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Advances in Brief

Differentially Expressed MHC Class II-associated Invariant Chain in Rat Stomach Pyloric Mucosa with *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine Exposure[1]

Chie Furihata,[2] Masashi Oka, Masami Yamamoto, Takashi Ito, Masao Ichinose, Kazumasa Miki, Masae Tatematsu, Yoshiyuki Sakaki, and Konrad Reske

Department of Molecular Oncology [C. F.] and Human Genome Center [T. I., Y. S.], Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, Japan; First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan [M. O., M. I., K. M.]; Laboratory of Pathology, Aichi Cancer Center Research Institute, 1-1, Kanokoden, Chigusa-ku, Nagoya 464, Japan [M. Y., M. T.]; and Institut für Immunologie, Johannes Gutenberg Universität, D-55101 Mainz, Germany [K. R.]

Abstract

Administration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a glandular stomach carcinogen, at the concentration of 100 μg/ml in drinking water for 8 days induced the appearance of a MHC class II-associated invariant chain in the target organ of stomach pyloric mucosa of male Lewis rats. The up-regulation of the MHC class II-associated invariant chain was revealed by fluorescent differential display analysis, reverse transcription-PCR, Northern blot, and histochemical staining. The appearance of MHC class II and MHC class I was also demonstrated by reverse transcription-PCR and Northern blot. The results suggest the involvement of MHC-controlled immune reactions in chemically-induced stomach carcinogenesis.

Introduction

We have studied various alterations in the initiating step of experimental rat stomach carcinogenesis (1–4). Previously, we reported that glandular stomach carcinogens induced: (*a*) c-*fos* and c-*myc* expression 30 min and 3 h after treatment, respectively (1); (*b*) unscheduled DNA synthesis and DNA single-strand scission after 2 h (2); (*c*) ornithine decarboxylase and replicative DNA synthesis after 24 h (3); and (*d*) a reduction of pepsinogen-1 2 weeks after treatment (4) in the rat stomach pyloric mucosa. Changes affecting the expression of other genes that are associated with carcinogenesis and cell proliferation are expected to occur in the initiation stage of stomach carcinogenesis. One novel approach for the search of differentially expressed transcripts is fluorescent differential display, an arbitrarily primed RT-PCR[3] fingerprinting technique (5). We applied the method to study changes in gene expression in the initiating stage of stomach carcinogenesis induced by MNNG. Consequently, we found the unexpected appearance of some immune system components, namely Ii, Ia, and MHC class I, in the target organ of rat stomach pyloric mucosa after exposure to MNNG.

Materials and Methods

Animal Exposure. Groups of five male 8-week-old Lewis rats (LEW/Crj; Charles River Japan, Inc., Yokohama, Japan) were given MNNG (Aldrich Chemical Co., Milwaukee, WI) or NaCl by gastric intubation. A third group of 6-week-old rats was given MNNG (100 μg/ml, the same concentration as that for long-term stomach carcinogenesis) in drinking water. A fourth group of 6-week-old rats was given 10% NaCl in the diet. Control rats were given distilled water instead of MNNG or NaCl. At the appropriate time, stomachs and small intestines were surgically removed and washed with cold PBS, and the mucosa was scraped with a blade, pooled, frozen in liquid nitrogen, and stored at −80°C until use. An additional group of 6-week-old rats was treated in a manner similar to that of the third and fourth groups, but the animals were sacrificed, and gastrointestinal tissues were fixed for histochemical examination.

RNA Isolation. Total RNAs were isolated by a modified acid-guanidine thiocyanate-phenol chloroform method using TRIzol (Life Technologies, Inc., Gaithersburg, MD) and ISOGEN (Nippon Gene, Toyama, Japan) reagents and stored at −80°C until use.

Differential Display Analysis. The first cDNA strand was prepared with 2.5 μg of total RNA and 50 pmol of $GT_{15}C$ by SuperScript II reverse transcriptase (Life Technologies, Inc.). The second strand (20 μl) was prepared with 0.02 of the first cDNA strand, 10 pmol of arbitrary 5′ primer (OPC-9, 5′-CTCACCGTCC-3′; Operon Technologies, Alameda, CA), and 5 pmol of $GT_{15}C$ by 1 unit of Taq DNA polymerase. The mixture was subjected to a low stringency PCR (5). PCR products were separated on a 5% polyacrylamide gel, stained with SYBR Green I (Molecular Probes, Eugene, OR), and visualized by scanning with a fluorescence image analyzer (FluorImager 575; Molecular Dynamics, Sunnyvale, CA). Gel pieces of the bands of interest were reamplified by PCR.

Cloning and Sequencing. Reamplified DNA fragments were cloned into the deoxyT-added *Eco*RV site of pT7Blue vector (Novagen, Madison, WI). After isolation, plasmid DNAs were sequenced using the BcaBEST dideoxy sequencing kit (Takara Shuzo Co., Ltd., Kyoto, Japan) with an ALFred DNA sequencer (Pharmacia LKB Biotechnology AB, Bromma, Uppsala, Sweden). Sequence data obtained were subjected to a homology search at nucleotide levels using the BLASTN program on the HGC computer at the Human Genome Center, Institute of Medical Science, University of Tokyo.

Analysis of the Obtained Clone. PCR with the specific primers and the first-strand cDNA was performed at high stringency (5). The amplified cDNAs were examined on a 5% polyacrylamide gel. For Northern blot analysis, total RNAs (30 μg) were resolved on a 1% agarose-2.2 M formaldehyde gel, blotted onto a Hybond-N membrane filter (Amersham, Buckinghamshire, United Kingdom), and then hybridized with a ^{32}P-labeled probe. Washed filters were exposed on an imaging plate and analyzed by BAS 2000A (Fuji Film, Tokyo, Japan). The specific oligonucleotide primers were as follows: Ii, 5′-TCACCGTCCCTGGACACAACAAAT-3′ (5′ primer) and 5′-CTACCTTTTATTGTCACGTGAACC-3′ (3′ primer) and 5′-CTCTGTCCTGGTGGCTCTGCTCTT-3′ (5′ primer) and 5′-AGTCTGGGTGGGCTGCTTCTCCTC-3′ (3′ primer); Ia, 5′-GTCTGGTAGGCATCGTCGTC-3′ (5′ primer) and 5′-TGGAGAAATGTCAAGCCGTAAGTG-3′ (3′ primer); MHC class I, 5′-TACCCAGAAAAGGAGAAGGAGAG-3′ (5′ primer) and 5′-GCCACAGCAGCAACGCAGAG-3′ (3′ primer); and β-actin, 5′-ATCGTGGGCCGCCCTAGGCA-3′ (5′ primer) and 5′-TGGCCTTAGGGTTCAGAGGGG-3′ (3′ primer).

Immunohistochemical Study. Immunohistochemical staining of Ii was performed (6) using mAb RG11 directed against the carboxyl-terminal segment of the rat invariant γ chain (7).

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[2] To whom requests for reprints should be addressed, at Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, Japan. Phone: 81-3-5449-5623; Fax: 81-3-5449-5423; E-mail: furi@ims.u-tokyo.ac.jp.

[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; mAb, monoclonal antibody; Ii, MHC class II-associated invariant chain; Ia, MHC class II.

Results

Differentially Expressed Ii. Fluorescent differential display analysis of RT-PCR of stomach pyloric mucosa cDNA shows that the band of 340 bp increased seven times in intensity at 2 weeks after the beginning of MNNG exposure (8 days of MNNG drinking and 6 days of tap water; Fig. 1*a*). The band was cloned and sequenced. The sequence was entirely identical to 324 bp of the 3′ end of Ii (1024–1348 from the origin of Ii cDNA). Fig. 1*b* shows that the band also increased after 27 days of MNNG drinking, although to a lesser extent than after 8 days of MNNG drinking. Fig. 1, *a* and *c*, shows that the band did not increase remarkably after NaCl administration by gastric intubation or at 2 weeks after the beginning of NaCl exposure.

RT-PCR with Specific Primers and Northern Blot. RT-PCR with specific primers and Northern blot confirmed that Ii increased severalfold in the stomach pyloric mucosa at 2 weeks after MNNG exposure (Fig. 2, *a-1* and *a-2*). The band increased slightly in the fundic mucosa. The abundant expression of Ii in the small intestine is shown as a positive control. We also examined the expression of related immune system components. Fig. 2 (*b-1, b-2, c-1,* and *c-2*) demonstrates increases in Ia and MHC class I in rat stomach pyloric mucosa at 2 weeks after MNNG exposure. Fig. 2 (*d-1* and *d-2*) shows control β-actin that confirmed the relative amount of RT-cDNA and total RNA.

Immunohistochemical Demonstration of Ii. Fig. 3 shows immunohistochemical staining of pyloric mucosa with an Ii-specific mAb. The staining pattern suggests that RG11-stained lymphocytes were increased in interstitial tissue in the pyloric mucosa at 2 weeks after MNNG exposure.

Discussion

The present results clearly showed that MHC components (Ia, Ii, and class I) appeared simultaneously in the stomach pyloric mucosa in the initiation stage of rat stomach carcinogenesis 2 weeks after MNNG administration (8 days of MNNG drinking and 6 days of tap water). The 6-day tap water period was allowed for restoration of the transient inflammation caused by MNNG.

Histochemical staining using a rat-specific mAb revealed antibody-stained lymphocytes in the interstitial tissue of the pyloric mucosa after MNNG exposure. The life span of surface mucous cells and pyloric gland cells in the rat stomach pyloric mucosa is 3 days and 11–13 days, respectively (6). Thus, most of the epithelial cells were replaced 2 weeks after the beginning of MNNG treatment. The results suggested that changes in epithelial cells triggered the entry of lymphocytes into the interstitial tissue in the pyloric mucosa. This is the first demonstration of interstitial tissue alteration in the pyloric mucosa during the initiation stage of stomach carcinogenesis. Up-regulation of Ii in the pyloric mucosa continued after 27 days of MNNG drinking. In contrast, up-regulation of MHC components was not observed in the stomach fundic mucosa as studied by fluorescent

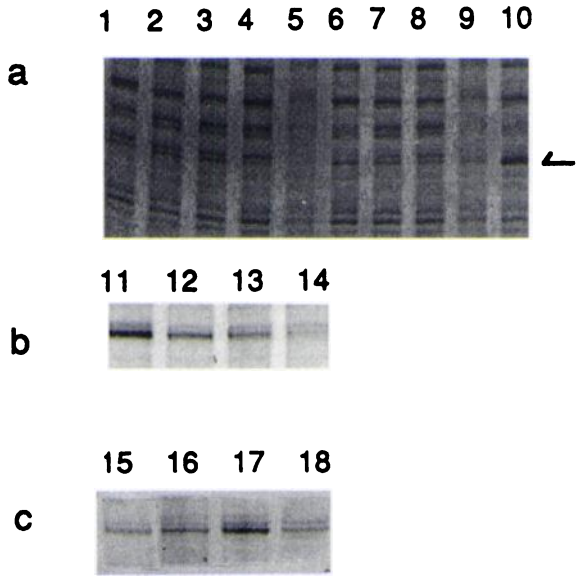

Fig. 1. Fluorescent differential display of PCR products from cDNA derived from total RNA of rat stomach pyloric mucosa. *a*, 0 and 30 min and 3, 20, and 48 h after NaCl gastric intubation (0.8 g/kg body weight), *Lanes 1–5*; 30 min and 3, 20, and 48 h after MNNG gastric intubation (50 mg/kg body weight), *Lanes 6–9*; and 2 weeks after the beginning of MNNG exposure [8 days of MNNG drinking (100 μg/ml) and 6 days of tap water), *Lane 10*. A 7-fold increase in the 340-bp band was observed in *Lane 10* (*arrow*). *b*, MNNG drinking at 2 weeks (8 days of MNNG and 6 days of tap water), 27 days, and 34 days (27 days of MNNG and 7 days of tap water) and 0, *Lanes 11–14*. *c*, 0 and 3 days of MNNG drinking, 2 weeks after MNNG administration (8 days of MNNG drinking and 6 days of tap water) and 2 weeks after NaCl administration (8 days of 10% NaCl in the diet and 6 days of tap water), *Lanes 15–18*.

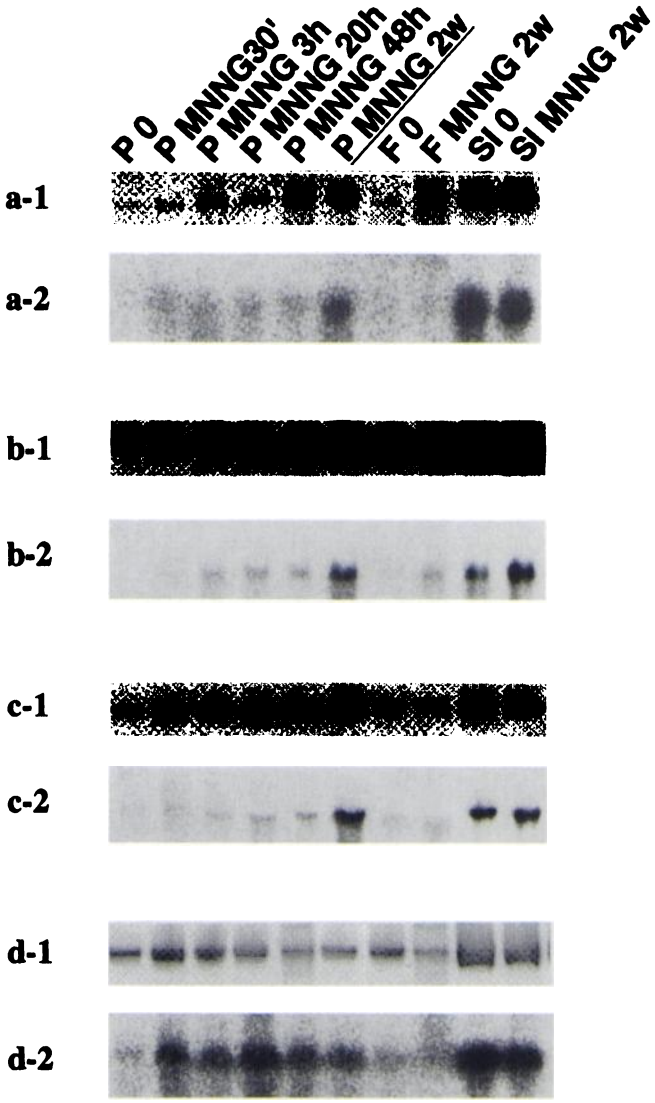

Fig. 2. RT-PCR with specific primers for Ii (*a-1*), Ia (*b-1*), MHC class I (*c-1*), and β-actin (*d-1*) and Northern blot for Ii (*a-2*), Ia (*b-2*), MHC class I (*c-2*), and β-actin (*d-2*), respectively, after MNNG administration. Total RNAs were from pylorus (*P*) at 0 and 30 min; 3, 20, and 48 h; and 2 weeks (30 μg/lane); fundus (*F*) at 0 and 2 weeks (30 μg/lane); and small intestine (*SI*) at 0 and 2 weeks (10 μg/lane). Increases in Ii, Ia, and MHC class I at 2 weeks in the pylorus (*P MNNG 2w*) were observed. β-Actin is shown as the control for RT-cDNA and total RNA amount.

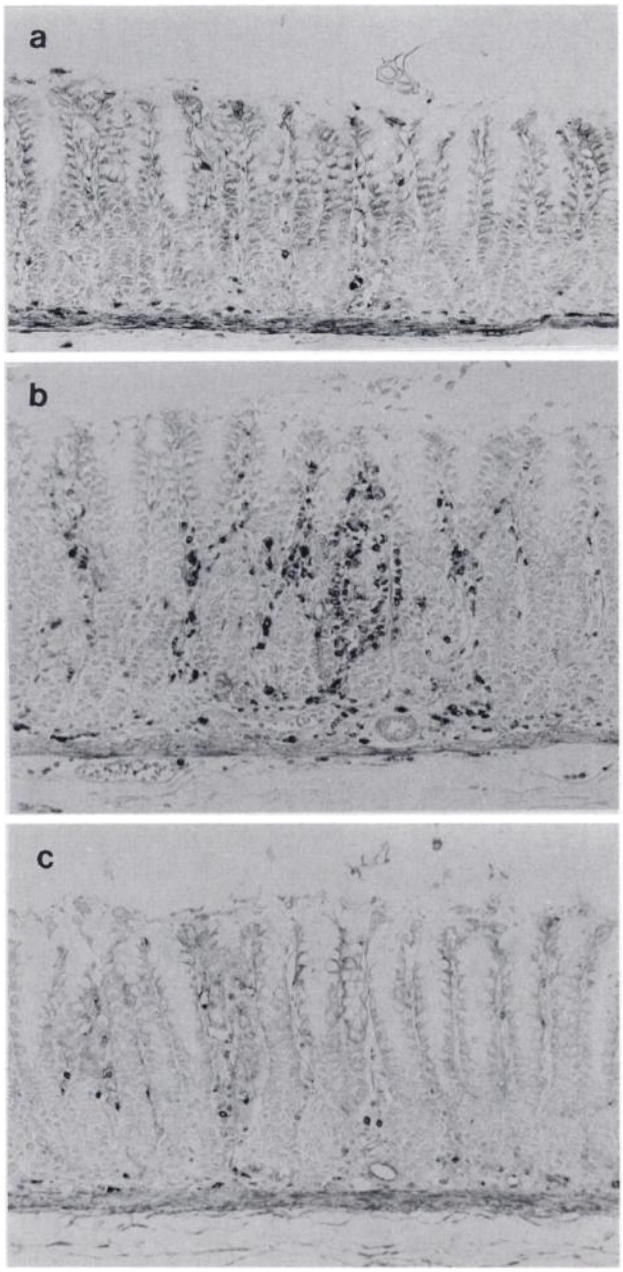

Fig. 3. Immunohistochemical staining of Ii in the pyloric mucosa after MNNG and NaCl administration. Samples were pylorus at time 0 (*a*), 2 weeks after MNNG administration (*b*), and 2 weeks after NaCl administration (*c*). Antibody (RG11 directed against rat Ii)-stained lymphocytes (*dark cells*) were increased in the interstitial tissue of the pyloric mucosa after MNNG treatment. The pylorus was hyperplastic in *b* and *c*. Magnification, ×200.

differential display, RT-PCR, Northern blot, and histochemical staining, suggesting that the effect of MNNG occurs specifically in the pyloric mucosa. Concerning the MNNG induction of adenocarcinomas, these tumors are numerous in the pyloric mucosa but rare in the fundic mucosa. Therefore, these results suggested that MHC components appeared only in the target organ of stomach carcinogenesis in the present study. The presence of Ia and Ii in normal small intestinal mucosa and the lack of Ia and Ii in normal stomach mucosa have also been observed in mice (8).

Conversely, NaCl, a glandular stomach tumor promoter, did not cause the appearance of MHC components at 2 weeks after administration. High concentrations of NaCl induced rapid damage of the surface mucous cell layer of the stomach mucosa, resulting in transient cell proliferation during recovery (9). Thus the *de novo* expression of MHC components is suggested to occur in response to the carcinogen but not to the tumor promoter of rat stomach carcinogenesis.

Altered MHC class I and II expression has been reported in several tumors (10, 11), and its relationship to progression and metastasis was proposed, but its role in the initiation stage was not hypothesized. The present results suggest early triggering of the immune system during the initiation stage of rat stomach carcinogenesis.

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