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-H2DCFDA, 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 produg designed to release NO after reactivating 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 (Semsoth 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 2-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-iminothiolane) was purchased from Pierce Chemical (Rockford, IL). Isoeptynitrite, 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 $-20^\circ$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 sulphydryl 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 PelleconXL filtration device (Millipore Corporation, Billerica, MA), and the final concentration adjusted to 2 mM NO-HSA. The sample was stored at $-80^\circ$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 NaNO2 (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% 1-naphthyl-2-hydrazinylamine-diamide, and 0.15% sodium dithionite with or without 0.09 mM HgCl2, 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 HgCl2 from values in the presence of HgCl2; 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% CO2 in air.

For detection of reactive oxygen species (ROS), C26 cells (1.0 × 104 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-H2DCFDA) (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
were monitored using flow cytometric analysis with rhodamine 123 staining. C26 cells (1.0 × 10⁶ 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 × 10⁶ 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 protease 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 × 10⁴ 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 AnNCrIcrj mice (17–20 g) were purchased from Charles River Italia (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 × 10⁶ 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 × b²), 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/paraformaldehyde 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 cryostat 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% H₂O₂ 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 Labelling 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-H₂O₂ 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 Tumor Cell Apoptosis 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 H₂O₂, O₂⁻, 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-H₂DCFDA), 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 mitochondria,
PBS, 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.

Fig. 2. Production of ROS in C26 cells after NO-HSA treatment. C26 cells were pretreated with CM-H$_2$DCFDA 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.

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-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone), from absorbance after incubation without caspase inhibitor. The absorbances among PBS-, HSA- and NO-HSA-treated cells were 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-
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 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

<table>
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<tr>
<th>Serum Biochemical Parameter</th>
<th>Saline</th>
<th>HSA</th>
<th>NO-HSA</th>
</tr>
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<tbody>
<tr>
<td>Total protein (g/dl)</td>
<td>5.23 ± 0.21</td>
<td>5.33 ± 0.06</td>
<td>5.55 ± 0.24</td>
</tr>
<tr>
<td>Cr (mg/dl)</td>
<td>0.13 ± 0.04</td>
<td>0.14 ± 0.01</td>
<td>0.18 ± 0.01</td>
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<tr>
<td>BUN (mg/dl)</td>
<td>15.25 ± 0.96</td>
<td>15.33 ± 1.53</td>
<td>13.75 ± 1.26</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>143.5 ± 62.9</td>
<td>91.7 ± 39.4</td>
<td>116.3 ± 69.1</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>169.8 ± 111.6</td>
<td>110.0 ± 81.2</td>
<td>161.3 ± 126.2</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>395.3 ± 4.7*</td>
<td>345.3 ± 4.7*</td>
<td>300.5 ± 23.0**</td>
</tr>
</tbody>
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* P < 0.05, saline vs. HSA.
** P < 0.01, saline vs. NO-HSA.
† P < 0.05, HSA vs. NO-HSA.

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.
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-nitrosothiols 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 NaNO2 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 reactive 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 O2 to produce NO2, which is then available for nitrosation reactions with intracellular thiols at the membrane- cytosol interface (Ramachandran 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. elucidated the detailed mechanism of apoptosis induced by NO-ASA. Intra- cellular 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-RasV12, 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-phenyl-ethyl 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 leukemia 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.

Antiangiogenic 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 antiangiogenic activity (Ishihara 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 present 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 activation of caspase-3 induced by actinomycin D in U937 cells) was 26 to 130 and 165 to 660 μM respectively, and 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) (Ishihara 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 iminodiacetate as a spacer. NO-HSA generated ROS in C26 cells, and it induced intrinsic 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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