[CANCER RESEARCH 62, 3138–3143, June 1, 2002]

Tumor-targeted Delivery of Polyethylene Glycol-conjugated D-Amino Acid Oxidase for Antitumor Therapy via Enzymatic Generation of Hydrogen Peroxide[1]

Jun Fang, Tomohiro Sawa, Takaaki Akaike, and Hiroshi Maeda[2]

Department of Microbiology, Kumamoto University School of Medicine, Kumamoto 860-0811, Japan

ABSTRACT

Hydrogen peroxide (H_2O_2) is a strong oxidant that induces apoptosis of tumor cells *in vitro*. Here, we investigated the antitumor activity of an H_2O_2-generating enzyme, D-amino acid oxidase (DAO), and its conjugate with polyethylene glycol (PEG; PEG-DAO). Compared with DAO, PEG-DAO showed improved pharmacokinetic parameters in mice after i.v. injection. PEG-DAO administered i.v. accumulated selectively in tumor tissue with insignificant accumulation in normal organs and tissues. To generate cytotoxic H_2O_2 at the tumor site, PEG-DAO was first administrated i.v. to tumor-bearing mice. After an adequate lag time, the substrate of DAO, D-proline, was injected i.p. This treatment resulted in significant suppression of tumor growth compared with tumor growth in control animals (not given treatment; $P < 0.001$). Similar treatment with native DAO showed no effect under the same conditions. Oxidative metabolites were significantly increased in solid tumors by administration of PEG-DAO followed by D-proline ($P < 0.002$, compared with the group receiving no treatment), as evidenced by thiobarbituric acid-reactive substance assay. This treatment did not affect results from the metabolites in the liver and kidney. These findings suggest that tumor-targeted delivery of DAO is accomplished by using pegylated enzyme and thereby taking advantage of the enhanced permeability and retention effect in solid tumor. PEG-DAO thus delivered together with D-proline produces remarkable antitumor activity via extensive generation of H_2O_2.

INTRODUCTION

ROS,[3] *e.g.*, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH), are potentially harmful byproducts of normal cellular metabolism that directly affect cellular functions (*e.g.*, development, growth, and aging) and survival as well as causing mutation (1). ROS are generated by all aerobic organisms and seem to be indispensable for signal transduction pathways that regulate cell growth (2) and reduction-oxidation (redox) status (1). However, overproduction of these highly reactive metabolites can initiate lethal chain reactions that involve oxidation and that damage cellular integrity and survival (1).

Among the ROS, H_2O_2 readily crosses cellular membranes and causes oxidative damage to DNA (3), proteins (4), and lipids by direct oxidation or via the transition metal-driven Haber-Weiss reaction to the extremely reactive hydroxyl radical (5). It was also reported that H_2O_2 induces apoptosis of many tumor cells *in vitro* (6–10) via activation of the caspase cascade. Of greater importance, many antitumor agents, such as vinblastine, doxorubicin, camptothecin, and inostamycin, exhibit antitumor activity via H_2O_2-dependent activation of apoptotic cell death, which suggests potential use of H_2O_2 as an antitumor principle (11).

H_2O_2 is relatively unstable and is a small water-soluble molecule. Those characteristics hamper the utility of H_2O_2 as an antitumor agent that might be selectively delivered to tumor. In fact, H_2O_2 used alone was ineffective when injected into tumor or into the circulation (12–16), perhaps because of its rapid clearance and decomposition by catalase in erythrocytes. Use of an H_2O_2-generating enzyme has been proposed as an alternative approach to developing an H_2O_2-dependent antitumor system. Nathan and Cohn (17) and Ben-Yoseph and Ross (18) reported that GO, which generates H_2O_2 during oxidation of glucose, showed antitumor activity in solid tumor models. However, regulation of H_2O_2 production by exogenously administered GO in tumor-bearing hosts is problematic because the availability of its substrates, oxygen and glucose, cannot be significantly modulated, with the possible induction of severe systemic side effects because of systemic generation of H_2O_2. In fact, GO administration to produce H_2O_2 required injection of antioxidants to minimize systemic toxicity (17, 18).

More recently, we developed a conjugate of PEG and XO (PEG-XO) for effective delivery of XO to tumor (19). Infusion of the substrate of XO to generate superoxide thus caused tumor regression (19). In the present study, along this line, we selected DAO (EC 1.4.3.3) because of its capacity for H_2O_2 generation. DAO is a flavoprotein that catalyzes the stereoselective oxidative deamination of D-amino acids to the corresponding α-keto acids. During this oxidation reaction, molecular oxygen (O_2) is used as an electron acceptor, and H_2O_2 is generated (20). D-Amino acids do not usually exist in mammalian organisms to a significant level (21). We therefore expected that DAO activity and hence generation of H_2O_2 could be regulated by exogenous administration of D-amino acids.

DAO does have a pharmacological drawback, however: a short *in vivo* half-life. The molecular size of DAO (M_r 39,000) is slightly smaller than the renal excretion threshold (M_r ~50,000), so it would be excreted gradually as observed previously for other small proteins or polymer drugs smaller than M_r 40,000 (22–27). To overcome this drawback, DAO was chemically modified by conjugation with a biocompatible polymer, PEG. This modification results in an increased *in vivo* half-life, a reduced antigenicity of the native protein, and an inhibition of proteolytic degradation, as reported previously (19, 27, 28).

As described in earlier reports, biocompatible macromolecules accumulate and remain in solid tumor because of the unique characteristics of the tumor vasculature and the impaired lymphatic clearance system. This phenomenon was named the EPR effect of macromolecules and lipids in solid tumor (22–35). Therefore, on the basis of the EPR effect, with DAO conjugated to PEG (PEG-DAO), we anticipated a high blood level of DAO for a long period and preferential accumulation of DAO in tumor. In this report, we describe the synthesis, accumulation in tumor, and antitumor activity of PEG-DAO.

Received 10/29/01; accepted 3/27/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by Grant-in-Aid 12217120 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

[2] To whom requests for reprints should be addressed, at Department of Microbiology, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860-0811, Japan. Phone: 81-96-373-5098; Fax: 81-96-362-8362; E-mail: msmaedah@gpo.kumamoto-u.ac.jp.

[3] The abbreviations used are: ROS, reactive oxygen species; DAO, D-amino acid oxidase; PEG, polyethylene glycol; GO, glucose oxidase; XO, xanthine oxidase; EPR, enhanced permeability and retention; TNBS, trinitrobenzenesulfonic acid; TBARS, thiobarbituric acid-reactive substance; AUC, area under the concentration *versus* time curve; LC_{50}, median lethal concentration.

MATERIALS AND METHODS

Materials. DAO (from porcine kidney), D-alanine, and D-proline were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Succinimide-activated PEG (MEC-50HS), with an average molecular size of M_r 5,000, was a kind gift from Nippon Oil & Fat Co. (Tokyo, Japan). Other reagents were of reagent grade and were used without further purification.

Animals. Male ddY mice, 6 weeks of age and weighing 30–35 g, were from SLC, Inc. (Shizuoka, Japan). All experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Kumamoto University School of Medicine.

Synthesis and Purification of PEG-DAO. DAO was subjected to gel filtration chromatography to purify the DAO monomer by using Sephadex G-100 (Pharmacia LKB, Uppsala, Sweden); the column size was ϕ20 mm × 50 cm; 50 mM sodium phosphate buffer (pH 7.4) was the mobile phase, and the flow rate was 15 ml/h. Under these conditions, DAO monomer was eluted to the 34–50-ml fraction, which was collected and condensed to ~1.5 mg/ml protein by use of the Amicon ultrafiltration system (YM-10 membrane; cutoff size, M_r 10,000).

Succinimidyl PEG was conjugated to the amino group of DAO by using nucleophilic succinimide-activated PEG as described in our previous report (19). In brief, to the DAO solution (1.5–2.0 mg/ml protein in 50 mM sodium phosphate buffer, pH 7.4), succinimide-activated PEG was added at a 3.5 molar excess of PEG/mol of free amino groups in DAO and allowed to react for 1 h at 4°C. The reaction mixture containing PEG-DAO thus obtained was then purified to remove free PEG and other low molecular weight reactants by ultrafiltration with the YM-10 membrane as mentioned above, using 10 times the volume of 0.01 M phosphate-buffered 0.15 M saline (PBS). PEG-DAO was stored in PBS containing 0.1 mM flavin adenine dinucleotide, a cofactor of DAO, at 4°C.

Determination of the Degree of PEG Conjugation. The extent of PEG conjugation with DAO was determined by the method using TNBS to measure the decrease in free amino groups (36). Glycine was used as a standard. The protein concentrations of both native DAO and PEG-DAO were quantified by using the DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). The molecular size of PEG-DAO thus estimated from the increase relative to PEG was confirmed by approximation via the size exclusion chromatography, described below.

Size Exclusion Chromatography. The preparation described above was further subjected to high-performance liquid chromatography for preparation as well as for analysis. For size exclusion chromatography, we used the fast protein liquid chromatography system (Pharmacia LKB) equipped with a Superose 6 HR 10/30 column (Pharmacia LKB). The mobile phase was 50 mM sodium phosphate buffer (pH 7.4), and monitoring was by absorption at 280 nm.

Enzyme Activity of DAO. Activity of DAO was determined via the horseradish peroxidase-coupled colorimetric assay with *o*-dianisidine as a substrate for horseradish peroxidase. In this assay, the substrate is reduced, and color develops with an absorption maximum of 460 nm (37). D-Alanine was used as the substrate with an initial concentration of 0.1 M. The enzyme reaction was carried out at 25°C in 0.1 M Tris-HCl buffer (pH 8.2), where 1 unit of DAO activity is defined as the rate of formation of 1 μmol of H_2O_2 per min.

***In Vitro* Cytotoxicity Assay.** *In vitro* cytotoxicity of DAO was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (38) with human colon cancer SW480 cells, which were plated in 96-well culture plates (3000 cells/well). Cells were cultured overnight in DMEM with 10% FCS. SW480 cells were then incubated in the presence of native DAO or PEG-DAO with a substrate (D-proline or D-alanine) for 24–48 h. Toxicity was quantified as the fraction of cells surviving relative to untreated controls.

***In Vivo* Distribution of PEG-DAO after i.v. Injection into Tumor-bearing Mice.** Mouse sarcoma S-180 cells (2×10^6 cells) were implanted s.c. in the dorsal skin of ddY mice. At 7–10 days after tumor inoculation when tumors reached a diameter of 5–7 mm but no necrotic areas were apparent, DAO activity in each tissue was measured to determine the distribution of native DAO and PEG-DAO after i.v. injection. The dose of DAO in both groups was 1.5 units/mouse given via the tail vein. At scheduled time points, from 1 min to 8 h, mice were killed, and blood samples were drawn from the inferior vena cava, after which mice were subjected to reperfusion with 10 ml of physiological saline containing heparin (5 units/ml) to remove blood components in the blood vessels of all tissues. Tumor tissues as well as normal tissues and organs including liver, kidney, spleen, intestine, heart, lung, muscle, and brain were collected and weighed. The enzyme activity of DAO in each tissue was then determined, based on formation of α-keto acid (pyruvic acid) from the reaction between the D-amino acid (D-alanine) and DAO (39).

The pharmacokinetic parameters of native DAO and PEG-DAO were determined by use of a two-compartment model, and the plasma half-life was estimated via the nonlinear least squares program MULTI (40), in which the AUC was calculated by using the trapezoidal rule and extrapolating to infinity. The total body clearance (*CL*) was calculated as:

$$CL\ (\text{ml/h}) = \text{dose (milliunits)}/AUC_{0\text{-}\infty}\ (\text{milliunits·h/ml})$$

***In Vivo* Antitumor Activity of PEG-DAO.** Mice with palpable S-180 tumors (4–5 mm in diameter; the tumors implanted as described above) were used to examine the antitumor activity of native DAO and PEG-DAO. Native DAO or PEG-DAO was injected (1.5 units/mouse) i.v., followed by i.p. administration (0.5 mmol/mouse) of D-proline twice daily at 2 and 4 h after native DAO or PEG-DAO injection. Tumor volume was estimated by measuring longitudinal cross section (*L*) and transverse section (*W*) and applying the formula $V = (L \times W^2)/2$.

TBARS Assay. Oxidative cellular damage was determined by assay of lipid peroxide formation via the TBA reaction (41). Native DAO or PEG-DAO was injected (1.5 units/mouse) i.v. to mice bearing S-180 tumors, after which, 2 and 4 h later, D-proline was administered i.p. (0.5 mmol/mouse). Mice were killed 12 h after the last administration of D-proline. After reperfusion of mice with physiological saline containing heparin, tumors, livers, and kidneys were collected and weighed, followed by homogenization and centrifugation. The TBARS assay was then carried out. The concentration of the product, malondialdehyde, was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (42).

Statistical Analysis. Student's *t* test was used to determine the significance between each experimental group. The difference was considered statistically significant when $P < 0.05$.

RESULTS

Synthesis and Characterization of PEG-DAO. Physicochemical and biochemical characteristics of native DAO and PEG-DAO are summarized in Table 1. The reaction resulted in 34.6% conjugation to the amino groups of DAO, which has a total of 13 amino groups; 88.7% of the original enzyme activity of native DAO was retained. The molecular size of the conjugate PEG-DAO was estimated to be M_r 63,000, according to the degree of attached PEG as determined by the TNBS assay. The increase in the apparent molecular size of PEG-DAO in aqueous medium was confirmed by size exclusion chromatography (Fig. 1).

***In Vitro* Cytotoxicity of PEG-DAO.** Cytotoxicity of DAO was first examined via an *in vitro* system using colon cancer SW480 cells. D-Proline was used as the substrate because it was reported that D-proline is the optimal substrate of DAO with a high turnover rate (43). PEG-DAO alone and D-proline alone showed no cytotoxicity against SW480 cells (data not shown). In contrast, PEG-DAO showed remarkable concentration-dependent cytotoxicity in the presence of D-proline (Fig. 2). This toxicity was completely nullified by the

Table 1 *Physicochemical and biochemical characteristics of native DAO and PEG-DAO*

Type of DAO	Feed ratio[a] (PEG/NH_2)	Free NH_2[b] (% conjugation)	M_r[c]	Enzyme activity (units/mg of protein, % of control)
Native DAO	0	13 (0)	39,000	7.25 (100)
PEG-DAO	3.5	8.5 (34.6)	63,000	6.43 (88.7)

[a] Molar excess of PEG added over amino groups of the enzyme.
[b] Determined by the TNBS method.
[c] Calculated from the loss of amino groups and attached PEG.

Fig. 1. Size exclusion chromatography of native DAO and PEG-DAO. The column was Pharmacia LKB Superose 6 HR (10-mm inner diameter, 30-cm length); the elution buffer was 50 mM sodium phosphate buffer (pH 7.4); absorbance was detected at 280 nm.

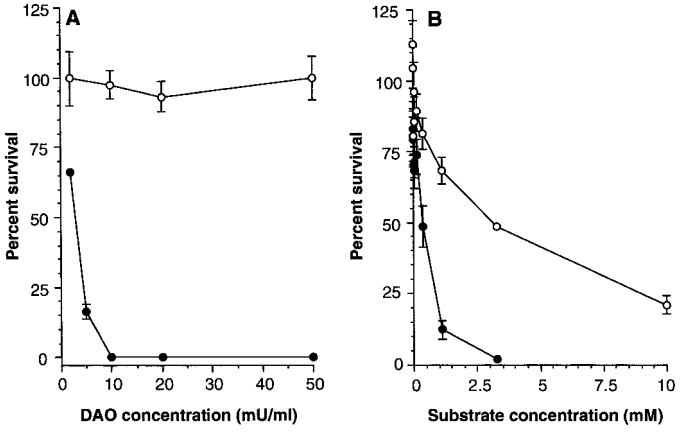

Fig. 2. *In vitro* cytotoxicity of PEG-DAO. *A*, cultured SW480 cells were exposed to increasing amounts of PEG-conjugated DAO in the presence of 50 mM D-proline for 24 h in the absence (●) or presence (○) of catalase (1000 units/ml). *B*, SW480 cells were incubated with 10 milliunits/ml PEG-DAO in the presence of D-alanine (○) or D-proline (●) for 48 h. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values were represented as means ($n = 6$); *bars*, SE.

addition of catalase (Fig. 2*A*), indicating that H_2O_2 plays a critical role in the observed cytotoxicity induced by the PEG-DAO/D-proline system. Similar results were obtained by using native DAO plus D-proline (data not shown). In addition, with the same concentration of PEG-DAO (10 milliunits/ml), D-proline showed much greater cytotoxic activity (LC_{50}, 0.3 mM) than did D-alanine (LC_{50}, 3.6 mM; Fig. 2*B*).

***In Vivo* Distribution of PEG-DAO after i.v. Injection.** After i.v. injection, native DAO was rapidly cleared from the circulation (Fig. 3), with a plasma half-life of 14 min (Table 2). PEG conjugation significantly prolonged the half-life to 36 min, based on the enzyme activity. The AUC value of PEG-DAO was ~2.9 times higher than that of native DAO, whereas urinary clearance of PEG-DAO decreased to about one-third of that of native DAO (Table 2).

As shown in Fig. 4*A*, significant endogenous DAO activity was detected in kidney and liver, which is consistent with a previous report (39). Administration of native DAO slightly increased the enzyme activity in plasma and tumor 4 h after injection (Fig. 4). However, PEG conjugation dramatically improved intratumor accumulation of DAO as well as plasma level of DAO after i.v. injection (Fig. 4), *i.e.*, 3.2-fold relative to native DAO and 7.4-fold to untreated control (background) in tumor; 2.4-fold relative to native DAO and 9.1-fold to untreated control in plasma. It is noteworthy that PEG-DAO administration showed no effect on the enzyme activity in normal organs and tissues including liver, kidney, spleen, intestine, heart, lung, muscle, and brain (Fig. 4). These findings suggest that tumor-targeted delivery of DAO can be achieved with relatively high efficiency by pegylating DAO.

***In Vivo* Antitumor Activity of PEG-DAO.** Native DAO or PEG-DAO was administrated i.v., and after an adequate lag time (*e.g.*, 2 or 4 h after each administration) to allow the accumulation of DAO in the tumor (see Fig. 4), the substrate D-proline was administered by i.p. injection. This treatment procedure allows generation of cytotoxic H_2O_2, predominantly at the tumor site.

As shown in Fig. 5, tumor growth was significantly suppressed in mice administered PEG-DAO plus D-proline. Growth suppression continued to at least 27 days after tumor implantation, which was 15 days after the last treatment with PEG-DAO and D-proline. In contrast, no significant antitumor effect was observed in mice treated with native DAO plus D-proline. The average tumor weights on the 27th day after tumor inoculation for the groups treated with PEG-DAO/D-proline and native DAO/D-proline and the control were 0.34 ± 0.11, 1.5 ± 0.15, and 1.59 ± 0.32 g, respectively (mean ± SE, $P < 0.001$; control *versus* PEG-DAO/D-proline group). In separate experiments,

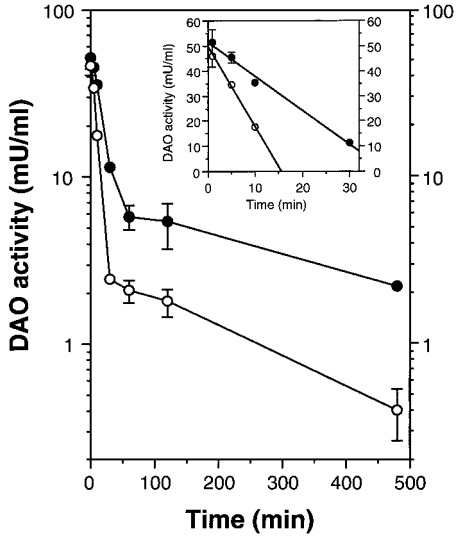

Fig. 3. Enzyme activity in plasma after i.v. injection of native DAO or PEG-DAO. Native DAO (○) or PEG-DAO (●; each at 1.5 units/mouse) was injected i.v. into S-180 tumor-bearing ddY mice. After scheduled periods, mice were killed, and plasma was collected. Enzyme activity in the plasma was quantified by measuring pyruvic acid formation after addition of D-alanine. See text for details. Results are expressed as means ($n = 3$–6); *bars*, SE. The *inset* shows the linear change in enzyme activity in plasma within 30 min after i.v. injection of native DAO or PEG-DAO.

Table 2 *Pharmacokinetic parameters of native DAO and PEG-DAO*

Material	$t_{1/2}$ (min)[a]	$AUC_{0-\infty}$ (mU·h/ml)[b]	CL (ml/h)[c]
Native DAO	14	1100	1.36
PEG-DAO	36	3147	0.48

[a] Half-life ($t_{1/2}$) required to reach to half-concentration of time zero by interpolation.
[b] Area under the plasma concentration *versus* time curve.
[c] Total body clearance.

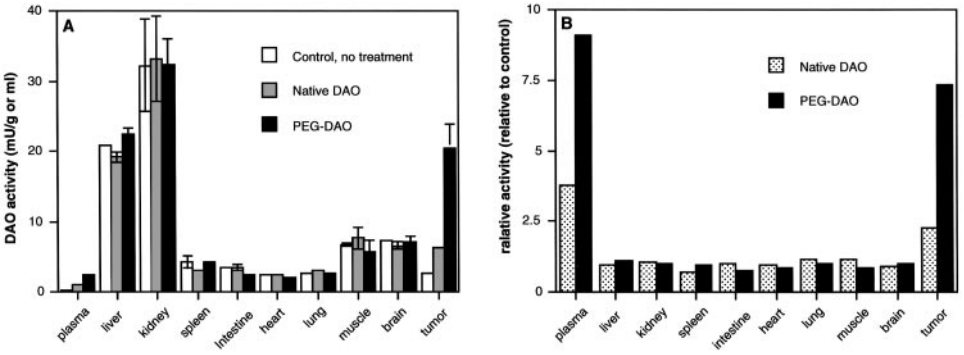

Fig. 4. Body distribution of native DAO and PEG-DAO in tumor-bearing mice. Native DAO or PEG-DAO (each at 1.5 units/mouse) was administrated i.v. After 4 h, mice were killed, and DAO activity in each tissue was measured as described in Fig. 3. *A*, DAO activity in various organs and tumor tissue. *B*, the ratio of activity of native DAO or PEG-DAO *versus* activity in untreated mice. Results are expressed as means ($n = 3–6$); *bars*, SE.

neither PEG-DAO nor D-proline alone showed any antitumor activity (data not shown).

Fig. 6 shows body weight changes in mice receiving different treatments. At the early stage of observation, especially during the first 5 days of treatment, a slight loss of body weight, in both the PEG-DAO/D-proline group and native DAO/D-proline group (there was no significant difference in body weight between these two groups), was observed compared with the control group (given no drugs). After the cessation of treatment, body weight of both groups increased gradually at a rate of growth comparable with that of the control mice. At later stage of observation, a significant difference in body weight was found between the PEG-DAO/D-proline group *versus* the native DAO/D-proline group and the control group ($P < 0.01$); however, it is mostly attributed to the difference of tumor weight between them.

Tumor-specific Oxidative Damage Caused by PEG-DAO plus D-Proline. Administration of PEG-DAO plus D-proline caused a significant increase in TBARS, a marker of oxidative cellular injury, in tumor tissue (Fig. 7*A*). In contrast, no significant increase in TBARS production was observed either in the control group (given no drugs) or in the native DAO treatment group. Also, PEG-DAO/D-proline treatment did not affect the level of TBARS in the liver and kidney, where intrinsic DAO activity with or without PEG-DAO treatment was high (Fig. 4). This finding suggests that PEG-DAO/D-proline treatment generates cytotoxic H_2O_2 selectively at the tumor site rather than in normal tissues. PEG-DAO had a greater effect on TBARS in the tumor tissue than did native DAO (Fig. 7*B*).

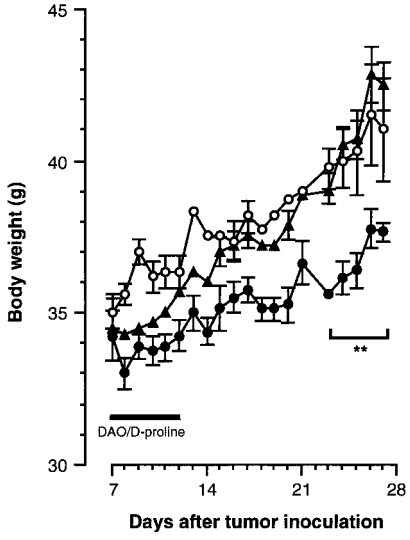

Fig. 6. Body weight changes in ddY mice treated with native DAO or PEG-DAO. The treatment protocol is the same as that described in Fig. 5. ○, control mice without any drug; ●, PEG-DAO plus D-proline; ▲, native DAO plus D-proline. Data are means ($n = 3–4$); *bars*, SE. **, $P < 0.01$ (PEG-DAO group *versus* control group and native DAO group).

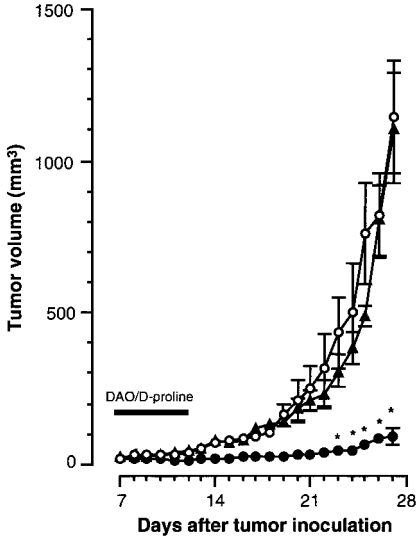

Fig. 5. Antitumor effect of PEG-DAO in the S-180 solid tumor model. S-180 cells (2×10^6 cells) were implanted s.c. in ddY mice. Seven days after inoculation, mice were treated with 1.5 units of native DAO (▲) or PEG-DAO (●) followed by D-proline (0.5 mmol, i.p., 2 and 4 h after DAO administration). ○, result for control mice (no treatment). Data are means ($n = 4–8$); *bars*, SE. *, $P < 0.001$ (PEG-DAO group *versus* control group and native DAO group). See text for details.

DISCUSSION

In the present study, we demonstrated the remarkable antitumor activity of pegylated DAO plus D-proline on mouse solid tumor model (Fig. 5). In regard to the side effects, although some side effects may be induced by PEG-DAO/D-proline treatment as evaluated by body weight change, it appears to be slight and transient (Fig. 6). This effect is similar to that observed in the case of PEG-conjugated XO (19). Key findings of the present study are as following: (*a*) tumor-targeted delivery of an H_2O_2-generating enzyme (DAO) can be accomplished; and (*b*) generation of H_2O_2 by exogenous administration of its substrate (D-proline) resulted in tumor regression.

To deliver DAO to tumor tissue, we used the concept of the EPR effect, which is applicable to macromolecules and lipids in a variety of solid tumors (22, 23, 25, 29–35). In the phenomenon termed the EPR effect, biocompatible macromolecules and lipids preferentially and spontaneously leak out of tumor vessels and remain accumulated in tumor tissues. This EPR effect is a molecular size-dependent phenomenon, and it operates when the molecular size is above M_r 40,000 (22–27). This dependency exhibits a reverse correlation to the

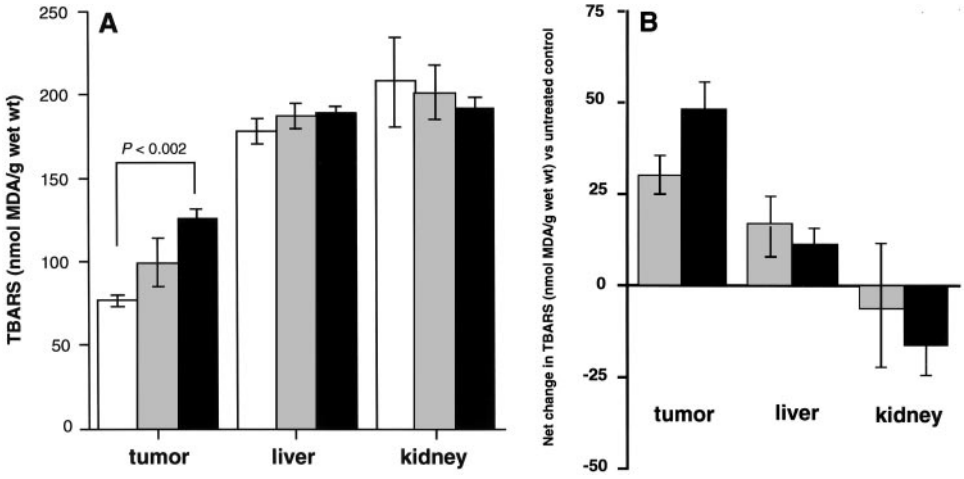

Fig. 7. TBARS in tumor tissue, liver, and kidney of tumor-bearing ddY mice after treatment with native DAO or PEG-DAO. D-Proline was administered (0.5 mmol/mouse) at 2 and 4 h after DAO injection (1.5 units/mouse). Twelve h after the last D-proline administration, mice were killed; tumor tissue, liver, and kidney were collected and then were subjected to the TBARS assay. *A*, malondialdehyde (*MDA*) production in tumor, liver, and kidney. *B*, net production of malondialdehyde in each tissue. Results are expressed as means (n = 3–6); *bars*, SE. □, control (no treatment); ▨, native DAO plus D-proline; ■, PEG-DAO plus D-proline.

renal clearance of the compounds (22, 33–35). Therefore, the EPR effect is observed for any biocompatible macromolecule with a molecular size larger than the renal excretion threshold.

As expected, native DAO (M_r 39,000) was rapidly cleared from the circulation after i.v. injection (Fig. 3 and *inset*) and did not accumulate well in tumor tissue (Fig. 4). To obviate this drawback, we prepared PEG-DAO, which showed an increased *in vivo* half-life (Fig. 3 *inset* and Table 2). More important, PEG-DAO accumulated more selectively in tumor tissue (Fig. 4). The relative concentration of DAO was about 7.4- and 9.1-fold higher in the tumor and plasma, respectively, after PEG-DAO injection compared with untreated control. These findings clearly indicate that PEG-DAO is delivered to tumor tissue according to the EPR effect. We previously found similar beneficial effects of polymer conjugation for several polymers (22, 25, 34) including pyran copolymer, succinylated keratin, copolymer of styrene and maleic acid, and poly(vinyl alcohol).

PEG-DAO alone or D-proline alone did not show any antitumor activity; the cytotoxic effect was observed only when DAO and D-proline were both present. The new type of cancer therapy described here depends on targeting the H_2O_2-generating enzyme (DAO) to the tumor site, the enzyme converting a pharmacologically inert prodrug (D-proline) to a highly cytotoxic metabolite, H_2O_2. From *in vitro* experiments, the LC_{50}s for D-proline and D-alanine in the presence of 10 milliunits/ml PEG-DAO were determined to be 0.3 and 3.6 mM, respectively (Fig. 2). More important, these values are much higher than those in plasma, *i.e.*, the plasma concentrations of D-proline and D-alanine in humans have been reported as 0.22 and 0.61 μM, respectively (44). Via tumor-targeted delivery of PEG-DAO, the generation of H_2O_2 could be regulated by exogenous administration of D-amino acids. This notion is further supported by a tumor-specific increase in TBARS, which is a marker of oxidative metabolites (Fig. 7).

Catalase plays a central function in the antioxidant defense of the organism, as do other enzymes such as superoxide dismutase and glutathione peroxidase, as H_2O_2 is degraded to H_2O (45). Catalase activity has been found to be significantly reduced in various tumor cells (46). Sato *et al.* (47) demonstrated that such reduced catalase activity is attributable to a marked depression of catalase gene expression at the level of transcription. In addition, down-regulation of catalase gene expression has been observed in the doxorubicin-resistant acute myelogenous leukemia subline (AML-2/DX100), which overexpresses multidrug resistance-associated protein (48). The multidrug resistance cell line AML-2/DX100 shows resistance to doxorubicin, daunorubicin, and vincristine but is paradoxically sensitive to H_2O_2. In addition, it was reported that macromolecular drugs are not subject to P-glycoprotein-dependent efflux (49, 50). These findings may lend further support to the validity of the present approach using H_2O_2-generating enzymes for antitumor treatment.

Regulation of redox status in tumor cells may provide a new area of cancer chemotherapy (18, 19, 51–53). We demonstrated here that i.v. injection of PEG-DAO plus D-proline (i.p.) exhibited marked antitumor activity by increasing ROS formation at the tumor site, whereas administration of native DAO/D-proline combination had no effect. As also described, we showed recently that another ROS-generating enzyme, PEG-XO, had remarkable antitumor activity (19). Stegman *et al.* (52) reported an alternative approach to cancer treatment based on gene transfection of DAO to tumor cells to increase intratumor ROS generation. However, successful regulation of gene expression *in vivo* has not yet been possible. Huang *et al.* (53) reported recently, however, that a certain superoxide dismutase inhibitor exhibits selective cytotoxicity to cancer cells, although no tumor-targeting principle is operative. Nevertheless, the oxidative burden makes tumor cells more susceptible to endogenously generated ROS. This finding suggests additive and/or synergistic effects of the inhibitor on the action of ROS-generating enzymes including PEG-DAO and PEG-XO.

In conclusion, we report here that administration of two nontoxic components, PEG-DAO followed after specified times by D-proline, resulted in tumor regression. Because of the EPR effect, tumor-targeted delivery of PEG-DAO was thus possible, and subsequent injection of D-proline selectively generated potent cytotoxic compound H_2O_2 at the tumor site. Consequently, effective antitumor activity by H_2O_2 can be accomplished without any apparent toxicity to normal tissues and organs.

ACKNOWLEDGMENTS

We thank Dr. Akinobu Hamada at the Faculty of Pharmacy, Kumamoto University, for helpful discussions about pharmacokinetic analysis. We also thank Judith Gandy for editing the manuscript and Chie Honda for typing.

REFERENCES

1. Davies, K. J. Oxidative stress: the paradox of aerobic life. Biochem. Soc. Symp., *61:* 1–31, 1995.
2. Sundaresan, M., Yu, Z. X., Ferrans, V. J., Irani, K., and Finkel, T. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. Science (Wash. DC), *270:* 296–299, 1995.
3. Beckman, K. B., and Ames, B. N. Oxidative decay of DNA. J. Biol. Chem., *272:* 19633–19636, 1997.
4. Berlett, B. S., and Stadtman, E. R. Protein oxidation in aging, disease, and oxidative stress. J. Biol. Chem., *272:* 20313–20316, 1997.
5. Halliwell, B., and Gutteridge, J. M. Free radicals, lipid peroxidation, and cell damage. Lancet, *2:* 1095, 1984.
6. Bladier, C., Wolvetang, E. J., Hutchinson, P., de Haan, J. B., and Kola, I. Response of a primary human fibroblast cell line to H_2O_2: senescence-like growth arrest or apoptosis? Cell Growth Differ., *8:* 589–598, 1997.

7. Matsura, T., Kai, M., Fujii, Y., and Yamada, K. Hydrogen peroxide-induced apoptosis in HL-60 cells requires caspase-3 activation. Free Radical Res., *30:* 73–83, 1999.
8. Yamakawa, H., Ito, Y., Naganawa, T., Banno, Y., Nakashima, S., Yoshimura, S., Sawada, M., Nishimura, Y., Nozawa, Y., and Sakai, N. Activation of caspase 9 and 3 during H_2O_2-induced apoptosis of PC12 cells independent of ceramide formation. Neurol. Res., *22:* 556–564, 2000.
9. Suhara, T., Fukuo, K., Sugimoto, T., Morimoto, S., Nakahashi, T., Hata, S., Shimizu, M., and Ogihara, T. Hydrogen peroxide induces up-regulation of Fas in human endothelial cells. J. Immunol., *160:* 4042–4047, 1998.
10. Ren, J. G., Xia, H. L., Just, T., and Dai, Y. R. Hydroxyl radical-induced apoptosis in human tumor cells is associated with telomere shortening but not telomerase inhibition and caspase activation. FEBS Lett., *488:* 123–132, 2001.
11. Simizu, S., Takada, M., Umezawa, K., and Imoto, M. Requirement of caspase-3 (-like) protease-mediated hydrogen peroxide production for apoptosis induced by various anticancer drugs. J. Biol. Chem., *273:* 26900–26907, 1998.
12. Green, H. N., and Westrop, J. W. Hydrogen peroxide and tumour therapy. Nature (Lond.), *181:* 128–129, 1958.
13. Sugiura, K. Effect of hydrogen peroxide on transplanted rat and mouse tumours. Nature (Lond.), *182:* 1310–1311, 1958.
14. Mealey, J., Jr. Regional infusion of vinblastine and hydrogen peroxide in tumor-bearing rats. Cancer Res., *25:* 1839–1843, 1965.
15. Kaibara, N., Ikeda, T., Hattori, T., and Inokuchi, K. Experimental studies on enhancing the therapeutic effect of mitomycin-C with hydrogen peroxide. Jpn. J. Exp. Med., *41:* 323–329, 1971.
16. Makino, S., and Tanaka, T. The cytological effects of chemicals on ascites sarcomas. II. Selective damage to tumor cells by $CaCl_3$ and H_2O_2. Gann, *44:* 39–46, 1953.
17. Nathan, C. F., and Cohn, Z. A. Antitumor effects of hydrogen peroxide *in vivo*. J. Exp. Med., *154:* 1539–1553, 1981.
18. Ben-Yoseph, O., and Ross, B. D. Oxidation therapy: the use of a reactive oxygen species-generating enzyme system for tumour treatment. Br. J. Cancer, *70:* 1131–1135, 1994.
19. Sawa, T., Wu, J., Akaike, T., and Maeda, H. Tumor-targeting chemotherapy by a xanthine oxidase-polymer conjugate that generates oxygen-free radicals in tumor tissue. Cancer Res., *60:* 666–671, 2000.
20. Yagi, K. Reaction mechanism of D-amino acid oxidase. Adv. Enzymol. Relat. Areas Mol. Biol., *34:* 41–78, 1971.
21. Konno, R., and Yasumura, Y. D-Amino acid oxidase and its physiological functions. Int. J. Biochem., *24:* 519–524, 1992.
22. Matsumura, Y., and Maeda, H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent SMANCS. Cancer Res., *46:* 6387–6392, 1986.
23. Maeda, H., Seymour, L. W., and Miyamoto, Y. Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics *in vivo*. Bioconjug. Chem., *3:* 351–362, 1992.
24. Kojima, Y., Haruta, A., Imai, T., Otagiri, M., and Maeda, H. Conjugation of Cu,Zn-superoxide dismutase with succinylated gelatin: pharmacological activity and cell-lubricating function. Bioconjug. Chem., *4:* 490–498, 1993.
25. Noguchi, Y., Wu, J., Duncan, R., Strohalm, J., Ulbrich, K., Akaike, T., and Maeda, H. Early phase tumor accumulation of macromolecules: a great difference in clearance rate between tumor and normal tissues. Jpn. J. Cancer Res., *89:* 307–314, 1998.
26. Seymour, L. W., Miyamoto, Y., Maeda, H., Brereton, M., Strohalm, J., Ulbrich, K., and Duncan, R. Influence of molecular weight on passive tumour accumulation of a soluble macromolecular drug carrier. Eur. J. Cancer, *31A:* 766–770, 1995.
27. Tsutsumi, Y., Kihara, T., Yamamoto, S., Kubo, K., Nakagawa, S., Miyake, M., Horisawa, Y., Kanamori, T., Ikegami, H., and Mayumi, T. Chemical modification of natural human tumor necrosis factor-α with polyethylene glycol increases its antitumor potency. Jpn. J. Cancer Res., *85:* 9–12, 1994.
28. Pyatak, P. S., Abuchowski, A., and Davis, F. F. Preparation of a polyethylene glycol: superoxide dismutase adduct, and an examination of its blood circulating life and anti-inflammatory activity. Res. Commun. Chem. Pathol. Pharmacol., *29:* 113–127, 1980.
29. Duncan, R. Polymer conjugates for tumour targeting and intracytoplasmic delivery. The EPR effect as a common gateway? Pharm. Sci. Technol. Today, *2:* 441–449, 1999.
30. Muggia, F. M. Doxorubicin-polymer conjugates: further demonstration of the concept of enhanced permeability and retention. Clin. Cancer Res., *5:* 7–8, 1999.
31. Marecos, E., Weissleder, R., and Bogdanov, A. Jr. Antibody-mediated *versus* nontargeted delivery in human small cell lung carcinoma model. Bioconjug. Chem., *9:* 184–191, 1998.
32. Maeda, H., Takeshita, J., and Kanamaru, R. A lipophilic derivative of neocarzinostatin. A polymer conjugation of an antitumor protein antibiotic. Int. J. Peptide Protein Res., *14:* 81–87, 1979.
33. Maeda, H., and Matsumura, Y. Tumoritropic and lymphotropic principles of macromolecular drugs. Crit. Rev. Ther. Drug Carrier Syst., *6:* 193–210, 1989.
34. Maeda, H., and Kojima, Y. Polymer-drug conjugates. *In:* R. Arshady (ed.), Desk Reference of Functional Polymers: Synthesis and Application, pp. 753–767. Washington, DC: American Chemical Society, 1997.
35. Maeda, H. SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy. Adv. Drug Delivery Rev., *46:* 169–185, 2001.
36. Fields, R. The rapid determination of amino groups with TNBS. Methods Enzymol., *25:* 464–468, 1972.
37. Bernt, E., and Bergmeyer, E. R. Inorganic peroxides. *In*: H. Bergmeyer (ed.), Methods of Enzymatic Analysis, Vol. 4, pp. 2246–2247. New York: Academic Press, 1974.
38. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J. Immunol. Methods, *65:* 55–63, 1983.
39. D'Aniello, A., D'Onofrio, G., Pischetola, M., D'Aniello, G., Vetere, A., Petrucelli, L., and Fisher, G. H. Biological role of D-amino acid oxidase and D-aspartate oxidase. Effects of D-amino acids. J. Biol. Chem., *268:* 26941–26949, 1993.
40. Yamaoka, K., Tanigawara, Y., Nakagawa, T., and Uno, T. A pharmacokinetic analysis program (MULTI) for microcomputer. J. Pharmacobiodyn., *4:* 879–885, 1981.
41. Ohkawa, H., Ohishi, N., and Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem., *95:* 351–358, 1979.
42. Buege, J. A., and Aust, S. D. Microsomal lipid peroxidation. Methods Enzymol., *52:* 302–310, 1978.
43. Dixon, M. M., and Kleppe, K. D-amino acid oxidase. II. Specificity, competitive inhibition and reaction sequence. Biochim. Biophys. Acta, *96:* 368–382, 1965.
44. Nagata, Y., Masui, R., and Akino, T. The presence of free D-serine, D-alanine and D-proline in human plasma. Experientia, *48:* 986–988, 1992.
45. Mates, J. M., Perez-Gomez, C., and Nunez de Castro. I. Antioxidant enzymes and human diseases. Clin. Biochem., *32:* 595–603, 1999.
46. Greenstein, J. P. Biochemistry of Cancer, Ed. 2, pp. 518–541. New York: Academic Press, 1954.
47. Sato, K., Ito, K., Kohara, H., Yamaguchi, Y., Adachi, K., and Endo, H. Negative regulation of catalase gene expression in hepatoma cells. Mol. Cell. Biol., *12:* 2525–2533, 1992.
48. Kim, H. S., Lee, T. B., and Choi, C. H. Down-regulation of catalase gene expression in the doxorubicin-resistant AML subline AML-2/DX100. Biochem. Biophys. Res. Commun., *281:* 109–114, 2001.
49. Miyamoto, Y., Oda, T., and Maeda, H. Comparison of the cytotoxic effects of the high- and low-molecular-weight anticancer agents on multidrug-resistant Chinese hamster ovary cells *in vitro*. Cancer Res., *50:* 1571–1575, 1990.
50. Miyamoto, Y., and Maeda, H. Enhancement by verapamil of neocarzinostatin action on multidrug-resistant Chinese hamster ovary cells: possible release of nonprotein chromophore in cells. Jpn. J. Cancer Res., *82:* 351–356, 1991.
51. Yoshikawa, T., Kokura, S., Tainaka, K., Naito, Y., and Kondo, M. A novel cancer therapy based on oxygen radicals. Cancer Res., *55:* 1617–1620, 1995.
52. Stegman, L. D., Zheng, H., Neal, E. R., Ben-Yoseph, O., Pollegioni, L., Pilone, M. S., and Ross, B. D. Induction of cytotoxic oxidative stress by D-alanine in brain tumor cells expressing *Rhodotorula gracilis* D-amino acid oxidase: a cancer gene therapy strategy. Hum. Gene Ther., *9:* 185–193, 1998.
53. Huang, P., Feng, L., Oldham, E. A., Keating, M. J., and Plunkett, W. Superoxide dismutase as a target for the selective killing of cancer cells. Nature (Lond.), *407:* 390–395, 2000.