INTRODUCTION

ROS, e.g., superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), 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$_2$O$_2$ 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$_2$O$_2$ induces apoptosis of many tumor cells in vitro (6–10) via activation of the caspase cascade. Of greater importance, many anti-tumor agents, such as vinblastine, doxorubicin, camptothecin, and instamycin, exhibit antitumor activity via H$_2$O$_2$-dependent activation of apoptotic cell death, which suggests potential use of H$_2$O$_2$ as an antitumor principle (11).

H$_2$O$_2$ is relatively unstable and is a small water-soluble molecule. Those characteristics hamper the utility of H$_2$O$_2$ as an antitumor agent that might be selectively delivered to tumor. In fact, H$_2$O$_2$ 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$_2$O$_2$-generating enzyme has been proposed as an alternative approach to developing an H$_2$O$_2$-dependent antitumor system. Nathan and Cohn (17) and Ben-Yoseph and Ross (18) reported that GO, which generates H$_2$O$_2$ during oxidation of glucose, showed antitumor activity in solid tumor models. However, regulation of H$_2$O$_2$ 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$_2$O$_2$. In fact, GO administration to produce H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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 (Mr 39,000) is slightly smaller than the renal excretion threshold (Mr ~50,000), so it would be excreted gradually as observed previously for other small proteins or polymer drugs smaller than Mr 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.
MATERIALS AND METHODS

Materials. DAO (from porcine kidney), α-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, 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 subject to gel filtration chromatography to purify the DAO monomer by using Sephadex G-100 (Pharmacia LKB, Uppsala, Sweden); the column size was filtration chromatography to purify the DAO monomer by using Sephadex G-100 (Pharmacia LKB, Uppsala, Sweden); the column size was 20 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% 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, 10,000).

Succinimidyld D-proline conjugation to the amino group of DAO by using nuclophilic 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 to 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 horse-radish peroxidase-coupled colorimetric assay with 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 H2O2 per min.

In Vitro Cytotoxicity Assay. In vitro cytotoxicity of DAO was determined with 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 α-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 × 106 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 α-amino acid (α-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:

\[ CL (\text{ml/h}) = \text{dose (milliunits)} / \text{AUC}_{0-\infty} (\text{milliunits-h/ml}) \]

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 \( V = \frac{L \times W^2}{2} \).

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 × 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 \( M, 63,000 \), 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>
<thead>
<tr>
<th>Type of DAO</th>
<th>Feed ratio of PEG (mmol)</th>
<th>Free NH2 (mmol)</th>
<th>Molar excess of PEG/mol of free amino groups</th>
<th>Enzyme activity (units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native DAO</td>
<td>3.5</td>
<td>8.5</td>
<td>39,000</td>
<td>7.25 (100)</td>
</tr>
<tr>
<td>PEG-DAO</td>
<td>3.5</td>
<td>8.5</td>
<td>63,000</td>
<td>6.43 (88.7)</td>
</tr>
</tbody>
</table>

\( a \) 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.

\( d \) Calculated from the loss of amino groups and attached PEG.

\( e \) Calculated from the loss of amino groups and attached PEG.

\( f \) Calculated from the loss of amino groups and attached PEG.

\( g \) Calculated from the loss of amino groups and attached PEG.

\( h \) Calculated from the loss of amino groups and attached PEG.

\( i \) Calculated from the loss of amino groups and attached PEG.

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\( k \) Calculated from the loss of amino groups and attached PEG.

\( l \) Calculated from the loss of amino groups and attached PEG.

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\( o \) Calculated from the loss of amino groups and attached PEG.

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\( u \) Calculated from the loss of amino groups and attached PEG.

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\( w \) Calculated from the loss of amino groups and attached PEG.

\( x \) Calculated from the loss of amino groups and attached PEG.

\( y \) Calculated from the loss of amino groups and attached PEG.

\( z \) Calculated from the loss of amino groups and attached PEG.
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$_2$O$_2$, 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,
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. 7A). 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$_2$O$_2$ 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. 7B).

**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$_2$O$_2$-generating enzyme (DAO) can be accomplished; and (b) generation of H$_2$O$_2$ 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); ■, PEG-DAO plus D-proline; ▪, native DAO plus D-proline.

The EPR effect is observed for any biocompatible macromolecule with a molecular size larger than the renal excretion threshold. As expected, native DAO (M, 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 H2O2-generating enzyme (DAO) to the tumor site, the enzyme converting a pharmacologically inert prodrug (D-proline) to a highly cytotoxic metabolite, H2O2. From the tumor site, the enzyme converting a pharmacologically inert prodrug to a potent cytotoxic metabolite is selectively localized.

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 H2O2 at the tumor site. Consequently, effective antitumor activity by H2O2 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


Tumor-targeted Delivery of Polyethylene Glycol-conjugated d-Amino Acid Oxidase for Antitumor Therapy via Enzymatic Generation of Hydrogen Peroxide

Jun Fang, Tomohiro Sawa, Takaaki Akaike, et al.


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