experimental groups.

Similar findings were observed in the proliferative region of the fundic glands (Fig. 2b, 2d, and 3b). The numbers of fundic gland cells with γ-H2AX-positive foci were 1.37 ± 0.23, 3.26 ± 0.47, 2.98 ± 0.42, 2.02 ± 0.22, and 2.06 ± 0.31 (mean ± SE) in the control, MNU, DMAB, DMN, and DMH groups, respectively. Statistical significance was observed in the MNU (P<0.005, Dunnett’s multiple comparisons test) and DMAB (P<0.01, Dunnett’s multiple comparisons test) groups compared with the control (P<0.005, ANOVA).

**γ-H2AX as a histological apoptotic marker**

In the fundic mucosa, surface epithelial cells showed peripheral-nuclear and pan-nuclear staining in the MNU group (Fig. 4 and 5), which was considered to indicate apoptosis as previously described. In contrast, such apoptotic staining was rarely observed in the control and other groups. The numbers of γ-H2AX-positive apoptotic cells per fundic gland were 0.18 ± 0.06, 1.74 ± 0.32, 0.16 ± 0.04, 0.06 ± 0.04, and 0.10 ± 0.03 (mean ± SE) in the control, MNU, DMAB, DMN, and DMH groups, respectively. Statistical significance (P<0.0001, ANOVA) was observed in the MNU group compared with the control (P<0.0001, Dunnett’s multiple comparisons test). The pyloric mucosa did not have a significant number of apoptotic cells (data not shown), so the analysis was omitted for it.

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**Fig. 3.** Number of cells with intranuclear γ-H2AX-positive foci in the proliferating region of pyloric and fundic glands. (a) Pyloric mucosa (**P<0.0005, ANOVA**). The number of positive cells in the MNU group is significantly higher than that in the control group (†††P=0.0001, Dunnett’s multiple comparisons test) group. (b) Fundic mucosa (⁎P<0.005). The numbers of positive cells in the MNU (††P<0.0005) and DMAB (†P<0.01) groups are significantly higher than that in the control group.

**Fig. 4.** γ-H2AX-positive apoptotic cells on the surface of the fundic mucosa. (a) Control group. (b–d) MNU groups. Apoptotic cells show pan- or peripheral-nuclear γ-H2AX staining. γ-H2AX immunostaining, original magnification 200×. (c and d) Digital zoomed to 6,300×.
Alteration in mRNA levels of histone H2AX, p21waf1, and pepsinogens

Quantitative RT-PCR was performed to evaluate the alteration in mRNA levels of histone H2AX (h2afx), p21waf1, and pepsinogens, including pgc and pga5 (Fig. 6). The relative expression levels of h2afx mRNAs were 0.62 ± 0.16, 0.79 ± 0.33, 0.62 ± 0.07, 0.99 ± 0.24 in the MNU, DMAB, DMN, and DMH groups, respectively, when compared with the control group value set at 1.00 ± 0.24 (mean ± SE) (P<0.05, ANOVA). A decreasing tendency of h2afx mRNA was observed in the MNU and DMN groups (P=0.06, Dunnett’s multiple comparisons test) compared with the control. For p21waf1, the levels were 1.66 ± 0.30, 1.74 ± 0.74, 2.27 ± 0.40, and 1.78 ± 0.75, respectively (control, 1.00 ± 0.22) (P<0.05, ANOVA). In contrast to h2afx, a significant increase in p21waf1 mRNA level was observed in the DMN group (P<0.05, Dunnett’s multiple comparisons test) compared with the control. For pgc, the values were 1.07 ± 0.17, 0.73 ± 0.41, 0.83 ± 0.22, and 0.97 ± 0.37, respectively (control, 1.00 ± 0.30), without significant changes. Finally, concerning pgc5, the values were 0.39 ± 0.14, 0.72 ± 0.17, 0.67 ± 0.11, and 0.58 ± 0.30, respectively (control, 1.00 ± 0.38) (P<0.05, ANOVA), showing a significant decrease in the MNU group (P<0.01, Dunnett’s multiple comparisons test).

Discussion

In this study, we have demonstrated that γ-H2AX, in combination with mRNA levels of h2afx, p21waf1, and pga5, could be a powerful marker for evaluating chemical genotoxicity in the gastric mucosa.
Various molecular biological methods, such as pulsed-field gel electrophoresis (PFGE) and the comet assay, have been developed for detecting DSBs. However, with these methods, it is relatively difficult to quantify DSBs at a single-cell resolution. H2AX is phosphorylated at Ser 139 at DSB sites within 30 mins after DSB occurrence, and rapid accumulation of γ-H2AX can be indirectly detected by visualizing DSB sites as foci in nuclei using immunofluorescence or immunohistochemical staining. Moreover, pan or peripheral staining of γ-H2AX in nuclei, which was observed in the surface area of the fundic mucosa in this study, reflects apoptosis and various biological consequences. Considering that γ-H2AX foci were also accumulated in the proliferating zone in the control group, although in small numbers, the specificity of γ-H2AX foci visualization for DSB quantification should be carefully considered, as physiological phosphorylation of H2AX could occur in mitotic processes without a DNA damage response. It would be necessary to analyze other DSB-related protein expressions, such as 53BP1, for accurate quantification of DSBs. Histone H2AX mRNA, h2afx, was shown to be downregulated in the MNU and DMN groups despite γ-H2AX foci formation of being higher in the former. The molecular mechanism of the downregulation of mRNA expression and increase in foci formation is unclear. Thus, analyzing the expression changes in h2afx mRNA level at multiple time points, from the start of the experiment to experimental day 28, is necessary. We speculate that h2afx mRNA expression is regulated at the transcriptional and posttranscriptional levels. Tang et al. have demonstrated that the h2afx gene showed higher expression in oocytes of Dicer mutant mice. Dicer processes precursor microRNA (pre-miRNA) into mature miRNAs, which may posttranscriptionally regulate the target miRNAs. Three miRNAs, miR-22-3p, miR-409-3p, and miR-543-3p, were significantly downregulated in genotoxic agent-treated mouse liver, in which miR-22 overexpression was associated with an increased number of γ-H2AX. Considering the complexity of these findings, it is necessary to further analyze transcriptional regulation of h2afx as well as to perform an immunohistochemical analysis of a phosphorylated form of H2AX, especially in the gastric mucosa.

The protein p21 (WAF1/CIP1) is a cyclin-dependent kinase inhibitor (CDI), and it plays a role in the G1/S checkpoint, acting as one of the guardians of the genome in collaboration with p53. Tsuyma et al. demonstrated that exposure to X-rays induces dose-dependent accumulation of p21 through a p53 function. It also induces p21 overexpression in meiotic spermatoocytes in mouse and rat tests. p21 also acts as a downstream effector of the wild-type p53 tumor suppressor in response to chemical genotoxic agents. The gastric mucosa is exposed to endogenously formed nitroso compounds including DMN via dietary intake, but no association was revealed between DMN intake and gastric cancer risk. Furthermore, DMN has been shown to not induce unscheduled DNA synthesis nor stimulation of replicative DNA synthesis either in the rat stomachs. However, in the current experiment, p21 mRNA was upregulated in the stomach mucosae of the DMN group, which indicated possible genotoxicity. 1-Nitrosoindole-3-acetonitrile (NIAN), a food derived compound, showed direct-acting mutagenicity and induced gastric cancer only with the strong promotional effects of H. pylori infection and inflammation in Mongolian gerbils. Thus, further detailed analysis would be required to assess the gastric genotoxicity of DMN and to explain upregulation of p21 mRNA.

Pepsinogen 1, a protein product of pepsinogen c (pgc) produced in pyloric glands and fundic mucosa neck cells, showed reduced expression upon continuous carcinogen treatment; pyloric glands exhibiting this were called pepsinogen-altered pyloric glands (PAPG). In transgenic mice, complete loss of p53 in the null genotype enhanced the alteration of its expression in short-term experiments, but the heterozygotes required relatively longer exposure to the carcinogen to have reduced expression of the Pgc protein. These results prompted us to evaluate the mRNA expression of pepsinogens. Although the pgc mRNA level was not altered in the current short-term experiment, pga5, possessed in fundic chief cells, showed downregulation of its mRNA level, which could be caused by direct exposure to MNU and could be associated with increased apoptosis due to mucosal damage. In this study, RNA was isolated from the border of fundic and pyloric glands. In future study, the difference in mRNA levels of pgc and pga5 should be analyzed by in situ hybridization in both pyloric and fundic glands using formalin-fixed and paraffin-embedded tissue.

The method shown in this study is also important from the point of view of animal welfare. Generally, to assess the carcinogenicity of genotoxic chemical substances, performing long-term experiments of up to 2 years using a large number of animals is indispensable. However, detection of γ-H2AX and other markers may enable researchers to reduce the number of animals and shorten the experimental period. In particular, it is effective to assess a large number of compounds at the same time.

In conclusion, in the present study, we confirmed upregulation of γ-H2AX and alteration in mRNA levels of h2afx, p21, and pgc5 for rapid evaluation of gastric genotoxicity. Although further careful analyses are needed, γ-H2AX in combination with other gene expression analyses could be a useful biomarker in a short-term experiment on gastric chemical evaluation.

Disclosure of Potential Conflicts of Interest: We have no conflicts of interest to be declared.

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