RESEARCH ARTICLE

Involvement of heme oxygenase-1 in Korean colon cancer

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Abstract Heme oxygenase-1 (HO-1) catabolizes heme into carbon monoxide, biliverdin, and free iron which mediate its protective effect against oxidative stress. The aim of the present study was to determine the expression level and activity of HO-1 in Korean colon cancer tissues and cell lines. HO-1 protein expression was higher (>1.5-fold) in tumor tissues than in adjacent normal tissues in 14 of 20 colon cancer patients, and HO-1 protein expression was closely correlated with HO-1 enzyme activity in cancer tissues. Immunohistochemical data confirmed that HO-1 protein was expressed at a higher level in colon cancer tissues than in normal mucosa. Furthermore, HO-1 mRNA and protein expression and enzyme activity were higher in the colon cancer cell lines Caco-2, SNU-407, SNU-1033, HT-29, and SW-403 than in the normal fetal human colon cell line FHC. Treatment with the HO-1 inhibitor zinc protoporphyrin decreased the viability of colon cancer cell lines. These data indicate that HO-1 may serve as a clinically useful biomarker of colon cancer and as a target for anticolon cancer drugs.

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Introduction

Nearly one million cases of colon cancer are diagnosed worldwide each year, and an increasing trend in the incidence of colon cancer in Asian countries, including Korea, has been reported in recent years [1, 2]. Recent epidemiologic studies have indicated that a western style diet is closely associated with a high incidence of colon cancer [3, 4]. The presence of iron ions in the colon increases the production of reactive oxygen species (ROS) from peroxides via the Fenton reaction, and the prooxidant environment of the colon may contribute to greater cancer susceptibility [5, 6]. However, the mechanism by which colon cancers acquire resistance to ROS-induced cell death is not well understood. Therefore, a precise understanding of the relationship between colon cancer and oxidative stress-induced molecules is very important. Heme oxygenase-1 (HO-1) represents a prime cellular defense mechanism against oxidative stress via the antioxidant function of its catalytic products including bilirubin, carbon monoxide, and concomitant induction of iron-sequestering ferritin [7, 8]. HO-1 expression is induced in response to oxidative stresses caused by various chemical or physiological factors in cells and tissues, reflecting the main role of this enzyme in the protection against oxidative injury [9–11]. In addition, HO-1 regulates cell proliferation, modulates the inflammatory response, and facilitates angiogenesis [12, 13]. HO-1 is expressed at higher levels in oral squamous carcinoma, pancreatic cancer, hepatoma, and colon cancer than in surrounding normal tissue [14–17], suggesting that highly HO-expressed cancer cells offer a growth advantage and provide cellular resistance against ROS-mediated anticancer therapy [18, 19]. A recent

research has identified that HO-1 could be considered as a biomarker and potential therapeutic target for advanced prostate cancer [20]. The present study investigates the involvement of HO-1 in Korean human colon cancers and colon cancer cell lines.

Materials and methods

Reagents

Primary rabbit polyclonal HO-1 antibody was purchased from Calbiochem (San Diego, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and zinc protoporphyrin (ZnPP) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Patient tissues

Twenty tissue samples from human colon cancer patients were obtained from Jeju National University Hospital (Jeju, Republic of Korea). This study was approved by the institutional review board for ethics of Jeju National University Hospital (IRB:2011-38) and by informed written consent from patients.

Cell culture

The human colon cancer cell lines Caco-2, SNU-407, SNU-1033, HT-29, and SW-403 were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea), and the normal fetal human colon cell line FHC was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2. SNU-407, SNU-1033, HT-29, and SW-403 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), streptomycin (100 μg/ml), and penicillin (100 units/ml). Caco-2 cells were cultured in MEM medium containing 10% heat-inactivated FCS, streptomycin (100 μg/ml), and penicillin (100 units/ml). Human colon FHC cells were cultured in a 1:1 mixture of Ham's F12 and DMEM containing HEPES (25 mM), cholera toxin (10 ng/ml; Calbiochem-Novabiochem Corp., La Jolla, CA, USA), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (100 ng/ml), and 10% FCS.

Western blot analysis

Cells were lysed on ice for 30 min in 100 μl of lysis buffer (120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP-40) and centrifuged at 13,000×g for 15 min. Supernatants were collected, and the protein concentration was determined. Aliquots containing 40 μg of protein were boiled for 5 min and electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes, which were subsequently incubated with HO-1 antibody overnight at 4°C. The membranes were further incubated with secondary anti-immunoglobulin-G-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

HO-1 activity

HO-1 enzyme activity in colon tissues was measured as described previously [20]. Briefly, colon tissues or cells were homogenized in 0.5 ml ice-cold 0.25 M sucrose solution containing 50 mM potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 200×g for 10 min, and the supernatants were further centrifuged at 15,000×g for 60 min. The pellet was then resuspended in 50 mM potassium phosphate buffer (pH 7.4), and the amount of protein was determined. The reaction mixture (200 μl) containing 0.2 mM of the substrate hemin, 500 μg/ml of cell lysate, 0.5 mg/ml rat liver cytosol as a source of biliverdin reductase, 0.2 mM $MgCl_2$, 2 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, 1 mM NADPH, and 50 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C for 2 h. The reaction was stopped with 0.6 ml of chloroform and, after extraction, the chloroform layer was measured spectrophotometrically at 464 nm. HO-1 activity is expressed as nanomole of bilirubin per milligram of protein.

Immunohistochemistry for HO-1

Tissue specimens were fixed in 10% buffered formalin and embedded in paraffin. The same paraffin-embedded tissues as those used for the original hematoxylin-and-eosin-stained sections were used for immunohistochemistry. Tissue blocks were cut into 3-μm-thick slices and mounted on Superfrost Plus-coated slides. Sections were then deparaffinized in xylene and rehydrated through a graded ethanol series. A standard immunohistochemical technique was performed using a Ventana BenchMark XT immunostainer with HO-1 antibody at a dilution of 1:100. Antigen retrieval on the immunostainer was done for 30 min. The HO-1 antibody was incubated at 37°C for 60 min, and 3,3′-diaminobenzidine was used as a chromogen; slides were counterstained with hematoxylin prior to mounting. All staining procedures were performed according to the manufacturer's recommendations. Intramucosal mononuclear cells in the tissue samples served as internal positive controls based on their strong staining intensity. Negative controls for nonspecific binding were obtained by omitting the primary antibody.

Fig. 1 Protein level and activity of HO-1 in colon cancer patients. **a** The expression of the HO-1 protein was detected using an HO-1-specific antibody. **b** HO-1 enzyme activity in colon tissues was measured as described in the "Materials and methods". HO-1 activity is expressed as nanomole of bilirubin per milligram of protein. **c** Immunohistochemistry for HO-1 in colon carcinoma tissues showed (*a*) hematoxylin–eosin staining (×100), (*b*) positive-stained mucosal histocytes and faint expression in normal epithelial cells (×200), (*c*) transition area from normal to carcinoma (×100), (*d*) and (*e*) diffuse moderate expression in carcinoma cells (×200), and (*f*) strongly positive carcinoma cells (×400)
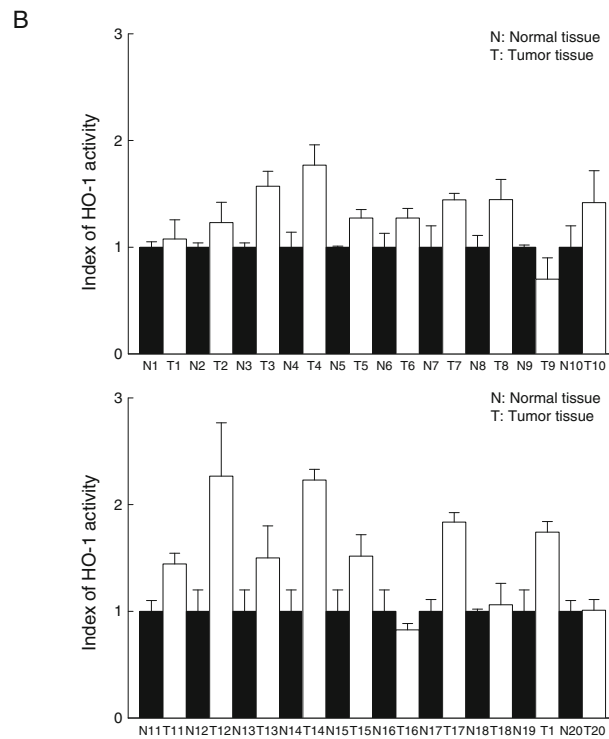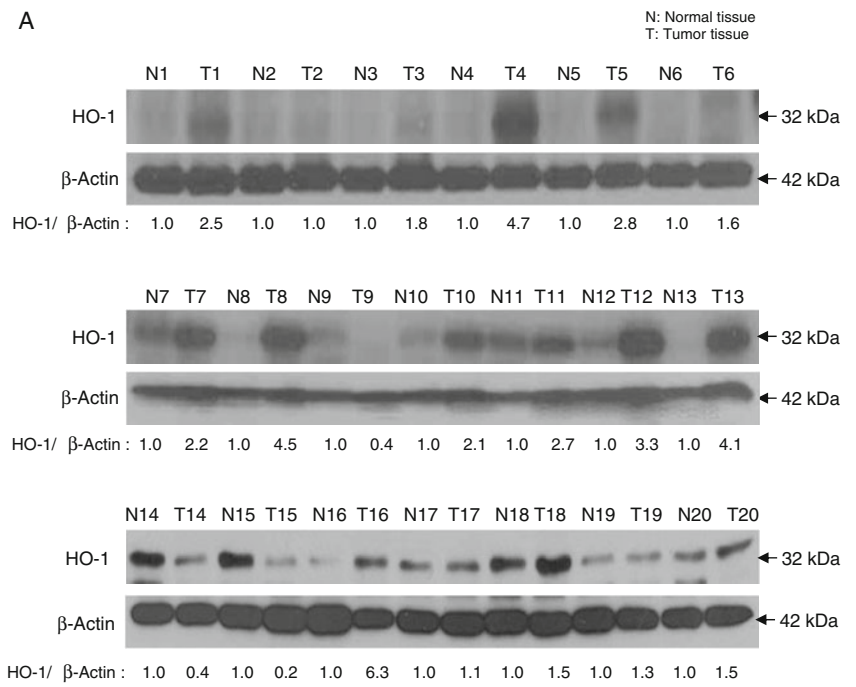

The immunohistochemical slides were evaluated and interpreted by a pathologist.

Reverse transcription polymerase chain reaction

Total RNA was isolated using Trizol (GibcoBRL, Grand Island, NY, USA). Reverse transcription polymerase chain reaction (RT-PCR) was performed as described previously [21]. PCR conditions for HO-1 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 35 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 60 s. The primer pairs (Bionics, Seoul, Republic of Korea) were as follows (forward and reverse, respectively): HO-1, 5′-GAGA ATGCTGAGTTCATG-3′ and 5′-ATGTTGAGCAGGA AGGC-3′; and GAPDH, 5′-AAGGTCGGAGTCAACGG ATTT-3′ and 5′-GCAGTGAGGGTCTCTCTCCT-3′. Amplified

Fig. 1 (continued)

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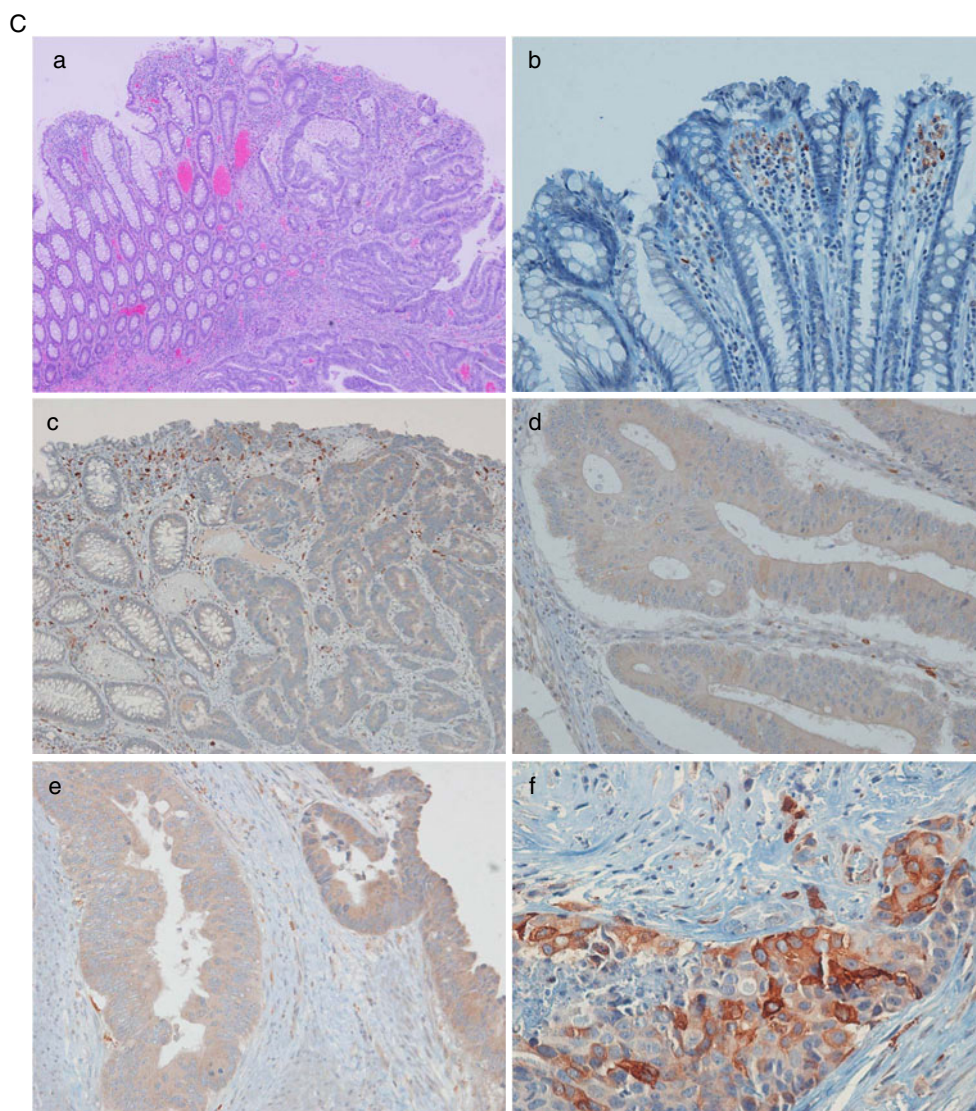
products were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

Quantitative real-time PCR

A total of 2 μg RNA was used in the first strand cDNA synthesis using the SuperScript kit (Invitrogen, Carlsbad, CA, USA). The cDNA was then diluted to a volume of 50 μl, of which 1 μl was used for amplification. Quantitative real-time PCR was performed with 2× SYBR Green Master Mix (Invitrogen, Carlsbad, CA, USA) and 900 nM primers for human HO-1 and 18 s RNA. The real-time PCR machine iQ5 (Bio-Rad Laboratories, Hercules, CA, USA) was used to amplify and detect the transcripts of interest. The real-time PCR parameters used were as follows: 50°C for 2 min, Taq activation at 95°C for 10 min, and 40 cycles of 95°C for 15 s and 55°C for 1 min. Reactions were performed in duplicate,

and specificity was monitored using melting curve analysis after cycling. Primers were designed using Bioneer (Seoul, Republic of Korea). The primers used were as follows (3′–5′): 18 s RNA, 5′-CAGCCACCCCAGATTGAGCA-3′ and 5′-TAGTAGCGACGGGCGGTGTG-3′; and HO-1, 5′-TGAGG AACTTTCAGAAGGGCC-3′ and 5′-TGTTGCGCTCA ATCCCTCC-3′. Data were analyzed using the iQ5 software package. The relative standard curve method was used to calculate relative mRNA abundance between samples, which was then presented as mean ± standard error of the mean of gene expression. All data were first tested for normality, and data with nonnormal distribution were subjected to square root transformation prior to statistical analyses. The comparative cycle threshold (Ct) method was used to calculate the relative changes in gene expression in the iQ5 real-time PCR system. The 2-delta-delta Ct value was calculated after 18 s RNA normalization [22].

Fig. 2 HO-1 mRNA and protein expression and protein concentration and activity in normal colon and cancer cell lines. The HO-1 mRNA level was detected using **a** RT-PCR and **b** real-time PCR analyses. *$p<0.05$, significantly different from FHC cells. **c** HO-1 protein level was detected using western blot analysis. **d** HO-1 enzyme concentration in colon cell lines was quantified using a human HO-1 ELISA kit. HO-1 concentration is expressed as nanogram per milliliter. *$p<0.05$, significantly different from FHC cells. **e** HO-1 enzyme activity in normal colon and cancer cell lines was measured as described in the "Materials and methods". HO-1 activity is expressed as nanomole of bilirubin per milligram of protein. *$p<0.05$, significantly different from FHC cells
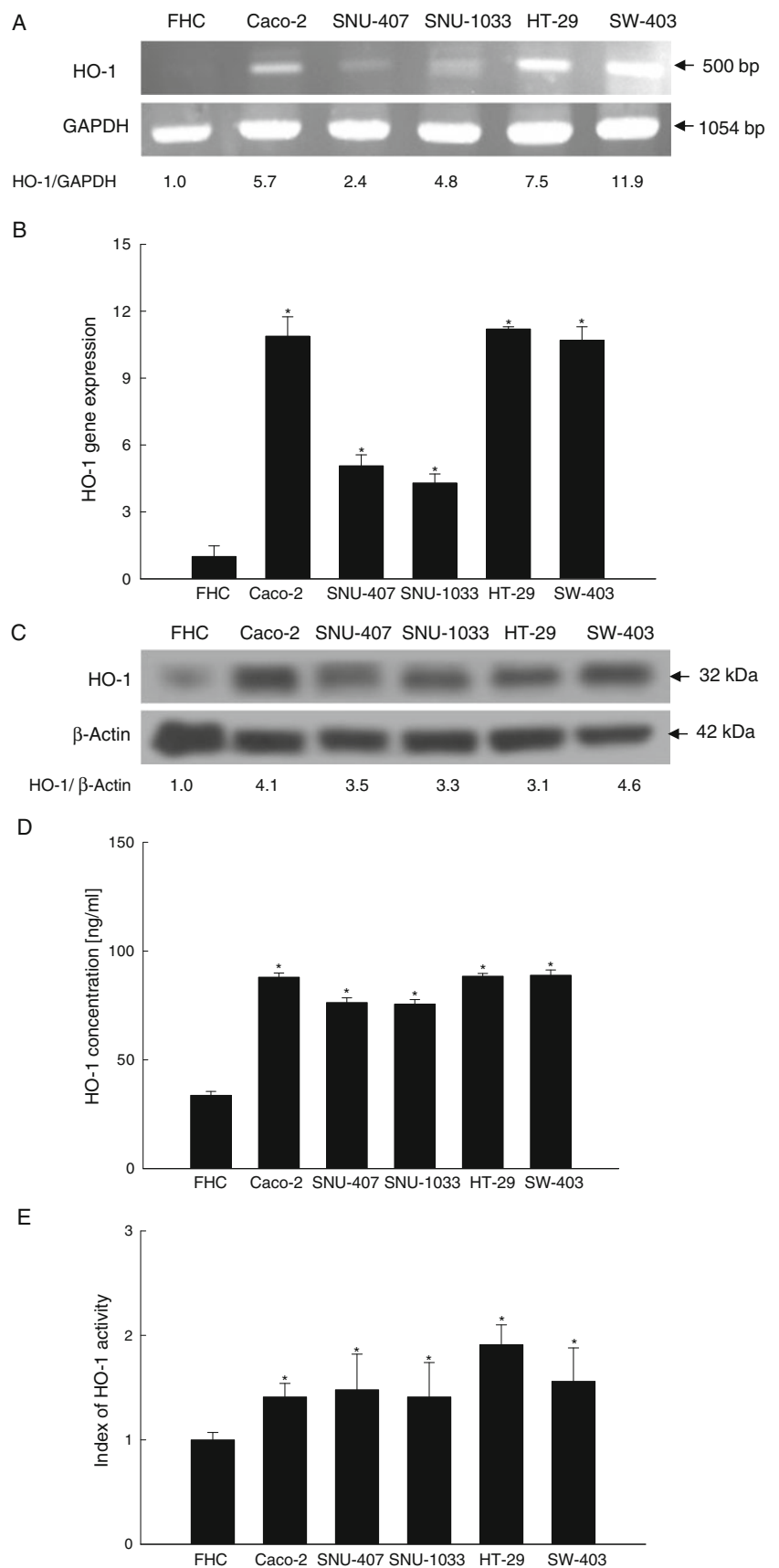
Quantification of HO-1 concentration

Cultured colon cancer cells were homogenized in RIPA buffer and 10 mM PMSF and centrifuged at $15,000 \times g$ for 15 min at 4°C to remove tissue debris. HO-1 production was quantified using a human HO-1 ELISA kit (Assay Designs Inc., Ann Arbor, MI, USA) following the manufacturer's protocol. Briefly, cell lysates were added to the coated plates and incubated for 1 h at room temperature with antihuman HO-1 antibody. After several washes, the plates were incubated with antirabbit IgG-horseradish peroxidase conjugate for 30 min and then treated with tetramethylbenzidine, a substrate for peroxidase. The reaction was stopped after 15 min, and the optical density at 450 nm was read using a microplate reader. HO-1 concentration is expressed as nanogram per milliliter.

Cell viability

The effect of the HO-1 inhibitor ZnPP on the viability of human colon cancer cells was determined using the MTT assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells [23]. Cells were treated with ZnPP at a concentration of 10 μM, and 48 h later, 50 μl of the MTT stock solution (2 mg/ml) was added to each well to obtain a total reaction volume of 200 μl. After incubation for 4 h, the plate was centrifuged at $800 \times g$ for 5 min, followed by aspiration of the supernatants. Formazan crystals present in each well were dissolved in 150 μl of dimethyl sulfoxide, and the absorbance at 540 nm was measured on a scanning multi-well spectrophotometer.

Statistical analysis

The results were subjected to analysis of variance using Tukey's test to analyze differences. The $p < 0.05$ was considered statistically significant.

Results

HO-1 protein expression and enzyme activity in normal and colon carcinoma tissues

HO-1 protein expression level and enzyme activity were assessed in colon cancer tissues from 20 patients using western blotting and enzyme activity assay. HO-1 protein expression was detected in 70% (14/20) of tumor tissues from colon cancer patients, and the expression level was more than 1.5-fold higher than that in the corresponding normal tissues (Fig. 1a). HO-1 enzyme activity was higher in tumor tissues than in normal tissues from colon cancer patients, almost in agreement with the western blot data (Fig. 1b). Immunohistochemical analysis

detected the faint expression of HO-1 protein in normal colonic epithelial cells (Fig. 1c(b)). The carcinoma cells showed diffusely increased expression in the cell cytoplasm (Fig. 1c(c–e)), while some of which reveal strong positivity (Fig. 1c(f)). Intramucosal mononuclear cells showed strong immunoreactivity in all specimens. These results show that HO-1 is highly expressed in tumor tissues from Korean colon cancer patients, suggesting that this protein could be used as a biomarker for cancer detection.

HO-1 mRNA and protein expression and activity in human colon cancer cell lines

The assessment of HO-1 expression in colon cancer cell lines by RT-PCR and western blotting showed that HO-1 mRNA (Fig. 2a, b) and protein expression (Fig. 2c) in the colon cancer cell lines Caco-2, SNU-407, SNU-1033, HT-29, and SW-403 were higher than those in the normal colon cell line FHC. HO-1 enzyme concentration and activity
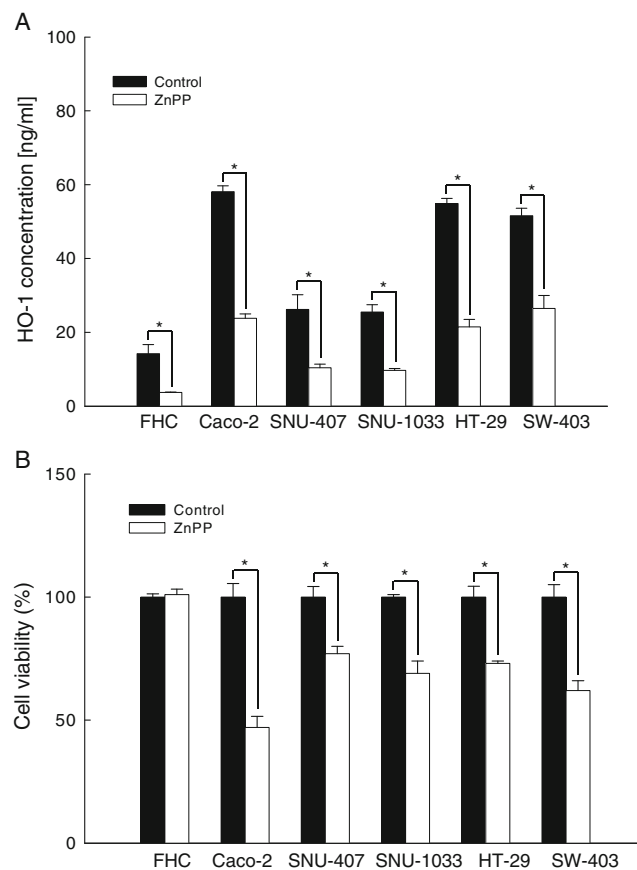
Fig. 3 The effect of the HO-1 inhibitor ZnPP on the cell viability of colon cell lines. **a** After ZnPP treatment for 48 h in colon cell lines, HO-1 enzyme concentration was measured and is expressed as nanogram per milliliter. *$p < 0.05$, significantly different from ZnPP-untreated cells in each cell line. **b** Cell viability was measured using the MTT assay. *$p < 0.05$, significantly different from ZnPP-untreated cells in each cell line

were also higher in cancer cell lines and were consistent with the pattern of HO-1 protein expression (Fig. 2d, e), although both HO-1 expression and activity level differed among the cell lines. Despite these differences in the expression pattern of different cell lines, the data indicate that HO-1 is a useful marker for the detection of colon cancer.

Downregulation of HO-1 levels decreased cell viability

The effect of the HO-1 inhibitor ZnPP [18] on cell viability was assessed in colon cell lines. ZnPP suppressed HO-1 concentration in the normal colon and cancer cell lines (Fig. 3a) and reduced the cell viability of colon cancer cell lines without affecting the viability of normal colon cells (Fig. 3b).

Discussion

HO-1 is a cytoprotective enzyme that is induced in response to a variety of stimuli including oxidative stress. HO-1 induction plays a role in resistance to apoptosis in various human cancer cells [24–26]. In the present study, HO-1 protein expression and activity were examined in tumor and normal specimens obtained from Korean colon cancer patients. HO-1 protein expression and enzyme activity were higher in colon cancer tissues than in the surrounding normal tissues. This was also similar to the case for colon cancer cell lines. The expression of HO-1 in colon cancer tissues was confirmed by histological analysis. Inhibition of HO-1 reduces tumor growth and increases sensitivity to chemotherapy [27, 28]. Overexpression of HO-1, which may exert beneficial effects in a number of pathological conditions [29], has been suggested to play a protective role in cancer cells. Malignant cancers express HO-1 and provide cellular resistance against ROS-mediated anticancer therapy [18, 19], and elevated levels of HO-1 protein are associated with neoplastic growth [29, 30]. This suggests that HO-1 acts as a survival factor for colon carcinoma cells. ROS are mutagenic and thereby induce carcinogenesis. Normally, ROS levels are controlled by an inducible antioxidant system that responds to cellular stress and are predominantly regulated by the transcription factor NF-E2-regulated factor 2 (Nrf2) and its repressor protein Kelch-like ECH-associated protein 1 (Keap1) [31]. In contrast to the acute physiological regulation of Nrf2, in neoplasia, there is evidence for increased basal activation of Nrf2. Recent research has identified that somatic mutations disrupt the Nrf2–Keap1 interaction to stabilize Nrf2 and increase the transcription of Nrf2 target genes, indicating that enhanced ROS detoxification and additional Nrf2 functions may be protumorigenic [32, 33]. HO-1, an inducible antioxidant enzyme, has been reported to be upregulated via activation of Nrf2 [34]. Thus, the upregulation of HO-1 in tumor tissue and colorectal cancer

lines may be due to the activation of Nrf2. Future studies will be necessary to determine the role of Nrf2 on HO-1 expression in colorectal cancer and its mechanism involved. HO-1 inhibition by ZnPP derivative enhances the cytotoxicity of hydroperoxides and anticancer drugs in SW-480 cells and mice bearing sarcoma S-180 cells [28]. In the present study, ZnPP enhanced cytotoxicity in cancer cell lines. In the human cancer cell line COLO-201, on the other hand, flavonoid-induced cytotoxicity was found to be mediated by the induction of HO-1 [35]. The relationship between HO-1 and cytotoxicity remains to be elucidated.

In conclusion, the present results indicate that HO-1 may serve as a clinically useful biomarker of colon cancer and a target for anticancer drugs.

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