medium containing 10% fetal calf serum (FCS), 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 x 10^4 cells/well) were cultured in 96-well plates in medium A for 12 h, washed twice with PBS, and incubated for an additional 30 min in HBSS containing 5 μM 5-(and-6)-chloromethyl-2',7'- dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Molecular probes). After washing twice with HBSS, the cells were cultured in HBSS containing 5% FCS 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 x 10^6 cells/well) were cultured in 6-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 (FACS Calibur; Becton Dickinson Biosciences, Franklin Lakes, USA).

For the determination of caspase-3 activity, cells were cultured to confluence in 6-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 cell lysis buffer, lysed by freeze-thawing and then incubated on ice for 15 min. The cell lysates were centrifuged at 15,000 rpm for 20 minutes at 4 °C, and the supernantant fraction was collected (cell extract). The caspase-3 activity in the cell extract was assessed using the colorimetric CaspASE™ Assay System (Promega), according to the manufacturer's instructions.

For the detection of DNA degradation (DNA ladder), C26 cells (1×10^6 cell/well) were cultured in 6-well plates. Cells were cultured for 12 h, washed twice with PBS, and 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 centrifuged at 4000 rpm for 10 min. The supernatant was again removed and the remaining pellet was incubated in 20 μl 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 15000 rpm for 5 min. The resulting supernatant (cell extract) was collected and incubated with 1 μl RNase A (10 μg/ml) for 30 min at 50 °C. 1 μl 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 the Cell Counting Kit-8 (WST-8),

which is based on the MTT assay. C26 cells were plated in 96-well plates at 1.0 x 10^4 cells/well and 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 medium A containing various concentrations of HSA or NO-HSA. After incubating the cultures for various lengths of time, 5 μl 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 (Calco, Como, Italy). The mice were housed in a 12/12 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 x 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 intravenous injection of saline, HSA (10 μmol/kg), or NO-HSA (10 μmol/kg), for 10 days from day 3 to 12 post-inoculation. Tumor volume was calculated using the formula 0.4 (a x b^2), where 'a' is the largest, and 'b' is the smallest, diameter of the tumor (Shimizu et al., 2005), and monitored from day 7 to 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 on days from 3 to 7 post-inoculation (the tumors in each group reached approximately 5 mm in diameter), blood samples were collected from the abdominal vena cava under diethylether anesthesia approximately 2 h after the daily injection and then the mice were sacrificed.

Tumors were dissected, fixed immediately with 2% periodate/lysine/ paraform-aldehyde fixative at 4°C for 5 h and 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), frozen in liquid nitrogen, and stored at -80°C. Five-μm tumor sections were prepared using a cryostatic microtome (HM500M, Microm, Walldorf, Germany), and 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 3 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 were incubated in 100 ml of permeabilization buffer on ice for 5 min. The slides were washed 3 times with 0.01 M phosphate buffered saline, and then were incubated with 50 ml of freshly prepared terminal deoxynucleotidyl transferase(TdT) reaction mixture (TdT enzyme 5 ml + Labeling Safe buffer 45 ml) at 37°C for 60 min. After the slides were washed 3 times with 0.01 M PBS, they were incubated in 70 ml of anti-FITC-HRP conjugate antibody at 37°C for 30 min. After the slides were washed 3 times with 0.01 M PBS, they were incubated in DAB-H_2O_2 reaction buffer at room temperature for 10

min. After the slides were washed 3 times with distilled water, they were stained with 3% methyl green for 10 min, dehydrated, penetrated and sealed (Gravrieli et al., 1992). Each slide then was visualized under a light microscope (Olympus, Tokyo, Japan), at a magnification of x 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 (DCF) 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 (Figure 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 mitochondrial 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 (Figure 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 with100 μM NO-HSA showed a larger increase in caspase-3 activity (Figure 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 (Figure 3C). Neither vehicle nor 100 μM HSA induced DNA fragmentation in C26 cells (Figure 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 (Figure 4A). The viability of C26 cells incubated with 50 μM NO-HSA significantly decreased with increasing incubation times (Figure 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.

NO-HSA exerts anti-tumor effect via the apoptotic pathway in vivo

To investigate the anti-tumor effect of NO-HSA *in vivo*, C26 tumor-bearing mice were intravenously injected 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 (Figure 5).

To clarify whether the suppression of tumor growth by NO-HSA is mediated via apoptosis, tumor tissues from C26 tumor-bearing mice injected 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 anti-tumor effect *in vivo* (Figure 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 U/l 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 anti-cancer agent. The vasodilating effect of NO-HSA was also evaluated in rats after intravenous injection at a dose of 10 μmol/kg (66 μmol of NO per kilogram). NO-HSA induced a decrease in the mean arterial blood pressure immediately after intravenous injection and the maximum

reduction effect was 32.8 ± 7.3 mmHg. In contast, 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 et al., 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 appears to act as a reservoir of NO *in vivo* (Stamler et al., 1992). Simon et al. 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, a NO-BSA conjugate prepared with the same method except that the BSA has been reduced with dithiothreitol and contains 19 mol of "S-NO" per mol of BSA, was a significantly more potent platelet inhibitor than polynitrosylated BSA described above (Simon et al., 1996). These findings show that nitrosylated BSA behave as a 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 non- 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 bovine serum albumin (BSA) by reduction with dithiothreitol and thiolation with N-acetylhomocysteine, thereby preparing poly-nitrosated BSA (12-15 mol NO/mol BSA) (Ewing et al., 1997). Marks et al. produced poly-nitrosated BSA (5.9

mol NO/mol BSA) by adding free sulfhydryl groups to the molecule and by treating the BSA with N-acetylhomocysteine thiolactone (Marks et al., 1995). However, the poly-nitrosated 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 poly-nitrosated HSA (NO-HSA) (6.6 mol NO/mol HSA), which did not form aggregates after non-reducing SDS-PAGE or native-PAGE (data not shown). Moreover, the far-UV 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, GSH reacts with organic nitrite esters to form GSNO, indicating that NO-NSAIDs release NO via S-nitrosothiol (Wong et al., 1999). Alternatively, the transfer of NO from NO-HSA to the cytosol could be inferred from a study by Ramachandran et al. The authors reported that NO is released from extracellular S-nitrosothiols by a cell-surface enzyme (protein disulfide isomerase) and 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 likely 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. Recently, Gao J et al. 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 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. Recently, Trachootham et al., 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. reported that cyaniding-3-rutinoside selectively induces accumulation of peroxides in HL-60 human leukemic cells, but not in normal

PBMC (Feng et al., 2007). Schumacker has proposed that ROS toxicity induced by certain chemo-therapeutic agents may be an effective means of selectively eradicating malignant cells (Shumacker, 2006). 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 likely 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 anti-cancer agent, given the unusual redox conditions typical of malignant cells.

Anti-apoptotic 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 et al., 1999). In a recent study using U937 human promonocytic cells, NO-R410C (a genetic variant of HSA) had anti-apoptotic activity (Ishima et al., 2007). Whether NO ultimately inhibits or promotes apoptosis likely depends on the cell, the signal, the source, the molecule, the amount, and the presence or absence of co-reactants. For example, the amount of NO bound to the carrier molecule appears to account for the discrepant results between previous investigations and the present study. The NO content of NO-R410C (in 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 26-130 and 165-660 μM, respectively. Mohr et al. reported that 10-100 μM GSNO inhibits the activation of caspase-3 induced by actinomycin D in U937 cells (Mohr et al., 1997). 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 (Mebmer et al.,

1995). 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 appears to be 100-200 μM.

Matsumura et al. 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 appeared to be due to reduced renal excretion of HSA compared with GSH 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, induced intrinsic apoptotic events, such as depolarization of mitochondoria 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 anti-cancer agent with few side effects.

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