Detection of \(N^\epsilon\)-(hexanoyl)lysine in the tropomyosin 1 protein in \(N\)-methyl-\(N'\)-nitro-
\(N\)-nitrosoguanidine-induced rat gastric cancer cells

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\(N^\epsilon\)-(hexanoyl)lysine, formed by the reaction of lysine with n-6 lipid hydroperoxide, is a lipid peroxidation marker during the initial stage of oxidative stress. The aim of the present study is to identify \(N^\epsilon\)-(hexanoyl)lysine-modified proteins in neoplastic transformed gastric mucosal cells by \(N\)-methyl-\(N'\)-nitro-\(N\)-nitrosoguanidine, and to compare the levels of these proteins between gastric mucosal cells and normal gastric cells. Much greater fluorescence of 2-[\(\epsilon\)-(4'-hydroxy)phenoxyl-3\(\epsilon\)-xanthene-3-on-9\(\epsilon\)]benzoic acid, an index of the intracellular levels of reactive oxygen species, was observed for gastric mucosal cells compared to normal gastric cells. \(N^\epsilon\)-(hexanoyl)lysine-modified proteins were detected by SDS-PAGE or two-dimensional electrophoresis and Western blotting using anti-\(N^\epsilon\)-(hexanoyl)lysine polyclonal antibody, and a protein band of between 30-40 kDa was clearly increased in gastric mucosal cells compared to normal gastric cells. Two \(N^\epsilon\)-(hexanoyl)lysine-modified protein spots in gastric mucosal cells were identified as the tropomyosin 1 protein by mass spectrometry using a MASCOT search. The existence of \(N^\epsilon\)-(hexanoyl)lysine modification in tropomyosin 1 was confirmed by Western blotting of SDS-PAGE-separated or two-dimensional electrophoresis-separated proteins as well as by the immunoprecipitation with anti-tropomyosin 1 antibody. These data indicate that \(N^\epsilon\)-(hexanoyl)lysine modification of tropomyosin 1 may be related to neoplastic transformation by \(N\)-methyl-\(N'\)-nitro-\(N\)-nitrosoguanidine in gastric epithelial cells.

Key Words: lipid peroxide, gastric cancer, hexanoyl-lysine, tropomyosin 1

Several in vivo cancer models support the hypothesis that lipid peroxidation plays a critical role in experimental carcinogenesis.1,2,4 In chemically induced mammary tumor animal models, high fat diets are associated with increased tumor incidence, and this effect is diminished by antioxidants (vitamin E and selenium), observations that would support a role of lipid peroxidation.4,5 Lipid peroxidation under excessive oxidative stress may play a crucial role in oxidative modification in the process of carcinogenesis, because reactive lipid-decomposition products trigger secondary modification of proteins and nucleic acids. Modification of DNA bases and proteins by reactive aldehydes, such as 4-hydroxy-2-nonenal (HNE), malondialdehyde, and acrolein, is thought to contribute to the mutagenic and carcinogenic effects associated with oxidative stress-induced lipid peroxidation.5 In addition to aldehyde-derived protein modification, a novel amide-type adduct, \(N^\epsilon\)-(hexanoyl)lysine (HEL), was chemically identified from a reaction mixture containing lysine and 13-hydroperoxyoctadecadienoic acid (13-HPODE).6 Kato et al.7 have demonstrated that HEL is formed by the reaction of lysine with n-6 lipid hydroperoxide, which is known to be an early product of the lipid peroxidation process, suggesting that HEL might be a lipo-oxidative stress marker during the initial stage of oxidative stress. HEL has been immunohistochemically identified in several tissues by using polyclonal or monoclonal antibodies to HEL.8,9,10 The excretion of HEL into human urine was confirmed by liquid chromatography tandem mass spectrometry (LC/MS/MS).11,12 However, there have been no studies investigating the role of 13-HPODE- or HEL-modified proteins in cancer tissues or cells.

We recently succeeded in the establishment of a novel, transformed cell line (RGK-1) derived from a normal gastric mucosal cell line (RGM-1) of Wistar rats after treatment with the alkylating carcinogen \(N\)-methyl-\(N'\)-nitro-\(N\)-nitrosoguanidine (MNNG).13 This cell line showed signs of neoplasia and transformation, in that it lost contact inhibition and formed tumors in nude mice. RGK-1 cells are the first MNNG-induced neoplastic mutant cells derived from a noncancerous, nonembryonic gastric epithelial cell line (RGM-1). This establishment of RGK-1 cells has made it possible to investigate gastric carcinogenesis using two paired cell lines: RGM-1 and RGK-1 cells. In the present study, HEL-modified proteins were detected by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and Western blotting using a novel monoclonal antibody against HEL, and determined by peptide mass fingerprinting using MALDI-TOF MS and the MASCOT search engine by comparing protein spots derived from RGM-1 and RGK-1 cells.

Materials and Methods

Antibodies. Anti-HEL polyclonal antibody was kindly provided by Professor Yoji Kato (University of Hyogo, Hyogo, Japan). Tropomyosin-1 (TPM1) monoclonal antibody (D12H4, XP Rabbit) and Horseradish peroxidase (HRP)-linked anti-rabbit IgG were purchased from Cell Signaling (Beverly, MA). Anti-actin antibody was purchased from Abcam Inc. (Cambridge, MA). HRP-linked anti-rabbit IgG was purchased from Cell Signaling Technology Inc.

Cell culture. We used the rat gastric mucosal cell line RGM-1 (RCB-0876 at Riken Cell Bank, Tsukuba, Japan), which was established by Matsui and Ohno,14 and an MNNG-induced

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mutant of a rat murine RGM-1 gastric epithelial cell line, which was named RGK-1. Procainamide was added to RGM-1 and RGK-1 cells and incubated at 37°C in a humidified chamber with 5% CO₂, and were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium/Ham’s F12 medium (Wako Pure Chem., Osaka, Japan) supplemented with 5% heat-inactivated fetal calf serum, 1% penicillin and streptomycin. The cell culture was subcultured every 3–4 days.

**Cellular microscopic fluorescence analysis.** RGM-1/RGK-1 cells were incubated on a Lab-Tec II slide chamber (Nalge Nunc International, Rochester, NY) at a concentration of 10⁵ cells/mL. The cells were then observed with a chilled CCD camera (AxioCam color, Zeiss, Jena, Germany) mounted to the manufacturer’s protocol. Protein concentrations were determined with a Plus One 2D Clean-up kit (GE Healthcare UK Ltd., Buckinghamshire, England) according to the manufacturer’s instructions.

**Sample preparation for proteinomics.** Cells were collected after washing with PBS and were lysed in Lysis buffer (8 M urea, 4% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris-HCl). The samples were centrifuged for 20 min at 12,000 × g, 24°C. Contaminants in the supernatants were removed using a Plus One 2D Clean-up kit (GE Healthcare UK Ltd., Buckinghamshire, England) according to the manufacturer’s protocol. Protein concentrations were determined by using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA).

**Western blotting.** The protein samples (10 μg) were supplemented with NuPAGE LDS Sample buffer containing Sample Reducing Agent (Invitrogen Japan K.K., Tokyo, Japan) and boiled for 10 min at 70°C. The protein samples were applied to 12% Bis-Tris Gels (Invitrogen Japan K.K.) at 200 V for 55 min. IPG strips, pH 4–7 (Invitrogen Japan K.K.) were rehydrated overnight at room temperature with rehydration buffer (8 M Urea, 2% CHAPS, 30 mM Tris-HCl). The samples were centrifuged at 12,000 × g, 25°C. The gels were run using an Xcise Proteomics System (Shimadzu Co., Kyoto, Japan). Isoelectric focusing was performed using a ZOOM IPG Runner system (Invitrogen Japan K.K., Tokyo, Japan). Isoelectric focusing was started at 175 V, and the voltage was gradually increased to 2,000 V for 45 min and further held at 2,000 V for 40 min. The strips were treated with initial equilibration buffer containing NuPAGE LDS Sample buffer and Sample Reducing Agent for 15 min and a second equilibration buffer containing NuPAGE LDS Sample buffer and iodoacetamide. The 2-DE was conducted on NuPAGE 4–12% BisTris Gel at 200 V for 55 min.

**Immunoprecipitation.** Immunoprecipitation was performed using a Protein A HP SpinTrap column (GE Healthcare UK Ltd.). The TPM1 antibody was incubated with Protein A Sepharose for 30 min at room temperature. After washing with PBS buffer, protein samples (800 μg) were incubated with the Protein A Sepharose for 1 h at room temperature. Immunoprecipitates were washed with PBS and boiled with NuPAGE LDS Sample buffer (2×) (Invitrogen Japan K.K.) for 10 min at 70°C. Immunoprecipitated proteins were subjected to Western blotting.

**Results**

**HPF dye fluorescence image.** We compared intracellular bioimages of ROS generation using HPF fluorescence dyes for RGM-1 and RGK-1 cells. As shown in Fig. 1, the HPF image showed clear fluorescence in the cytosol around the nucleus. Much greater fluorescence was observed in RGK-1 cells compared to RGM-1 cells.

**Detection of HEL in proteins from RGM-1 and RGK-1 cells.** To detect HEL-modified proteins in RGM-1 and RGK-1 cells, whole proteins from cells were separated by SDS-PAGE or 2-DE, and analyzed by Western blotting using anti-HEL polyclonal antibody. As shown in Fig. 2, HEL-modified proteins were detected in RGM-1 and RGK-1 cells, and the protein band between 30–40 kDa was increased in RGK-1 cells compared with RGM-1 cells. Furthermore, as shown in Fig. 3, HEL-modified protein spots were increased in RGK-1 cells compared to RGM-1 cells.

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**Fig. 1.** Phase-contrast micrographs of RGM-1 cells (a) and mutant RGK-1 cells (b). HPF fluorescence of RGM-1 cells (c) and mutant RGK-1 cells (d) was detected with 490 and 515 nm bandpass filters for excitation and emission, respectively.
Identification of HEL-modified proteins in RGK-1 cells.

To identify the two HEL-modified protein spots in RGK-1 cells, the protein spots were excised from 2-DE gel stained with CBB, and analyzed by mass spectrometry. The data obtained using mass spectrometry (Fig. 4) were searched using the MASCOT search engine, and both protein spots were identified as the TPM1 protein with a MASCOT score of 65 and 39% sequence coverage.

Detection of HEL-modified TPM1 protein in RGK-1 cells.

To confirm the presence of HEL-modified TPM1 in RGM-1 and RGK-1 cells, we detected TPM1 and HEL using Western blotting of SDS-PAGE-separated or 2-DE-separated proteins. As shown Fig. 5a, the protein band between 30–40 kDa reacted with both anti-TPM1 antibody and anti-HEL antibody. The TPM1 protein expression level was increased in RGK-1 cells compared with RGM-1 cells and was modified by HEL in RGK-1 cells. Moreover, as shown Fig. 5b, the HEL-modified protein spots reacted with anti-TPM1 antibody.

To establish the existence of HEL modification of the TPM1 protein, immunoprecipitation with anti-TPM1 antibody was performed using proteins from RGM-1 and RGK-1 cells, and the immunoprecipitated proteins were analyzed using Western blotting with anti-HEL antibody and anti-TPM1 antibody. As shown Fig. 5c, the TPM1 protein was modified by HEL in RGK-1 cells. Moreover, the TPM1 protein expression level was increased in RGK-1 cells compared to RGM-1 cells.

**Fig. 2.** Detection of HEL-modified proteins from RGM-1 and RGK-1 cells by SDS-PAGE. Protein extracts were separated by SDS-PAGE and were analyzed by Western blotting with anti-HEL polyclonal antibody.

**Fig. 3.** Detection of HEL-modified proteins from RGM-1 and RGK-1 cells by 2-DE. Proteins extracts were separated using 2-DE. The 2-DE gels were transferred onto nitrocellulose membranes and stained with Deep Purple Total Stain to detect total proteins. The membranes were subjected to Western blotting with anti-HEL antibody.
Discussion

In the present study, we observed much greater HPF fluorescence in transformed RGK-1 compared to normal gastric mucosal cells, detected HEL-modified proteins by immunoblotting using an anti-HEL-antibody, and confirmed that an HEL-modified TPM1 protein was present only in RGK-1 cells based on peptide mass fingerprinting using MALDI-TOF MS and the MASCOT search engine as well as immunoprecipitation. In contrast, the expression level of TPM1 in RGM-1 was decreased compared to RGK-1 cells, and HEL-modified TPM1 was not detected. These data indicate that HEL modification in TPM1 is related to neoplastic transformation by NMMG in the gastric epithelial RGM-1 cell line.

To compare the intracellular generation of ROS between the two cell types, HPF fluorescence was used in the present study. It has been reported that HPF is a fluorescence dye that clearly and precisely detects intracellular ROS generation from the two cell types, HPF fluorescence was used in the present study. HPF fluorescence was used in the present study. After the in-gel digestion, the peptides were analyzed by AXIMA-CFR plus. Mass spectra were used for protein identification using the MASCOT Search Engine. Both spots were identified as TPM1.

It has also been shown that the formation of HEL is a good marker for oxidative modification by oxidized n-6 fatty acids such as linoleic acid and arachidonic acid. Kato et al. evaluated muscular oxidation injury induced by excessive exercise using a novel anti-HEL antibody, and showed that flavonoid supplementation clearly reduced the HEL-positivity in muscular tissues, suggesting that this compound would be useful for reducing oxidation injury. We also recently demonstrated that HEL-modification of carnitine palmitoyltransferase I (CPT I), a rate-limiting mitochondrial enzyme in the oxidation of fatty acids in muscle, was increased by exercise, while astaxanthin, an antioxidative carotenoid, prevented this increase, indicating that the prevention of oxidative modification of CPT I is one of the mechanisms that might explain the effect of astaxanthin on muscle metabolism. In the present study, we succeeded in detecting HEL-modified proteins by immunoblotting using anti-HEL-antibody. Among these spots, we demonstrated the existence of HEL modification of TPM1 protein by MALDI-TOF peptide mass fingerprinting and a MASCOT database search in the 2-DE gel obtained from RGK-1 cells. To further confirm the presence of HEL-modified TPM1, we detected TPM1 and HEL by Western blotting in the protein sample obtained by immunoprecipitation with anti-TPM1 antibody. To the best of our knowledge, TPM1 is the first protein for which HEL modification has been determined by the 2D-PAGE and MS.

Although TPM isoforms have been known to function in the regulation of muscle contraction, the functional significance of the multiple TPM isoforms present in nonmuscle cells, especially in cancer cells, remains largely unknown. To date, decreases in TPM1 expression levels have been reported in human breast cancer, prostate cancer, and esophageal squamous cell carcinoma, while increases in TPM1 expression have been observed in urinary bladder cancer, inflammation-related colon cancer, and gastric adenocarcinoma. Interestingly, such opposite expression regulation for different TPM isoforms has often been found in tumors, implying that these isoforms may have different functions in cell transformation. These data suggest that the diametrically opposed regulations of TPM isoforms may cause an imbalance in normal phenotypes of epithelial microfilaments and lead to malignant morphological changes of aberrant cells.

The most striking observation from the present study was that HEL-modified TPM1 was identified even though the total protein levels in transformed gastric cancer cells were higher than those in normal gastric cells. It has been reported that the regulation of TPM1 expression appears to be mediated by TPM1 promoter methylation, histone deacetylation, and microRNA-21. In addition to these regulations, the present study indicates the
possibility that post-translational modification of the TPM1 protein under excessive oxidative stress may affect its function. As shown in previous studies, oxidative stress-induced modification of several proteins by HNE addition, carbonylation, or tyrosine nitration impairs their functions.\textsuperscript{17,31,32} As shown in our previous study, HEL modification affected the function of the CPT1 protein by inhibiting its interaction with cofactor protein FAT/CD36. The present study extends our knowledge about the regulation of TPM1, a tumor suppressor protein. Thus, in addition to epigenetic and translational regulations, TPM1 is also regulated by post-translational modification. It is our expectation that the molecular mechanism of HEL modification and the functional influence of this modification on the TPM1 protein will be identified in the near future.

Fig. 5. Detection of HEL and TPM1 in RGM-1 and RGK-1 cells. Whole proteins from cells were separated by SDS-PAGE (a) or 2-DE (b) and analyzed by Western blotting with anti-HEL polyclonal antibody and anti-TPM1. Proteins from RGM-1 and RGK-1 cells were immunoprecipitated by anti-TPM1 antibody. Immunoprecipitated proteins were subjected to Western blotting with anti-HEL polyclonal antibody and anti-TPM1 antibody (c).

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**Abbreviations**

| Acronym | Description           |
|---------|-----------------------|
| CAN     | acetonitrile          |
| 4-CHCA  | α-cyano-4-hydroxycinnamic acid |
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