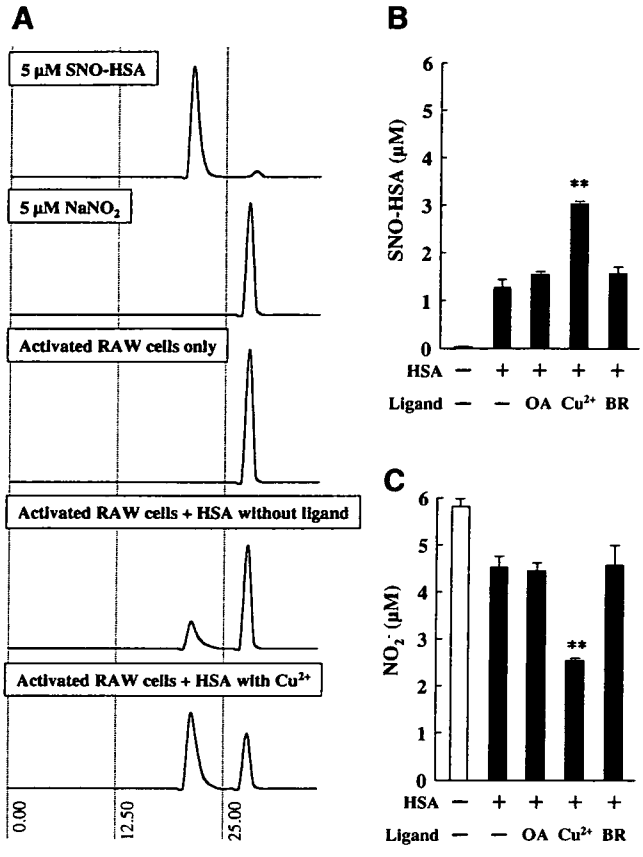

Fig. 4. Effect of high-affinity binding of OA, Cu^{2+} and BR on S-nitrosylation of HSA by activated RAW264.7 cells. (A) Elution profiles for the standard solutions (5 μM SNO-HSA and 5 μM $NaNO_2$) and for different cell media made by the HPLC-flow reactor system. The retention time for SNO-HSA and NO_2^- is about 21 and 27 min, respectively. (B) Production of SNO-HSA from HSA with and without bound ligand. (C) Production of NO_2^- in the presence of HSA with and without bound ligand. Data are expressed as means ± SEM ($n = 4$–6). $^{**}P < 0.01$, as compared with HSA without bound ligand.

disorders (BR) and Wilson's disease (Cu^{2+}). Thus, several physiological, clinical and pathological situations can affect the concentration of the quantitatively important SNO-HSA via modified high-affinity binding of OA, BR or Cu^{2+}.

In addition to HSA, other proteins possessing a free cysteine residue can be targets for S-nitrosylation, e.g. α_1-protease inhibitor [20]. Many of these proteins also bind ligand(s) the amounts of which can change in different biological or pathological conditions. Therefore, the present observations made with HSA could be general effects also applying to other proteins binding ligands and NO.

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Design and Evaluation of S-Nitrosylated Human Serum Albumin as a Novel Anticancer Drug

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ABSTRACT

In recent studies, the cytotoxic activity of NO has been investigated for its potential use in anticancer therapies. Nitrosated human serum albumin (NO-HSA) may act as a reservoir of NO in vivo. However, there are no published reports regarding the effects of NO-HSA on cancer. Therefore, the present study investigated the antitumor activity of NO-HSA. NO-HSA was prepared by incubating HSA, which had been sulfhydrylated using iminothiolane, with isopentyl nitrite (6.64 mol NO/mol HSA). Antitumor activity was examined in vitro using murine colon 26 carcinoma (C26) cells and in vivo using C26 tumor-bearing mice. Exposure to NO-HSA increased the production of reactive oxygen species in C26 cells. Flow cytometric analysis using rhodamine 123 showed that NO-HSA caused mitochondrial depolarization. Activation of caspase-3 and DNA fragmentation were observed in C26 cells after incubation with 100 μM NO-HSA for 24 h, and NO-HSA inhibited the growth of C26 cells in a concentration-dependent manner. The growth of C26 tumors in mice was significantly inhibited by administration of NO-HSA compared with saline and HSA treatment. Immunohistochemical analysis of tumor tissues demonstrated an increase in terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive cells in NO-HSA-treated mice, suggesting that inhibition of tumor growth by NO-HSA was mediated through induction of apoptosis. Biochemical parameters (such as serum creatinine, blood urea nitrogen, aspartate aminotransferase, and alanine aminotransferase) showed no significant differences among the three treatment groups, indicating that NO-HSA did not cause hepatic or renal damage. These results suggest that NO-HSA has the potential for chemopreventive and/or chemotherapeutic activity with few side effects.

Although cancer primarily arises from disorders of cell proliferation, it also may arise from disruptions in programmed cell death signaling pathways, resulting in decreased apoptosis of cancerous cells (Okada and Mak, 2004). Therefore, induction of apoptosis in neoplastic cells is a very effective therapy for tumor eradication (Meng et al., 2006). However, this type of chemotherapy often has negative side effects, such as transient cell cycle arrest, senescence, and autophagy. Drug delivery systems that facilitate selective apoptosis of neoplastic cells have been suggested as a way of overcoming this problem (Kaufmann and Gores, 2000; Kondo et al., 2005).

NO is a unique diffusible molecular messenger that occupies a central role in mammalian pathophysiology (Brune et al., 1998). Its multiple actions include vascular smooth muscle relaxation (Moncada et al., 1986; Ignarro, 1989), inhibition of platelet aggregation (Azuma et al., 1986), effects on neurotransmission (Garthwaite, 1991), and regulation of immune function (Marletta et al., 1988). Alternatively, under some circumstances, NO is cytotoxic (Laval and Wink, 1994). NO causes cellular iron losses and inhibits DNA synthesis, mitochondrial respiration, and aconitase activity in L10 hepatoma cells (Hibbs et al., 1988). In addition, NO reacts with

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; ASA, aspirin; HSA, human serum albumin; DTPA, diethylenetriaminepentaacetic acid; HBSS, Hanks' balanced salt solution; rHSA, recombinant human serum albumin; PAGE, polyacrylamide gel electrophoresis; C26, murine colon 26 carcinoma; ROS, reactive oxygen species; PBS, phosphate-buffered saline; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; Cr, serum creatinine; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BSA, bovine serum albumin; GSNO, S-nitrosoglutathione; R410C, genetic variant of human serum albumin mutated at position 410.

superoxide anion (which is produced by activated macrophages and other cells), to form peroxynitrite. This by-product of NO is a potent chemical oxidant, which alters protein function and damages DNA (Beckman and Crow, 1993). These effects are part of the nonspecific host defense, which facilitates killing of tumor cells and intracellular pathogens. In addition, the cytotoxicity arising from long-lasting NO generation has been attributed to induction of apoptosis (Brune et al., 1998).

In recent studies, the cytotoxic activity of NO has been studied to assess its therapeutic potential in cancer treatment. NO-donating nonsteroidal anti-inflammatory drugs (NSAIDs), especially NO-aspirin (NO-ASA), have been investigated as promising chemopreventive agents (Williams et al., 2001; Kashfi et al., 2002; Fabbri et al., 2005). NO-ASA consists of traditional ASA to which an NO-releasing moiety is bound via a spacer. This agent induces oxidative stress by increasing intracellular peroxide and O_2^-, thereby inducing apoptosis via activation of the intrinsic apoptosis pathway (Gao et al., 2005). JS-K is a prodrug designed to release NO after reacting with glutathione transferase, which induces double-stranded DNA breaks, activates DNA damage response pathways, and induces apoptosis in human multiple myeloma cells both in vitro and in vivo (Kiziltepe et al., 2007).

Human serum albumin (HSA) is an abundant circulating protein, and the nitrosated form serves as a reservoir of NO (Stamler et al., 1992). Therefore, NO-HSA is an NO donor that is currently being investigated for its potential therapeutic applications. For example, administration of NO-HSA to animals with ischemia-reperfusion injury minimizes the tissue damage that occurs after reperfusion (Semsroth et al., 2005). In a balloon-injured rabbit femoral artery model, locally delivered NO-HSA preferentially binds to sites of vessel injury and inhibits both platelet accumulation and the subsequent development of neointimal hyperplasia (Marks et al., 1995). NO-HSA also shows potent antibacterial activity and inhibits the proliferation of cultured human vascular smooth muscle cells (Ishima et al., 2007). However, there are no reports describing the effects of NO-HSA on cancer.

Accordingly, the present study evaluated the antitumor activity of NO bound to HSA (NO-HSA) via an *S*-nitrosothiol linkage using iminothiolane as a spacer. The molecular events related to induction of apoptosis by NO-HSA were studied in vitro, and the antitumor activity of NO-HSA was studied in vivo using a murine model of C26 colon carcinoma.

Materials and Methods

Chemicals. Traut's reagent (2-imminothiolane) was purchased from Pierce Chemical (Rockford, IL). Isopentyl nitrite, diethylenetriaminepentaacetic acid (DTPA), and Cell Counting Kit-8 (WST-8) were purchased from Wako Pure Chemicals (Osaka, Japan). RPMI 1640 medium, Hanks' balanced salt solution (HBSS), and RNase A were obtained from Sigma-Aldrich (St. Louis, MO). Proteinase K was obtained from Roche Applied Science (Indianapolis, IN). All other reagents used were of the highest grade available from commercial sources.

Expression and Purification of Recombinant HSA. rHSA was produced using a yeast expression system as described previously (Matsushita et al., 2004). In brief, for constructing the HSA expression vector pPIC9-HSA, native HSA coding region was incorporated into the methanol-inducible pPIC9 vector (Invitrogen, Carlsbad, CA). The resulting vector was introduced into the yeast species *Pichia pastoris* (strain GS115) to express rHSA. Secreted rHSA was isolated from the growth medium by a combination of precipitation with 60% (w/v) $(NH_4)_2SO_4$ and purification on a Blue Sepharose CL-6B column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) followed by Phenyl HP column (GE Healthcare). Isolated protein was defatted by using the charcoal procedure described by Chen (1967), deionized, freeze-dried, and then stored at −20°C until use. The resulting rHSA (treated with dithiothreitol) exhibited a single band on SDS-PAGE. Density analysis of protein bands stained with Coomassie Brilliant Blue showed that its purity was more than 97%.

Synthesis of NO-HSA. Terminal sulfhydryl groups were added to the HSA molecule by incubating 0.15 mM rHSA with 3 mM Traut's reagent in 100 mM potassium phosphate buffer containing 0.5 mM DTPA, pH 7.8, for 1 h at room temperature. The resultant modified rHSA then was *S*-nitrosylated by 3-h incubation with 15 mM isopentyl nitrite at room temperature (Fig. 1). The resulting NO-HSA was concentrated, exchanged with saline using a PelliconXL filtration device (Millipore Corporation, Billerica, MA), and the final concentration adjusted to 2 mM NO-HSA. The sample was stored at −80°C until use.

Determination of S-Nitrosylation Efficiency. The amount of the *S*-nitroso moieties of NO-HSA was quantified using a 96-well plate. First, 20-μl aliquots of NO-HSA solution and $NaNO_2$ (standard) were incubated with 0.2 ml of 10 mM sodium acetate buffer, pH 5.5, containing 100 mM NaCl, 0.5 mM DTPA, 0.015% *N*-1-naphthylsthylene-diamide and 0.15% sulfanilamide with or without 0.09 mM $HgCl_2$, for 30 min at room temperature. Then, the absorbance was measured at 540 nm. The number of moles of NO per mole of HSA was obtained by subtracting the values in the absence of $HgCl_2$ from values in the presence of $HgCl_2$; the value thus obtained, was 6.64 ± 0.54 mol NO/mol HSA.

Cellular Experiments with C26 Cells. C26 cells, which were donated by the Institute of Development, Aging and Cancer, at Tohoku University (Sendai, Miyagi, Japan), were cultured at 37°C in RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, and 10 μg/ml streptomycin (medium A). Trypsin (0.25%) EDTA solution was used to detach the cells from the culture flask for plating and passing the cells. All cell culture experiments were performed at 37°C in a humidified atmosphere of 5% CO_2 in air.

For detection of reactive oxygen species (ROS), C26 cells (1.0×10^4 cells/well) were cultured in 96-well plates in medium A for 12 h, they were washed twice with PBS, and then they were incubated for an additional 30 min in HBSS containing 5 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen, Carlsbad, CA). After washing twice with HBSS, the cells were cultured in HBSS containing 5% fetal calf serum for 15 min followed by the addition of PBS, 50 μM HSA, or 50 μM NO-HSA. After incubation, fluorescence was measured using a plate reader (excitation wavelength, 485 nm; emission wavelength, 535 nm). The change in fluorescence was calculated by subtracting the fluorescence at 0 h from the fluorescence measured at the indicated times. The fluorescence intensities of cells incubated with PBS, 50 μM HSA, and 50 μM NO-HSA at 0 h were 201.3, 166.1, and 181.3, respectively.

Changes in the mitochondrial membrane potential of C26 cells

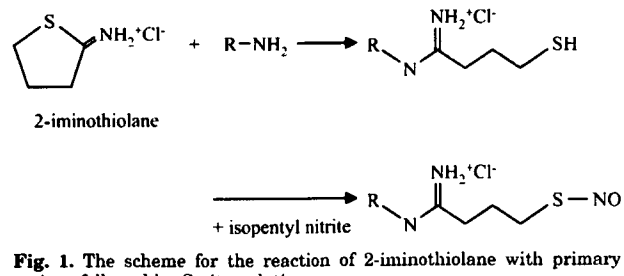

Fig. 1. The scheme for the reaction of 2-iminothiolane with primary amines followed by *S*-nitrosylation.

were monitored using flow cytometric analysis with rhodamine 123 staining. C26 cells (1.0×10^6 cells/well) were cultured in six-well plates for 12 h, washed twice with PBS, and incubated with PBS and either 100 μM HSA or various concentrations of NO-HSA in medium A for 24 h. The cells were then trypsinized, washed twice with PBS, and incubated for 15 min with 5 μg/ml rhodamine 123. The mean fluorescence intensity of rhodamine 123 in the cells was measured using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

For the determination of caspase-3 activity, cells were cultured to confluence in six-well plates, washed twice with PBS, and incubated with medium A containing 100 μM HSA or various concentrations of NO-HSA. Cells were incubated for 24 h, trypsinized, and washed with 0.2 ml of ice-cold PBS. The cell pellet was resuspended in 15 μl of cell lysis buffer, it was lysed by freeze-thawing, and then it was incubated on ice for 15 min. The cell lysates were centrifuged at 15,000 rpm for 20 min at 4°C, and the supernatant fraction was collected (cell extract). The caspase-3 activity in the cell extract was assessed using the colorimetric CaspASE Assay System (Promega, Madison, WI), according to the manufacturer's instructions.

For the detection of DNA degradation (DNA ladder), C26 cells (1.0×10^6 cell/well) were cultured in six-well plates. Cells were cultured for 12 h, they were washed twice with PBS, and then they were incubated with PBS and either 100 μM HSA or various concentrations of NO-HSA for 24 h. The cells then were trypsinized, collected, and centrifuged at 4000 rpm for 10 min. After removing the supernatant, the cell pellet was resuspended in 0.2 ml of PBS, and then it was centrifuged at 4000 rpm for 10 min. The supernatant was again removed, and the remaining pellet was incubated in 20 μl of 10 mM Tris-HCl buffer, pH 7.8, containing 2 mM EDTA and 0.5% SDS (cell lysis buffer) for 10 min at 4°C, followed by centrifugation at 15,000 rpm for 5 min. The resulting supernatant (cell extract) was collected and incubated with 1 μl of RNase A (10 μg/ml) for 30 min at 50°C. One microliter of proteinase K (10 μg/ml) was added to the cell extract, followed by a 1-h incubation at 50°C. The resulting DNA extract was electrophoresed in a 2.0% agarose gel, followed by staining of the gel with ethidium bromide and visualization of the DNA bands using ultraviolet illumination.

The cell viability assay was performed using WST-8, which is based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. C26 cells were plated in 96-well plates at 1.0×10^4 cells/well, and they were cultured for 32 h in medium A. Then, the cells were washed twice with PBS and incubated in a total volume of 0.2 ml of medium A containing various concentrations of HSA or NO-HSA. After incubating the cultures for various lengths of time, 5 μl of WST-8 solution was added to each well, and the cells were incubated for an additional 2 h at 37°C. The number of surviving cells was determined by measuring the absorbance at 450 nm. Cell viability was calculated as the percentage of the control value (without HSA or NO-HSA) (Ishiyama et al., 1996).

Animal Experiments. Five-week-old male BALB/c AnNCrlCrlj mice (17–20 g) were purchased from Charles River Italica (Calco, Italy). The mice were housed in a 12-h light/dark cycle in a humidity-controlled room. Mice were acclimated for at least 5 days before being used in experiments.

For tumor induction, mice were inoculated with C26 cells (1.0×10^6 cells/mouse) by a subcutaneous injection into the dorsal skin. Three days after inoculation, C26 carcinoma-bearing mice were randomly divided into three groups: control, HSA, and NO-HSA. The mice received a daily i.v. injection of saline, HSA (10 μmol/kg), or NO-HSA (10 μmol/kg) for 10 days from day 3 to day 12 after inoculation. Tumor volume was calculated using the formula $0.4 (a \times b^2)$, where a is the largest diameter and b is the smallest diameter of the tumor (Shimizu et al., 2005), and volume was monitored from day 7 to day 17 after inoculation. Variance in each group was evaluated using the Bartlett test, and differences in mean tumor volume were evaluated using the Tukey-Kramer test.

Some animals in each of the three treatment groups were used for immunohistochemical analysis and serum biochemistry. When the mice received five times per day from day 3 to day 7 after inoculation (the tumors in each group reached approximately 5 mm in diameter), blood samples were collected from the abdominal vena cava under diethyl ether anesthesia approximately 2 h after the daily injection, and then the mice were sacrificed.

Tumors were dissected, they were fixed immediately with 2% periodate/lysine/paraform-aldehyde fixative at 4°C for 5 h, and then they were washed with a graded series of sucrose solutions in PBS (10, 15, and 20%). After immersion in 20% sucrose in PBS to inhibit ice crystal formation, the tissues were embedded in O.C.T. compound (Sakura Fine Technochemical, Tokyo, Japan), they were frozen in liquid nitrogen, and then they were stored at −80°C. Five-micrometer tumor sections were prepared using a cryostatic microtome (HM500M; Microm, Walldorf, Germany), and they were mounted on poly-L-lysine-coated slides. The slides were stained using the TUNEL method and an in situ apoptosis detection kit (Takara-Bio Co. Ltd., Shiga, Japan). The slides were washed three times with 0.01 M phosphate buffer, pH 7.4, containing 0.9% NaCl, followed by application of methanol containing 0.3% H_2O_2 to inactivate endogenous peroxidase and incubation at room temperature for 30 min. The slides were washed 3 times with 0.01 M phosphate-buffered saline, and then they were incubated in 100 ml of permeabilization buffer on ice for 5 min. The slides were washed three times with 0.01 M phosphate-buffered saline, and then they were incubated with 50 ml of freshly prepared terminal deoxynucleotidyl transferase reaction mixture (5 ml of terminal deoxynucleotidyl transferase enzyme + 45 ml of Labeling Safe buffer) at 37°C for 60 min. After the slides were washed three times with 0.01 M PBS, they were incubated in 70 ml of anti-fluorescein isothiocyanate-horseradish peroxidase conjugate antibody at 37°C for 30 min. After the slides were washed three times with 0.01 M PBS, they were incubated in 3,3-diaminobenzidine-H_2O_2 reaction buffer at room temperature for 10 min. After the slides were washed three times with distilled water, they were stained with 3% methyl green for 10 min, dehydrated, penetrated, and sealed (Gavrieli et al., 1992). Each slide then was visualized under a light microscope (Olympus, Tokyo, Japan), at a magnification of 400×.

Serum was separated by centrifugation. Routine clinical laboratory techniques were used to determine the concentrations of total protein, serum creatinine (Cr), blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in serum. Variance in each group was evaluated using the Bartlett test, and differences were evaluated using the Tukey-Kramer test.

Results

NO-HSA Induces Cell Death via Apoptosis in Vitro. Apoptosis is induced by a variety of factors. Among them, it is well known that intracellular accumulation of ROS, such as H_2O_2, O_2^-, and peroxynitrite, causes apoptosis. Moreover, production of ROS also plays a major role in NO-ASA-induced apoptosis. To examine whether NO-HSA promoted ROS production in C26 cells, a fluorescent probe (CM-H_2DCFDA), which undergoes conversion to 2′,7′-dichlorofluorescein in the presence of intracellular ROS, was used. Addition of NO-HSA to C26 cells increased the amount of ROS compared with treatment with vehicle or HSA (Fig. 2). In addition, the ROS in the C26 cell culture medium increased with time after addition of NO-HSA. This result suggests that NO-HSA promotes a signal cascade leading to apoptosis by increasing intracellular production of ROS.

To evaluate the effect of NO-HSA on mitochondrial function, C26 cells were loaded with a mitochondria-selective fluorescent cation (rhodamine 123) to monitor the mitochon-

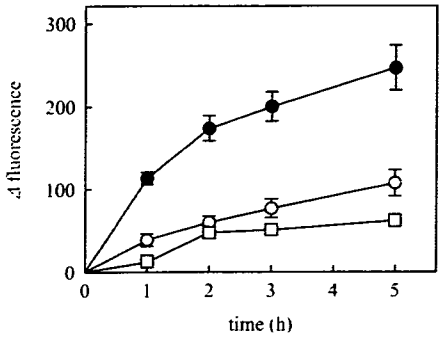

Fig. 2. Production of ROS in C26 cells after NO-HSA treatment. C26 cells were pretreated with CM-H_2DCFDA for uptake into C26 cells and hydrolysis by cellular esterase, followed by addition of either PBS (open circles), 50 μM HSA (open squares), or 50 μM NO-HSA (closed circles). Excitation of the probes was done at 485 nm, and emission was measured at 535 nm. Change in fluorescence was calculated by subtracting the fluorescence at 0 h from that at the indicated times. The fluorescence intensities of the PBS, 50 μM HSA, and 50 μM NO-HSA groups at 0 h were 201.3, 166.1, and 181.3, respectively. Results are the mean ± S.D. of three separate experiments.

drial membrane potential. Compared with vehicle, in cells treated with 50 or 100 μM NO-HSA rhodamine 123, fluorescence was decreased by 75%, whereas treatment with 25 μM NO-HSA or 100 μM HSA did not affect rhodamine 123 fluorescence compared with vehicle (Fig. 3A). These observations indicate that NO-HSA induces depolarization of the mitochondrial membrane.

Caspase-3 is a cell death protease that is involved in the downstream execution phase of apoptosis. During this phase of apoptosis, cells undergo morphological changes, such as DNA fragmentation, chromatin condensation, and formation of apoptotic bodies. Compared with the effect of vehicle, cells treated with 25 or 50 μM NO-HSA had slightly increased caspase-3 activity, and cells treated with 100 μM NO-HSA showed a larger increase in caspase-3 activity (Fig. 3B). Cells treated with HSA had the same level of caspase-3 activity as cells treated with PBS.

To further confirm that NO-HSA induced apoptosis in C26 cells, DNA fragmentation, which is a typical morphological change in the execution phase of apoptosis, was examined. DNA fragmentation was observed in C26 cells treated with 100 μM NO-HSA, but not in C26 cells treated with 25 or 50 μM NO-HSA (Fig. 3C). Neither vehicle nor 100 μM HSA induced DNA fragmentation in C26 cells (Fig. 3C). These findings suggest that NO-HSA induces apoptosis by increasing ROS production, activating caspase-3, and hyperpolarizing the mitochondrial membrane potential.

To determine the effect of NO-HSA on cell growth, the viability of C26 cells was examined after treatment with HSA or various concentrations of NO-HSA. NO-HSA inhibited growth of C26 cells in a concentration-dependent manner; cell growth was suppressed by 71, 80, and 85% after a 48-h incubation with 25, 50, and 100 μM NO-HSA, respectively (Fig. 4A). The viability of C26 cells incubated with 50 μM NO-HSA significantly decreased with increasing incubation times (Fig. 4B). NO-HSA inhibited growth to a greater extent than did HSA, which had only a weak inhibitory effect. These results suggest that NO-HSA inhibits cell growth of C26 cells by inducing apoptosis.

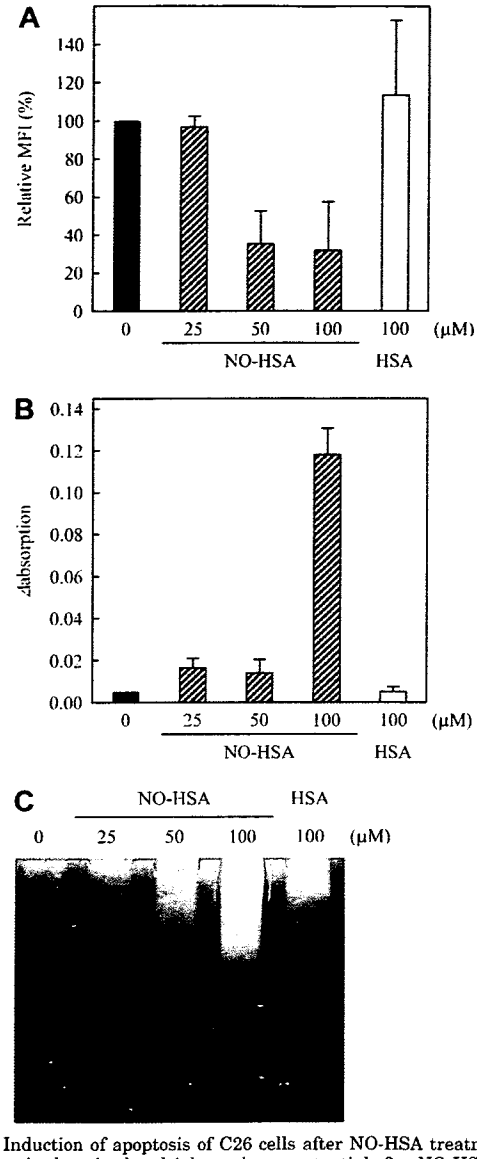

Fig. 3. Induction of apoptosis of C26 cells after NO-HSA treatment. A, alteration in the mitochondrial membrane potential after NO-HSA treatment. C26 cells were cultured with PBS, 100 μM HSA, or various concentrations of NO-HSA for 24 h, followed by addition of rhodamine 123. The amounts of cell-associated rhodamine 123 were determined as described under *Materials and Methods*. Results are the mean ± S.D. of three separate experiments. B, activation of caspase-3 after NO-HSA treatment. C26 cells were incubated with PBS, 100 μM HSA, or various concentrations of NO-HSA for 24 h. Caspase-3 activity was estimated by monitoring *p*-nitroaniline (absorbance at 405 nm) released from the substrate upon cleavage by caspase-3. Change in absorbance was calculated by subtracting absorbance after incubation with caspase inhibitor (*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone), from absorbance after incubation without caspase inhibitor. The absorbances among PBS-, HSA- and NO-HSA-treated cells incubated with *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone were almost identical (0.170 ± 0.17). Results are the means ± S.D. of three separate experiments. C, DNA fragmentation after NO-HSA treatment. C26 cells were incubated with PBS, 100 μM HSA or various concentrations of NO-HSA for 24 h. DNA fragmentation was detected as described under *Materials and Methods*.

NO-HSA Exerts Antitumor Effect via the Apoptotic Pathway in Vivo. To investigate the antitumor effect of NO-HSA in vivo, C26 tumor-bearing mice received i.v. injec-

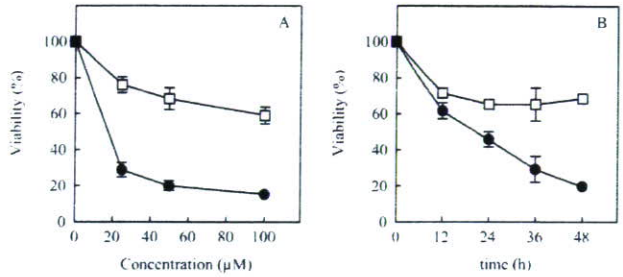

Fig. 4. Effect of NO-HSA on C26 cell viability. A, C26 cells were treated for 48 h with various concentrations of HSA (open squares) or NO-HSA (closed circles). B, C26 cells were incubated for the indicated times with 100 μM HSA (open squares) or 100 μM NO-HSA (closed circles). Cell viability was determined as described under *Materials and Methods*. Results are the mean ± S.D. of three separate experiments.

tions with saline, HSA, or NO-HSA. Mean tumor area increased with time in the saline-treated group. In the HSA-treated group, tumor growth was suppressed, compared with that in the control group, but the difference was not statistically significant. In contrast, tumor growth was significantly inhibited by administration of NO-HSA (Fig. 5).

To clarify whether the suppression of tumor growth by NO-HSA is mediated via apoptosis, tumor tissues from C26 tumor-bearing mice receiving injections with NO-HSA were examined using immunohistochemistry. In NO-HSA-treated mice, there were more TUNEL-positive cells than in the saline- and HSA-treated animals. In addition, the tumor tissue architecture was less defined in animals treated with NO-HSA than in the other groups, suggesting that NO-HSA induced apoptosis in C26 tumor cells and thus exerted an antitumor effect in vivo (Fig. 6).

To evaluate the side effects of NO-HSA treatment, several serum biochemical parameters were measured in tumor-bearing mice treated with saline, HSA, or NO-HSA (Table 1). There were no significant differences in total protein, Cr, BUN, AST, or ALT among the three groups, suggesting that

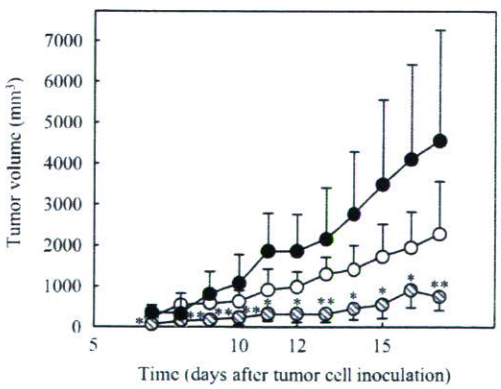

Fig. 5. Effect of NO-HSA on tumor growth in C26 tumor-bearing mice. C26 tumor-bearing mice were given daily i.v. injections of saline (5 ml/kg), HSA (10 μmol/5 ml/kg), or NO-HSA (10 μmol/5 ml/kg) for 10 days from day 3 to day 12 after inoculation with tumor cells. Tumor size was measured and tumor volume was calculated according to the formula: $V = 0.4a^2b$, where a is the smallest, and b is the largest, superficial diameter. Results are means ± S.D; $n = 10$ animals per experimental group. *, statistically significant reduction compared with saline ($P < 0.01$) or HSA ($P < 0.05$) at the corresponding time. **, statistically significant reduction compared with saline ($P < 0.01$) or HSA ($P < 0.01$) at the corresponding time.

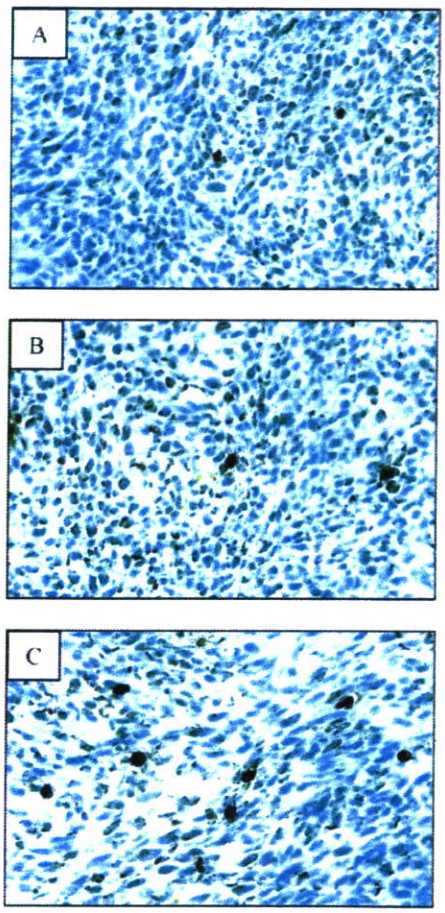

Fig. 6. Immunohistochemical staining of tumor tissues of C26 tumor-bearing mice receiving i.v. injections with NO-HSA using the TUNEL method. C26 tumor-bearing mice were given daily i.v. injections of saline (5 ml/kg) (A), HSA (10 μmol/5 ml/kg) (B), or NO-HSA (10 μmol/5 ml/kg) (C) for 5 days from day 3 to day 7 after inoculation with tumor cells. TUNEL staining, performed as described under *Materials and Methods*, shows apoptotic cells in the tumor tissue of mice treated with NO-HSA.

TABLE 1

Serum biochemical parameters of C26 tumor-bearing mice treated with saline, HSA, or NO-HSA

C26 tumor-bearing mice received i.v. injections five times per day with saline (5 ml/kg), HSA (10 μmol/5 ml/kg), or NO-HSA (10 μmol/5 ml/kg) from day 3 to day 7 after inoculation with tumor cells. Blood samples were collected from the abdominal vena cava under anesthesia with diethyl ether approximately 2 h after the last treatment injection, and mice were sacrificed. Serum biochemical parameters were measured using routine clinical laboratory techniques.

Serum Biochemical Parameter	Saline	HSA	NO-HSA
Total protein (g/dl)	5.23 ± 0.21	5.33 ± 0.06	5.55 ± 0.24
Cr (mg/dl)	0.13 ± 0.04	0.14 ± 0.01	0.16 ± 0.01
BUN (mg/dl)	15.25 ± 0.96	15.33 ± 1.53	13.75 ± 1.26
AST (U/l)	143.5 ± 62.9	91.7 ± 39.4	116.3 ± 69.1
ALT (U/l)	169.8 ± 111.6	110.0 ± 81.2	161.3 ± 126.2
ALP (U/l)	385.3 ± 18.3	345.3 ± 4.7*	300.5 ± 23.0**,*

* $P < 0.05$, saline vs. HSA.
** $P < 0.01$, saline vs. NO-HSA.
* $P < 0.05$, HSA vs. NO-HSA.

NO-HSA did not cause kidney or liver damage. However, compared with the control group, mice treated with HSA had significantly lower serum levels of ALP (345.3 ± 4.7 versus 385.3 ± 18.3 U/l). Moreover, the serum concentration of ALP

in mice treated with NO-HSA was 300.5 ± 23.0 U/l, which was significantly lower than the control ($P < 0.01$) and HSA ($P < 0.05$) groups. In general, ALP levels increase in several types of cancer, such as liver, lung, and bone cancer; thus, the present findings suggest that NO-HSA is an effective anticancer agent. The vasodilating effect of NO-HSA was also evaluated in rats after i.v. injection at a dose of 10 μmol/kg (66 μmol of NO per kg). NO-HSA induced a decrease in the mean arterial blood pressure immediately after i.v. injection and the maximum reduction effect was 32.8 ± 7.3 mm Hg. In contrast, HSA had no significant effect on the blood pressure. The fall in pressure returned to the initial levels in 30 min (data not shown).

Discussion

There have been many trials of NO as a therapeutic agent, because of its powerful biological activity (Moncada and Higgs, 1993). However, the in vivo half-life of NO (~0.1 s) is often too short to capitalize on its potential biological actions. The half-life of NO can be prolonged by adding *S*-nitrosothiol moieties with cysteine residues of proteins. For example, nitrosated HSA seems to act as a reservoir of NO in vivo (Stamler et al., 1992). Simon et al. (1996) incubated bovine serum albumin (BSA) with 200-fold excess concentration of $NaNO_2$ under acidic condition to synthesize polynitrosylated BSA that is highly modified at the thiol group of cysteine, hydroxyl group of tyrosine and amines (38 mol NO/mol BSA). The polynitrosylated BSA has been shown to exhibit antiplatelet activity. However, polynitrosylated *S*-NO-BSA, an NO-BSA conjugate prepared with the same method except that the BSA has been reduced with dithiothreitol and it contains 19 mol of "*S*-NO" per mol of BSA, was a significantly more potent platelet inhibitor than polynitrosylated BSA described above. These findings show that nitrosylated BSA behaves as an NO donor; in particular, the poly(*S*-nitroso) derivative could be by far the most potent compound. One molecule of HSA contains 35 cysteine residues, 34 of which form 17 nonreactive disulfide bonds, and one of which (Cys-34) forms a reactive free thiol (Peters, 1985). Thus, the number of NO molecules that can be bound to HSA is limited because only one free cysteine per HSA molecule is available for conjugation. Ewing et al. increased the number of free sulfhydryl groups on BSA by reduction with dithiothreitol and thiolation with *N*-acetylhomocysteine, thereby preparing polynitrosated BSA (12–15 mol NO/mol BSA) (Ewing et al., 1997). Marks et al. (1995) produced polynitrosated BSA (5.9 mol NO/mol BSA) by adding free sulfhydryl groups to the molecule and by treating the BSA with *N*-acetylhomocysteine thiolactone. However, the polynitrosated BSAs prepared in these studies formed aggregates as a result of intermolecular disulfide formation. Aggregate formation results in molecular heterogeneity, which limits the therapeutic application of *S*-nitroso residues. In the present study, iminothiolane, which reacts with primary amines to introduce sulfhydryl groups while maintaining charge properties similar to the original amino groups, was selected as the thiolation reagent. Iminothiolane was used to produce polynitrosated HSA (NO-HSA) (6.6 mol NO/mol HSA), which did not form aggregates after nonreducing SDS-PAGE or native-PAGE (data not shown). Moreover, the far-UV CD spectra of NO-HSA were nearly identical to those of HSA (data not shown). Therefore, NO-HSA is expected to be clinically applicable as a biocompatible pharmacological agent, although further study is required to clarify other potential issues, including the antigenicity of this protein.

NO-NSAIDs have been extensively investigated as therapeutic agents for cancer due to their ability to release NO, thereby promoting apoptosis. NO-NSAIDs are categorized as organic nitrate esters, which are readily reduced to organic nitrite esters by cytosolic enzymes. Subsequently, glutathione reacts with organic nitrite esters to form GSNO, indicating that NO-NSAIDs release NO via *S*-nitrosothiol (Wong and Fukuto, 1999). Alternatively, the transfer of NO from NO-HSA to the cytosol could be inferred from a study by Ramachandran et al. (2001). They reported that NO is released from extracellular *S*-nitrosothiols by a cell surface enzyme (protein disulfide isomerase) and that it accumulates in the cell membrane where it reacts with O_2 to produce N_2O_3, which is then available for nitrosation reactions with intracellular thiols at the membrane-cytosol interface (Ramachardran et al., 2001). Therefore, it is possible that NO-HSA also releases NO by the intracellular formation of *S*-nitrosothiol, suggesting that the species of NO released within the cell by *S*-nitrosothiols, as well as the reactive substances (such as ROS) derived from the released NO, would not differ significantly between NO-NSAIDs and NO-HSA. In support of this hypothesis, NO-HSA caused depolarization of the mitochondrial membrane potential, activation of caspase-3 and DNA fragmentation in the present study, consistent with the effects of NO-NSAIDs. Additional studies are needed to determine the details of the molecular events and the systematic pathways affected by NO-HSA, but the mechanism of action should be similar to that of NO-NSAIDs. In a recent study, Gao et al. (2005) elucidated the detailed mechanism of apoptosis induced by NO-ASA. Intracellular accumulation of ROS is a key proximal event in NO-ASA-induced apoptosis, and it correlates with the effect on tumor cell growth (Gao et al., 2005). In the present study, NO-HSA induced accumulation of ROS in tumor cells, suggesting that increased ROS production may be an important proximal event leading to induction of apoptosis.

The results of the in vivo study showed that NO-HSA significantly suppressed tumor growth by inducing apoptosis, without adverse changes in serum biochemical parameters in treated mice. In a recent study, Trachootham et al. (2006), using immortalized cell lines and their oncogenic progeny transfected with *H-Ras*^{V12}, demonstrated that cancer cells typically produce more ROS than normal cells. Moreover, the pro-oxidant status of cancer cells increases their susceptibility to treatment with agents that cause oxidative stress, as demonstrated in a study using β-phenylethyl isothiocyanate (Trachootham et al., 2006). In addition, Feng et al. (2007) reported that cyaniding-3-rutinoside selectively induces accumulation of peroxides in HL-60 human leukemic cells, but not in normal peripheral blood mononuclear cells (Feng et al., 2007). Schumacker (2006) has proposed that ROS toxicity induced by certain chemotherapeutic agents may be an effective means of selectively eradicating malignant cells. In the present study, we presumed that although NO reacts with superoxide anion to form peroxynitrite (a potent oxidant and nitrating agent), these highly reactive oxidant species are probably produced at higher

levels in C26 cells compared with normal cells. Therefore, NO-HSA may show selective cytotoxicity for tumor cells and not affect normal cells. These findings strongly suggest that NO-HSA is a promising therapeutic anticancer agent, given the unusual redox conditions typical of malignant cells.

Antiapoptotic effects of NO have been observed in a variety of cells, including T cells, hepatocytes, endothelial cells, neurons, ovarian follicle cells, eosinophils, thymocytes, and embryonic kidney cells (Liu and Stamler, 1999). In a recent study using U937 human promonocytic cells, NO-R410C (a genetic variant of HSA) had antiapoptotic activity (Ishima et al., 2007). Whether NO ultimately inhibits or promotes apoptosis probably depends on the cell, the signal, the source, the molecule, the amount, and the presence or absence of coreactants. For example, the amount of NO bound to the carrier molecule seems to account for the discrepant results between previous investigations and the present study. The NO content of NO-R410C (in the previous study) and NO-HSA (in this study) were 1.3 and 6.6 mol NO/mol HSA, respectively, and the *S*-nitroso moiety concentrations in vitro were 26 to 130 and 165 to 660 μM, respectively. Mohr et al. (1997) reported that 10 to 100 μM GSNO inhibits the activation of caspase-3 induced by actinomycin D in U937 cells. In contrast, apoptosis (characterized by DNA fragmentation and morphological changes) was observed in U937 cells treated with GSNO at concentrations in excess of 250 μM (Messmer et al., 1996). Based on the results of the present study and those of previously published investigations, the critical NO threshold concentration between promotion and inhibition of apoptosis seems to be 100 to 200 μM.

Matsumura et al. (1987) examined the accumulation of differently sized proteins within tumor tissues of tumor-bearing mice. Macromolecules containing HSA tended to accumulate in tumor tissues, apparently due to hypervascularization and enhanced vascular permeability (even to macromolecules) of the tumors, with little export of macromolecules from the tumor tissue via blood or lymphatic vessels (Matsumura et al., 1987). Therefore, NO-HSA may be a useful agent for targeting chemotherapeutics to tumor tissue. However, the short half-life of NO has been one of the greatest obstacles to therapeutic application of NO donors. Consequently, the pharmacokinetic properties of NO-HSA in mice were measured to determine the biological fate of NO. The apparent half-life of *S*-nitroso moieties in NO-HSA was estimated to be 18.9 min (data not shown), which is similar to that of NO-R410C, but much longer than that of the low-molecular-weight NO donor GSNO (4.2 min) (Ishima et al., 2007). In the present study, the difference in NO half-life between NO-HSA and GSNO seemed to be due to reduced renal excretion of HSA compared with glutathione due to its larger molecular size, suggesting that HSA may be a useful NO carrier in vivo.

In summary, NO-HSA was synthesized by inducing *S*-nitrosothiol linkages using iminothiolane as a spacer. NO-HSA generated ROS in C26 cells, and it induced intrinsic apoptotic events, such as depolarization of mitochondrial membrane potentials, activation of caspase-3, and induction of DNA fragmentation. Moreover, NO-HSA inhibited proliferation of tumor cells in vitro in a concentration-dependent manner. In the in vivo experiments, NO-HSA also strongly inhibited tumor growth by inducing apoptosis, with no side effects. The results of the present study suggest that NO-HSA has promise as a new generation anticancer agent with few side effects.

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REGULAR ARTICLE

Platelet responsiveness to *in vitro* aspirin is independent of COX-1 and COX-2 protein levels and polymorphisms

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KEYWORDS
Aspirin resistance;
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Abstract

Aspirin's inhibitory effect on platelet function has been shown to be highly heterogeneous. However, due to the considerable individual variation in pharmacokinetics after aspirin intake, it has been difficult to investigate the mechanism of aspirin resistance empirically. Our objective was to examine whether platelet responsiveness to *in vitro* aspirin treatment could be affected by cyclooxygenase (COX)-1/2 protein levels in platelets or single-nucleotide polymorphisms (SNPs), which could possibly change specific activity of enzymes and/or aspirin susceptibility. Collagen/epinephrine closure time (CEPI-CT) of PFA-100 in blood from 178 healthy males was assessed with/without aspirin. Platelet COX-1 protein levels and the sequences of COX-1 gene exons were examined in three groups categorized by CEPI-CT: PR (Poor responders to aspirin), 10 people showing the shortest CEPI-CT under aspirin; GR–High or GR–Low (good responders to aspirin with high or low platelet basal reactivity), 10 people showing CEPI-CT over 300 s under aspirin and having the

Abbreviations: COX, cyclooxygenase; SNPs, single nucleotide polymorphisms; CEPI-CT, collagen/epinephrine closure time; CADP-CT, collagen/ADP closure time; PR, Poor responders to aspirin; GR–High, Good responders to aspirin with high platelet basal reactivity; GR–Low, Good responders to aspirin with low platelet basal reactivity; VWF:Ag, von Willebrand factor antigen; VWF:RCo, VWF ristocetin cofactor activity.

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shortest or longest basal CEPI-CT, respectively. We analyzed the three groups, representing phenotypic extremes, aiming to increase statistical power to investigate the possible relevance of COXs to platelet response to aspirin. Western blot analysis revealed that COX-1 was abundantly expressed in platelets at comparable levels among the three groups, whereas COX-2 was undetectable. The frequencies of nonsynonymous COX-1/2 SNPs were unlikely to explain the difference in aspirin responsiveness considering the observed genotype frequencies and wide individual variation in platelet response. These results suggest that heterogeneity in platelet responsiveness to *in vitro* aspirin is independent of COX-1/2 protein levels and SNPs.

Introduction

Acetylsalicylic acid (aspirin) has been shown to be one of the most effective antiplatelet agents and is now commonly used to prevent arterial vascular events. Results of a recent meta-analysis revealed that aspirin reduces the risk of secondary cardiovascular events by approximately 25% [1]. In some patients, however, a lack of platelet inhibition has been demonstrated despite aspirin therapy [2–4]. At present, there is no consensus regarding the definition of "aspirin resistance", but it is roughly categorized into two types: "clinical failure", which means inability of aspirin to protect patients from vascular events; and "laboratory aspirin resistance", which means impaired platelet sensitivity to aspirin detected by *ex vivo/in vitro* laboratory tests. Importantly, previous some studies have suggested that "laboratory aspirin resistance" increases the risk of "clinical failure" [5,6].

Despite considerable efforts, the mechanism of aspirin resistance remains unclear. One important reason would be the considerable individual variation in pharmacokinetics after aspirin intake [7–9]. This heterogeneity in aspirin blood concentration makes it difficult to investigate the mechanism of aspirin resistance empirically. In our previous report [10], we characterized individuals showing a poor response to the *in vitro* effect of aspirin by using PFA-100, a useful and widely used apparatus for monitoring aspirin non-responders [11]. The individuals who responded poorly to 10 μM aspirin tend to have higher basal platelet aggregability, higher plasma adiponectin levels, and higher prevalence of diabetes. Notably, maximum inhibition of platelet aggregation (CEPI-CT > 300 s) was observed for almost all subjects when their blood samples were incubated with a higher concentration of aspirin (30 μM), suggesting that "laboratory aspirin resistance", and consequently "clinical failure" could be overcome by a higher dose of aspirin.

There are two isoforms of cyclooxygenase, the molecular targets of aspirin, coded by distinct genes [12,13]. COX-1 is constitutively expressed in most human cells and tissues and plays several important physiological roles. In platelets, aspirin irreversibly acetylates serine 530 of COX-1, resulting in inhibition of proaggregatory thromboxane A_2 generation [1]. On the other hand, COX-2 is normally undetectable in most tissues and is rapidly induced by various stimuli, such as mitogens and cytokines [14]. Generally, expression level of proteins, rather than mRNA, determines the phenotype [15], but there are few reports exploring the relevance of COX protein level and aspirin response [16].

In this study where aspirin was added *in vitro* (*i.e.* aspirin exposure to platelet was kept constant for excluding the individual pharmacokinetic variation), we aimed to investigate whether aspirin responsiveness can be influenced by COX-1/2 protein levels in platelets or by polymorphisms in the COX-1/2 gene that cause amino acid substitutions, both of which could possibly change specific activity of enzymes. Furthermore, we investigated the possible expression of COX-3 and other splicing variants of COX-1 [17], because they also potentially produce prostanoids.

Materials and methods

Subjects

All experiments were performed with the permission of the ethics committees of the research institutes (DAIICHI SANKYO Co., Ltd., Tokyo, Japan and Keio University Hospital, Tokyo, Japan) and the gene-analyzing institutes (Toyobo, Osaka, Japan and TAKARA BIO, Otsu, Japan). Informed written consent was obtained from all subjects before enrollment. Three hundred and twenty-five male subjects were initially enrolled at the time of their regular health check-ups. 32 subjects who had any apparent hematologic, cardiovascular, hepatic, or renal diseases, and those who had taken any NSAIDs or cold medicines within 10 days of testing were then excluded, and 115 were excluded due to instrument error of PFA-100 either with or without 10 μM aspirin. Finally, 178 subjects were available for the analyses.

Table 1 Demographic and biochemical characteristics in PR, GR–High, and GR–Low groups

	All	PR	GRó High	GRó Low	P[a]
N	178	10	10	10	Ö
CEPI-CT at vehicle (s)	150±50	102±16	99±6	282±18	Ö
CEPI-CT at 10 M aspirin (s)	268±61	113±15	300	300	Ö
CADP-CT (s)	99±23	88±17	82±9	122±19	b0.001
Age (y)	47±5	48±3	45±4	44±4	0.127
BMI (kg/m^2)	24.0±2.9	22.5±2.6	22.5±2.4	25.2±2.3	0.028
Current smoker (n)	77	3	8	4	0.061
RBC (x10^4/μL)	471±32	468±38	457±36	467±24	0.740
WBC (/μL)	6000±1600	5500±1000	7000±2000	5400±1100	0.031
Platelet (x10^4/μL)	24.3±4.5	25.5±5.1	22.8±5.2	26.0±6.0	0.382
Hematocrit (%)	45.1±2.4	44.4±2.3	45.3±1.9	44.2±2.2	0.518
Hemoglobin (g/dL)	14.8±0.9	14.3±0.8	14.9±0.6	14.7±0.8	0.244
Fibrinogen (mg/dL)	240±58	228±35	251±43	217±37	0.158
Total protein (g/dL)	7.22±0.37	6.86±0.37	7.04±0.43	7.24±0.28	0.085
Creatinine (mg/dL)	0.88±0.10	0.86±0.06	0.89±0.06	0.84±0.08	0.328
GOT (AST) (U/L)	23.8±12.0	18.9±3.9	23.7±10.8	29.2±21.8	0.285
GPT (ALT) (U/L)	28.2±17.2	18.7±5.0	26.2±16.0	35.8±32.5	0.211
γ-GTP(U/L)	59.7±59.8	31.4±10.7	56.8±44.6	50.4±37.0	0.239
VWF:Ag (%)	111±44	123±40	135±38	83±25	0.006
VWF:RCo (%)	93.1±39.6	110±35	113±45	65±35	0.015
sVCAM-1 (ng/mL)	516±167	562±164	565±151	480±95	0.315
Adiponectin (μg/mL)	7.6±3.2	11.0±5.2	8.1±3.7	6.9±2.2	0.066
HbA1c (%)	5.2±0.6	5.1±0.4	5.1±0.3	5.3±0.5	0.419
HDL cholesterol (mg/dL)	55.6±13.1	57.7±12.6	60.5±18.5	54.0±11.4	0.609
LDL cholesterol (mg/dL)	130±31	137±17	121±25	138±25	0.170
TXB_2 (ng/mL)[b]					
At vehicle	54.1±23.6	53.2±21.6	56.9±18.6	52.3±34.5	0.931
At 10 μM aspirin	25.5±15.8	29.5±11.5	23.9±4.7	28.9±38.9	0.876

Values are given as means±S.D. or number.

*$P<0.05$, **$P<0.01$ (*vs* GR–Low, Tukey test).

CEPI-CT, collagen/epinephrine-induced closure time; CADP-CT, collagen/ADP-induced closure time; VWF:Ag, von Willebrand factor antigen; VWF:RCo, VWF ristocetin cofactor activity.

[a]χ^2 test for categorical variables, and one-way ANOVA for continuous ones.

[b]Collagen-induced thromboxane B_2 biosynthesis, as described in Materials and methods.

PFA-100 testing

We evaluated PFA-100 CEPI-CT as previously described [10]. Briefly, aspirin (0.2% ethanol as vehicle or 10 μM, final concentration) was added to whole blood (0.38% sodium citrate, final concentration) and incubated for 30 min at room temperature.

Thromboxane generation

Thromboxane B_2 (TXB_2) biosynthesis during 2-μg/mL collagen-induced aggregation of platelet-rich plasma (PRP) with or without 10 μM aspirin was measured, as described previously [10].

DNA sequence analysis of COX-1 gene and SNP search of COX-2

Genomic DNA was extracted from whole citrated blood using MagExtractor (Toyobo).

For DNA sequence analysis of the COX-1 gene in the 30 subjects of PR, GR–High, or GR–Low, all 11 exons and exon–intron boundary sites were amplified by PCR with appropriate primer sets. The amplified products were purified and sequenced in both directions by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed with a 3730*xl* DNA analyzer (Applied Biosystems) or MegaBACE (Amersham Biosciences, USA).

To determine the frequency of SNPs in the COX-2 gene in our subjects, 6 known nonsynonymous SNPs (M1I, R228H, P428A, E488G, V511A, G587R) from the NCBI dbSNP build 123 (http://www.ncbi.nlm.nih.gov/SNP/) were genotyped in 25 subjects randomly selected from among the 325 enrollees by the single nucleotide primer extension method. Only one heterozygote was found for G587R, and then its genotype was determined for all enrollees.

Measurement of COX-1 and COX-2 protein levels in platelets

We investigated the COX-1/2 protein levels in the three groups categorized by CEPI-CT: PR (poor responders to aspirin), 10 people who showed the shortest CEPI-CT under aspirin; GR–High or GR–Low (good responders to aspirin with high or low platelet reactivity), 10 people showing CEPI-CT over 300 s under aspirin and having the shortest or longest basal CEPI-CT, respectively. The characteristics of the three groups are summarized in Table 1.

One mL of PRP adjusted to 30×10^4 platelets/μL with 5 mM EGTA and 1 μM PGE_1 was centrifuged at 12,000 rpm for 5 min. The pellet was resuspended with cold wash buffer (10 mM HEPES, pH 7.4, 138 mM NaCl, 2.9 mM KCl, 5.5 mM glucose, 12 mM $NaHCO_3$, 5 mM EGTA, and 1 μM PGE_1) twice and dissolved in 50 μL of Laemmli sample buffer (Bio-Rad Laboratories, CA, USA) with 5% β-mercaptoethanol. Then, the sample was boiled at 95 °C for 5 min and stored at −80 °C until assayed. Total protein concentration was determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For the quantification of COX-1 protein, total protein (1 μg) was separated by SDS-PAGE and then transferred onto an Immun-Blot™ polyvinylidene difluoride (PVDF) membrane with a semidry transfer unit. The membrane was blocked with BlockAce (Dainippon Pharmaceutical, Osaka, Japan) overnight at room temperature. Subsequently, membranes were treated with anti-COX-1 polyclonal antibody C24 (IBL, Gunma, Japan) at a dilution of 1:100 and concomitantly with anti-GPIIb sc-15328 (Santa Cruz, CA, USA) at a dilution of 1:5000 at room temperature for 90 min, followed by 60 min incubation with horseradish peroxidase-conjugated anti-rabbit IgG (17502; IBL) at a dilution of 1:100 at room temperature. Immunoreactive bands were visualized by using a peroxidase stain kit for immunoblotting (Nacalai Tesque Inc., Kyoto, Japan). Membranes were scanned and quantified with NIH image software (version 1.63; National Institutes of Health, Bethesda, Maryland, USA). COX-1 protein levels were determined from a standard curve generated with known amounts of ovine COX-1 standard (Cayman Chemical, MI, USA). The value was normalized by the density of the internal control sample on each blotting membrane and also by the density of platelet-specific GPIIb to adjust the amount of platelet protein loaded. The determinations were made in triplicate for each sample.

For the measurement of COX-2 protein levels in platelets, 15 μg of total platelet protein was subjected to SDS-PAGE and transferred onto a PVDF membrane by semidry blotting. As positive control, 1 ng of recombinant human COX-2 standard (Cayman Chemical) was used. Membranes were probed with anti-COX-2 monoclonal antibody (Cat# V10419; Biomeda Corp., Foster City, CA, USA) raised against a synthetic peptide corresponding to the C-terminus of rat COX-2 at a 1:200 dilution. Detection was performed using ECL plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK) or the FAS-1000 Lumino Imaging Analyzer (Toyobo).

Statistical analysis

Results are expressed as means ± S.D. For continuous variables, data were analyzed by one-way ANOVA and Tukey test. For the categorical variables,

differences between groups were detected by the χ^2 test. Differences were considered significant when P values were less than 0.05. These analyses were performed with SAS release 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

The means ± SD of basal CEPI-CT were 102 ± 16 s, 99 ± 6 s, and 282 ± 18 s in PR, GR–High, and GR–Low groups, respectively (Table 1). As described in our earlier report [10], they were strongly correlated with VWF:Ag or VWF:RCo. In the current study, the GR–Low group had significantly longer CADP-CT and higher BMI compared with the other groups, and the GR–High group had higher level of white blood cells. No significant differences were observed in age, current smoking status, blood pressure, platelet counts, HbA1c, and LDL cholesterol among the three groups.

COX-1 and COX-2 protein levels in platelets

All COX-1 levels were well within the quantitative range of standard curve. As shown in Fig. 1, there were no significant differences in platelet COX-1 levels among the PR, GR–High, and GR–Low groups after the normalization with platelet-specific GPIIb, which is expressed at stable levels in healthy subjects [18]. This result was consistent with that before the normalization. The regression analysis revealed no significant correlation between COX-1 protein levels and TXB_2 production during the collagen-induced aggregation with or without incubation of PRP with aspirin.

To investigate the relevance of COX-2 expression with heterogeneity of aspirin response, we determined COX-2 protein levels in platelets from healthy subjects by Western blot analysis. As shown in Fig. 1, COX-2 protein was undetectable in all subjects, although 1 ng of human recombinant COX-2 standard was detected.

The antibodies for COX-1 (C24) and COX-2 (V10419) did not crossreact with 5 ng of recombinant human COX-2 standard and 50 ng of ovine COX-1 standard, respectively (data not shown).

Sequencing all exons and exon/intron boundaries of the COX-1 gene

All 11 exons and their surroundings of the COX-1 gene were screened for polymorphisms in this population. As shown in Table 2, we identified five SNPs in exons; three were nonsynonymous SNPs (R8W, R179H, D258G) and the two others were

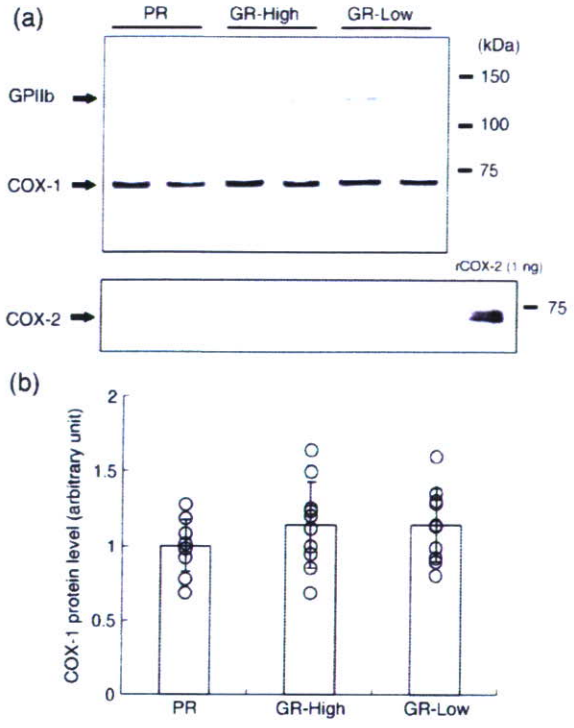

Figure 1 (a) Representative Western blot of GPIIb (top), COX-1 (middle), and COX-2 (bottom) in platelets from healthy subjects. One μg (upper membrane for GPIIb and COX-1) or 15 μg (lower membrane for COX-2) of total protein was separated by SDS-PAGE, transferred to PVDF membranes, and probed with sc-15328 (GPIIb specific), C24 (COX-1 specific), or V10419 (COX-2 specific) antibodies. (b) Densitometric quantification of COX-1 levels (in arbitrary units), normalized for GPIIb protein content, from PR, GR–High, and GR–Low groups. Data were shown as the means ± SD from triplicate determinations. No significant differences in COX-1 expression levels were observed among the three groups by one-way ANOVA.

synonymous (Q41Q, G213G). R179H and D258G were novel nonsynonymous COX-1 SNPs. One subject was a double heterozygote for two SNPs, Q41Q and D258G. The remaining three SNPs (R8W, R179H, G213G) were found in independent subjects. No SNPs were found in exon–intron boundaries of the COX-1 gene. P17L (C50T, rs3842787), a common SNP in Caucasian [19–21], was not found in this population.

Single nucleotide polymorphisms in the COX-2 gene

To investigate the frequency of nonsynonymous SNPs in the Japanese population, 25 enrollees were randomly selected from among all the subjects enrolled and genotyped at 6 potential loci selected from the dbSNP database. One subject was a

Table 2 Summary of SNPs in exons of the COX-1 gene in the Japanese population

Contig position[a]	Nucleotide change	dbSNP accession #	Amino acid change	PR		GR–High		GR–Low	
				WT	Hetero	WT	Hetero	WT	Hetero
32454683	C/T	rs1236913	R8W	10	0	9	1	10	0
32461410	G/A	rs3842788	Q41Q	9	1	10	0	9	1
32464893	G/A	–	R179H	10	0	10	0	9	1
32464996	C/A	rs5788	G213G	9	1	9	1	10	0
32465211	A/G	–	D258G	10	0	10	0	9	1

db SNP, NCBI Single Nucleotide Polymorphism Database; WT, Wild type; Hetero, Heterozygote.
[a] GenBank accession # NT_008470.17.

heterozygote for G587R (rs3218625), and this SNP was further investigated in subjects enrolled. But all 30 subjects in the three groups were homozygous for the G/G genotype.

Discussion

Despite the large number of studies on aspirin resistance, its mechanism still remains unclear. One possible reason for this is the substantial individual variation in pharmacokinetics after aspirin intake [7–9]. Moreover, even after repeated oral administration of low-dose aspirin (80 mg/day), the degree of platelet inhibition was highly variable, as determined by serum TXB_2 levels [7]. These data suggest that platelets are exposed to different concentrations of aspirin when administered orally, making it difficult to investigate the mechanism of aspirin resistance empirically. To exclude the involvement of individual pharmacokinetic variation after aspirin intake and to focus on platelet susceptibility to aspirin, platelet functions were evaluated after *in vitro* incubation with a fixed dose of aspirin.

The degree of platelet inhibition by aspirin greatly differs depending on the method of determination or agonists. In some platelet function tests, including collagen- or ADP-induced optical aggregation assay, platelet function is not completely inhibited even when aspirin abolishes COX-1 enzymatic activity completely [22], indicating poor sensitivity to aspirin's effect. In contrast, PFA-100 is known to be quite sensitive and appropriate for the detection of aspirin response [23]. We have previously described that a poor response to 10 μM of *in vitro* aspirin was significantly associated with higher basal platelet activities, an increased level of adiponectin, and the presence of diabetes mellitus [10]. Furthermore, we showed that the platelet functions of poor responders were inhibited with 30 μM aspirin, roughly equal to the peak plasma concentration after taking 650 mg of aspirin [9,24], suggesting that "laboratory aspirin resistance" could be circumvented with a higher dose.

So, we hypothesized that quantitative and qualitative variations in the COX-1 protein could alter the platelet response to aspirin, and the expression levels of COX-1 protein in platelets and genetic variants of the COX-1 gene-coding region among the three groups (PR, GR–High, and GR–Low) were investigated. In general, a 'comparison of extremes' approach increases the statistical power in the ability to detect SNPs affecting phenotype [25–27]. In this study, analysis of PR and GR–High groups, representing the phenotypic extremes, is most expected to reveal potential differences in the quantity (protein levels) and/or quality (nonsynonymous SNPs) of cyclooxygenases.

Although expression level of proteins, rather than mRNA, is important to determine the phenotype [15], there are few reports regarding the relevance of COX protein level and aspirin response [16]. To assess the relevance of COX-1 protein levels to the *in vitro* aspirin response, we qualified its expression in platelets by Western blot analysis. COX-1 protein is abundantly expressed in platelets (equivalent to approximately 2.2 ng ovine COX-1 in 1 μg of total platelet protein). As described above, the three groups had similar levels of COX-1 protein after the normalization with GPIIb. However, COX-2 was undetectable in all groups even when 15 μg of total platelet protein was loaded (Fig. 1). The level of platelet COX-2 was less than 1 ng in 15 μg of total platelet protein, which means that the expression level of COX-2 is far lower than that of COX-1 in our healthy subjects. Although some reports have suggested that the presence of platelet COX-2 might cause the aspirin resistance under certain clinical conditions [28,29], it has been well described that COX-1 is the only functional isoform in platelets from healthy subjects [30,31], supported by the fact that celecoxib or NS-398, COX-2 selective inhibitors, had no effect on platelet aggregation or the production of TXB_2 [32,33]. In terms of enzymatic activity, an *in vitro* study revealed that the COX-2 isoform exhibits specific activity equivalent to that of COX-1 in converting arachidonic acid to PGH_2 [34]. Our result

that COX-2 protein expression is undetectable is consistent with these previous observations. Taken together, COX-2 is assumed to have only a minor role, if any, in platelet function and activation under physiological conditions.

SNPs accompanying an amino acid substitution in COX-1 may alter the catalytic activity or the affinity for aspirin, and SNPs in the exon–intron boundaries may influence mRNA splicing. From DNA sequence analysis of all exons and exon–intron boundaries, five SNPs in the exons of COX-1 gene were revealed (Table 2), showing that the frequency of SNPs is relatively low in the Japanese population compared with Caucasian or African American populations [19–21,35]. Of the five SNPs, three substituted amino acids and the other two were synonymous. The three nonsynonymous polymorphisms are unlikely to be responsible for poor or good responses to *in vitro* aspirin, because the allele frequencies of these SNPs (1.7% each, one heterozygote per 30 subjects at each locus) are similar among the three groups and are too low to account for the wide variability in aspirin responsiveness among the three groups. Furthermore, these residues are not located near the active site or acetylating site according to three-dimensional structural information [36,37]. According to the previous reports, the relevance of COX-1 mutations to 'clinical' or 'laboratory' aspirin resistance is unclear, because they have yielded controversial results. Maree et al. showed that the haplotype, including the minor-842G allele in the promoter region, appeared to contribute 'laboratory aspirin resistance' determined by arachidonic acid-induced platelet aggregation [38]. On the other hand, Halushka et al. showed that patients who were heterozygote for the C50T allele, which is in complete linkage with A-842G in Caucasian, showed greater inhibition of PGH_2 formation by aspirin compared with the wildtype [20]. Furthermore, no common COX-1 SNPs were found in 'clinical' aspirin non-responder with ischemic stroke despite taking aspirin [21]. From the DNA sequence analysis, we have not found the C50T in our population ($n=30$), as was reported in other Japanese population [39], suggesting that C50T/A-842G unlikely to affect *in vitro* effect of aspirin at least in this *in vitro* study.

Recently, COX-3 or other splicing variants of COX-1 have been proposed as a possible factor in aspirin resistance, because some of these variants have COX activity [17]. C24, an anti-COX-1 antibody raised against a synthetic peptide (ADPGAPTPVNPC) near the N terminus, which is conserved in COX-3 and splicing variants [17], was presumed to detect those splicing variants near the COX-1 band. However, no bands, other than a single 70-kDa band of COX-1, were observed (Fig. 1). This suggests that COX-3 or other splicing variants are not the major isoforms of cyclooxygenases in platelets in normal subjects.

COX-independent pathways have often been noted as mechanisms for aspirin resistance. Frelinger et al. reported that low-dose aspirin substantially reduces serum TXB_2 production during blood coagulation, but the remaining TXB_2 formation was not inhibited by further *in vitro* addition of aspirin or indomethacin [40], suggesting that there is a mechanism(s) for 'laboratory aspirin resistance' other than the COX pathway. Prostaglandin H_2 produced by regenerated nucleated cells [5], reduced sensitivity of platelets from diabetic patients to aspirin [41], or platelet hyperreactivity to agonists [10,42] have been also proposed as a cause of aspirin resistance. Further prospective studies are needed to confirm these results.

Several limitations of the present study should be acknowledged. First, this study was performed in a subset of individuals with high or low sensitivity to *in vitro* aspirin. Although extreme selection strategies can markedly increase power to detect the loci responsible to phenotype [25–27], the degree of association between sensitivity and COX might be changed with a different subset of individuals. To minimize variance, we determined genotype by sequencing of both strands and protein levels in triplicate from a standard curve with internal standard on each membrane. Second, we assessed *in vitro* laboratory aspirin resistance using PFA-100, and concluded that it is not caused by heterogeneity in COX-1/2 protein levels and common SNPs. This conclusion was derived from a particular setting (*i.e. in vitro* incubation of aspirin, healthy subjects, and PFA-100). Therefore, extrapolation of our results to all aspirin resistance (especially clinical aspirin resistance) should be made with caution.

Taken together, our results suggest that individual variations in the response of platelets to fixed concentrations of aspirin do not result from differences in COX-1 protein levels in platelets, changes in COX-1 catalytic activity, and/or aspirin susceptibility. Furthermore, other isoforms such as COX-2 or putative COX-3 are expressed at undetectable levels in platelets from healthy subjects and should be insignificant factors in affecting aspirin responsiveness.

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