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L-Amino acid oxidase (LOX) modulation of melphalan activity against intracranial glioma

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Abstract These studies evaluated the efficacy of sequential pretreatment with L-amino acid oxidase (LOX) and LOX antiserum in the modulation of melphalan activity against intracranial glioma in athymic nude mice. LOX produced statistically significant ($P < 0.01$) depletion of the large neutral amino acids isoleucine, leucine, methionine, phenylalanine, tyrosine, and valine in murine plasma at doses of 100 and 200 μg administered intravenously. Polyclonal anti-LOX antibody was successfully produced in mice, rabbits, and goats subsequent to immunization with LOX. Staphylococcal protein A-purified rabbit anti-LOX serum inhibited approximately 50% of LOX activity in vitro relative to control samples. This antiserum was used in vivo to inactivate LOX after it had depleted the large neutral amino acids, thereby preventing LOX-mediated catabolism of melphalan. Inoculation of three mice with rabbit anti-LOX serum after the treatment with LOX (100 μg) reduced LOX activity by 100%, 89%, and 100% at 6 h compared with reductions of 80%, 59%, and 52% over the same period in animals receiving LOX alone. In three separate studies using groups of eight to ten mice bearing intracranial human glioma xenografts, pretreatment with LOX followed by anti-LOX serum increased the antitumor activity of melphalan as compared with treatments with melphalan plus LOX, melphalan plus anti-LOX serum, or melphalan alone.

Key words Melphalan · L-Amino acid oxidase · Large neutral amino acids · Glioma

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Introduction

Melphalan, a bifunctional alkylating agent combining the amino acid phenylalanine and the nitrogen mustard mechlorethamine, was originally synthesized in 1953 to exploit endogenous cell membrane transport systems and thus enhance delivery of this cytotoxic agent into tumor cells [1]. Melphalan is transported into cells and across the blood-brain barrier (BBB) via the facilitated transport system for large neutral amino acids (LNAAs) [3, 7, 8, 21]. Unlike LNAA transport across the plasma membranes of cells in most tissues, LNAA transport of melphalan into brain is limited by competitive inhibition with endogenous LNAAs [12–17]. Although it is a relatively poor competitor for this LNAA transport system [7], melphalan has, nonetheless, significant antineoplastic activity against several central nervous system (CNS) tumors, including medulloblastoma [5].

In studies designed to exploit the competitive nature of LNAA transport, Groothius et al. [9] examined the effect of amino acid depletion on melphalan transport into murine brain and human glioma xenografts growing in athymic nude mice. Enhanced melphalan transport across the BBB was achieved by lowering murine plasma LNAA levels with an overnight fast followed by

a protein-free diet. Rich et al. [19] proposed that a more rapid and profound amino acid depletion might facilitate increased melphalan transport across the BBB, enhancing melphalan uptake by intracranial glioma xenografts and thus achieving greater antitumor activity. Previous work [24] has suggested that plasma amino acids can be depleted by the administration of L-amino acid oxidase (LOX), an amino acid : oxygen oxidoreductase purified from the venom of *Crotalus adamanteus* [23]. LOX efficiently converts most LNAAs to their respective keto acids and the resulting keto acids would not be expected to compete for the LNAA transport pathway. Rich et al. [19] were able to deplete plasma LNAAs substantially with LOX, but were unable with LOX alone to achieve significant enhancement of melphalan cytotoxicity against intracranial human glioma xenografts. Further studies established that melphalan was a substrate of LOX, resulting in the generation of the keto acid derivative of melphalan. A small but significant ($P < 0.05$) increase in survival following melphalan treatment was achieved only when LOX-induced amino acid depletion was combined with an overnight fast and subsequent protein-free diet [19].

We postulated that LOX may enhance melphalan delivery if, after optimal depletion of LNAAs and before melphalan administration, LOX could be inactivated. To examine this hypothesis, we developed an anti-LOX antiserum and investigated the efficacy of a protocol in which plasma LNAAs are depleted with LOX, anti-LOX antibodies are administered to inactivate LOX, and melphalan is then given. We showed that this protocol enhances melphalan activity against intracranial glioma xenografts in a murine model.

Materials and methods

LOX preparation

From *Crotalus adamanteus* venom, purchased from the Miami Serpentarium (Miami, Fl.), LOX was purified and twice crystallized by the procedure outlined by Wellner and Meister [23]. The protein content of each batch of LOX was determined by the method described by Lowry et al. [11]. Concentrations of LOX used in these experiments ranged from 6.9 to 18.3 mg/ml.

LOX immunization protocols and schedules

Each of six BALB/c mice was inoculated subcutaneously (s.c.) with 50 μg LOX in 0.2 ml of complete Freund's adjuvant and was bled on days 16 and 41 after the primary immunization. Four specific-pathogen-free rabbits were inoculated s.c., each with 500 μg LOX in a solution of 0.5 ml saline and 0.5 ml complete Freund's adjuvant. The rabbits were bled on days 22 and 36 after the primary immunization. They were boosted with 500 μg LOX and 1 mg *Salmonella minnesota* in 0.5 ml saline and 0.5 ml incomplete Freund's adjuvant 53 days after the primary immunization and bled on days 40 and 79 after the secondary immunization. A third boost with 500 μg LOX in 0.5 ml saline and 0.5 ml complete Freund's adjuvant was given 102 days after the secondary immunization, and rabbits were bled on days 7 and 14 after the third immunization. One goat was immunized with 500 μg LOX in 1.0 ml saline and 1.0 ml complete Freund's adjuvant injected s.c. into four separate sites. The goat was bled on day 36 after the primary immunization. A secondary immunization was given by s.c. injection of 500 μg LOX and 2 mg *S. minnesota* in 1.0 ml saline and 1.0 ml complete Freund's adjuvant into four sites 60 days after the primary immunization, and the goat was bled on days 48, 85, and 90 after the secondary immunization. A third immunization was given by s.c. injection of 500 μg LOX in 1.0 ml saline and 1.0 ml incomplete Freund's adjuvant into four sites on day 93 after the secondary immunization. The goat was bled on day 31 after the third immunization.

ELISA titration of anti-LOX antisera

Successful production of polyclonal antibodies in mice, rabbits, and the goat was verified by enzyme-linked immunosorbent assay (ELISA) titration. Immunoglobulin titers were determined by an ELISA method. LOX was diluted to a concentration of 1 μg/μl in 0.1 m*M* Na_2CO_3 (pH 9.6). Each well of a 96-well plate was coated with 50 μl of the LOX solution, and the plate was incubated overnight at 4 °C. The plate was then washed five times with a rinse buffer (115 m*M* phosphate containing 0.05% gelatin and 0.05% Brij 35, Sigma, St. Louis, Mo.) and blocked with the same buffer for 10 min at room temperature. The antiserum was serially diluted (range 1/250 to 1/128 000), and 50 μl of each dilution was applied in triplicate to a LOX-coated well and allowed to incubate for 1 h at room temperature. The plate was again washed five times with a rinse buffer. A secondary antibody (50 μl biotinylated donkey antigoat, goat antimouse, or goat antirabbit) was applied to each well and allowed to incubate for 1 h at room temperature. The plate was again rinsed five times. Streptavidin-alkaline phosphatase (50 μl) was applied to each well and allowed to incubate for 1 h, followed by rinsing five times. The plates were developed by the addition of 100 μl/well of 10% diethanolamine buffer (pH 9.6) with 5 m*M* $MgCl_2$ and 4 mg/ml *p*-nitrophenylphosphate. Absorbance was read with a Flow Laboratories ELISA plate reader, Model MCC/340, at 405 nm.

SpA purification

Immunoglobulins were purified from rabbit (bled day 40 after secondary immunization and day 14 after third immunization) and goat (bled days 48 and 85 after secondary immunization) anti-LOX antisera. Antisera were diluted with equal volumes of 3 *M* NaCl and 1.5 *M* glycine buffer (pH 8.9) and filtered through a 0.22-μm Millistak filter (Millipore Corporation, Bedford, Mass.). After the diluted sera were passed through a 15 × 2.5-cm column (7.5 g staphylococcal protein A (SpA)/Sepharose 4B; Sigma), the resin was rinsed with 10–15 volumes of 1.5 *M* NaCl and 0.75 *M* glycine buffer (pH 8.9). The column immunoglobulins were then eluted with 0.55 *M* glycine buffer (pH 3.0) containing 0.85% NaCl. Fractions were immediately neutralized by the addition of 1 *M* Tris buffer (pH 9.0), and fractions containing antibody were pooled and dialyzed against 0.115 *M* phosphate buffer (pH 7.4). High-pressure liquid chromatography analysis of SpA-purified antisera confirmed the purity of eluted fractions. The protein concentration of antibody was determined by the method of Lowry et al. [11]. Antibody was sterilized by passing through a 0.22-μm Millipore Millex-GV filter, and was stored at 4 °C.

Assay for LOX activity using radiolabeled amino acid

L-[^{14}C]Leucine was purchased from New England Nuclear and had a specific activity of 11.5 GBq/mmol (310.8 mCi/mmol) and a

concentration of 0.1 mCi/ml (0.32 μmol/ml, 0.042 mg/ml). All other reagents were purchased from Sigma. Each 100-μl reaction mixture consisted of 40 μl of 1 *M* Tris HCl buffer at pH 7.5, 10 μl 20 m*M* L-[^{14}C]leucine and unlabeled L-leucine mixture (0.32 μCi total), 10 μl bovine catalase (12 000 IU/ml), and 40 μl plasma sample containing LOX. Each mixture was allowed to react for 60 min at 37 °C. The reaction was stopped by the addition of 100 μl cold (20%) trichloroacetic acid. After 5 min on ice, each reaction mixture was centrifuged at 12 000 rpm (Beckman microfuge, Beckman, Fullerton, Calif.) for 1 min at 4 °C. A portion of the supernatant (180 μl) was loaded onto a small column (0.5 × 2 cm) of Bio-Rad Dowex 50W-X8 (200–400 mesh, hydrogen form) cation exchange resin. Each column was washed with 3.82 ml deionized water, and the keto acid effluents were collected in test-tubes. The LOX activity in a plasma sample was determined by examining the amount of keto acid formed, which was determined in 2 ml of the column effluent using a scintillation counter. The amount of keto acid product was calculated on the basis of the specific activity of the L-[^{14}C]leucine in the reaction mixture. One unit of LOX was that amount which oxidized 1 μmol L-leucine/per minute.

Assay of L-amino acid oxidase activity by H_2O_2 formation

LOX activity was also assayed by quantitating the hydrogen peroxide produced in the conversion of LNAAs to their respective keto acids. All reagents were purchased from Sigma unless otherwise noted. Each well of a 96-well plate contained a 200-μl reaction mixture consisting of 50 μl 4 m*M* L-leucine, 50 μl plasma sample with LOX, and 100 μl of either anti-LOX serum diluted in a 20-m*M* Tris NaCl buffer (pH 7.4) containing 0.05% bovine serum albumin and 10 m*M* aminotriazole, or diluent alone. Each plate was incubated in a humidity chamber for 1 h at 37 °C. From each reaction mixture, 25 μl was transferred to another 96-well plate and combined with 200 μl Pierce PeroXOquant reagent (Pierce PeroXOquant quantitative peroxidase assay kit, lipid compatible formula, Pierce Chemical Company, Rockford, Ill.). The plate was incubated for 12–15 min at room temperature, and absorbance was read in a Flow Laboratories ELISA plate reader, Model MCC/340, at 620 nm. LOX activity and percentage inhibition were calculated. An H_2O_2 standard curve was developed so that data from each plasma sample could be translated into moles of H_2O_2 produced per hour, thus determining LOX activity.

In vitro inhibition of LOX activity with anti-LOX antisera

The PeroXOquant H_2O_2 detection assay was found to be simpler, more efficient, and equally accurate in the assessment of LOX activity as compared with the traditional radiolabeled amino acid assay and thus was used to assess LOX inhibition by unpurified mouse, rabbit, and goat anti-LOX antisera and SpA-purified rabbit and goat anti-LOX antisera. Serum aliquots of unpurified antisera or 0.1–10 μg/ml SpA-purified anti-LOX were diluted in 20 m*M* Tris NaCl buffer (pH 7.4) to 100 μl and added to the reaction mixture, which contained 30 ng LOX. Absorbance values were compared with an H_2O_2 standard curve, and the amount of H_2O_2 produced by LOX at each concentration of anti-LOX antisera was determined. Percentage inhibition was calculated using normal mouse, rabbit, and goat sera as controls for the respective raw anti-LOX antisera, normal SpA-purified rabbit antiserum for SpA-purified rabbit anti-LOX antiserum, and normal goat raw antiserum for SpA-purified goat anti-LOX antiserum.

Animals

Male or female athymic BALB/c mice (*nu*/*nu* genotype, 6 weeks old or older) housed at Duke University Cancer Center Isolation Facility were used for all xenograft studies unless otherwise noted. They were maintained as previously described [2].

Dose-dependent response of plasma amino acids to LOX

Each group consisted of three BALB/c athymic mice, which received a dose of 0 μg, 100 μg, or 200 μg LOX intravenously (i.v.) through a tail vein injection at t = 0 h. The LOX doses were based on values determined to be optimal by Rich et al. [19]. Mice were serially bled from the eye with a heparinized microtiter pipette at 0, 1, 2, 3, 4, 6, and 8 h after LOX injection. Blood volume was maintained by giving mice a 1-ml bolus of normal saline, s.c., to the neck scruff at t = 1.5 h after LOX administration. Each 40-μl blood sample was centrifuged at 7000 rpm for 10 min. A 20-μl plasma sample was then removed and transferred to an Eppendorf tube containing 80 μl 5% 5′-sulfosalicylic acid. After the sample was spun at 6000 rpm for 10 min, it was stored at − 80 °C. Samples were shipped on Dry Ice™ to the Medical College of Wisconsin, where plasma amino acid levels were measured on a Beckman analyzer. For each LNAA, an average plasma level at a given time-point after LOX administration was determined from the three plasma samples per group. Percentage depletion of LNAA was calculated using, as a reference, the plasma LNAA value of the same animal prior to receiving LOX.

In vivo inhibition of LOX activity with anti-LOX antisera

Each BALB/c athymic mouse (three/group) received a dose of 100 μg LOX followed 4 h later by either saline (1.0 ml) i.p. or SpA-purified rabbit anti-LOX serum (1.0 ml at 5 mg/ml) i.p. Mice were serially bled from the eye at 0, 1, 2, 3, 4, and 10 h after LOX injection. The PeroXOquant H_2O_2 detection assay was used to measure LOX activity.

Intracranial tumor model

D-54 MG is a Duke University subline of A-172 derived from a malignant glioma by Giard et al. [6]. D54 MG human glioma xenografts were grown s.c. in athymic BALB/c mice and excised. Necrotic areas were removed, and grossly minced tumor was passed through a tissue sieve. The homogenate was combined with an equal volume of 1% methylcellulose, loaded into a 500-μl Hamilton syringe and injector (Hamilton Syringe Co., Reno, Nev.), and 5 μl of the cell mixture was injected intracranially into the right frontal hemisphere of each mouse. Mice were randomly assigned to treatment or control groups containing eight to ten mice each and treated on day 6 after tumor injection. The survival of the mice was monitored as a measure of response to therapy. The results were analyzed statistically using the Wilcoxon log rank test [19].

Drugs for therapy study

Melphalan, kindly provided by Glaxo-Wellcome (Research Triangle Park, N.C.), was administered in a single intraperitoneal (i.p.) injection in 17% dimethyl sulfoxide at a dose of 71 mg/m^2 or 35.5 mg/m^2, which corresponds to 100% or 50%, respectively, of the dose lethal to 10% of treated animals (LD_{10}) [4].

In vivo treatment of intracranial D-54 MG with LOX, anti-LOX antibodies, and melphalan

Four experiments were performed to assess the therapeutic effect of LOX and anti-LOX antibodies on the antitumor activity of

melphalan at 100% and 50% of the LD_{10}. In each experiment, LOX was given at t = 0 h by tail vein injection at a dose of 100 μg. An average of 0.2 ml (2.53 mg/ml) SpA-purified goat anti-LOX antiserum was administered by i.p. injection at t = 3 h. Melphalan was dosed i.p. at various times (t = 4, 5, 6, 8, 9 h) at either 100% or 50% of the LD_{10} as presented in Table 1. Response of xenografts was assessed by survival analysis.

Results

Time-course of depletion of LNAAs by LOX

Effectiveness of depletion of LNAAs (isoleucine, leucine, methionine, phenylalanine, tyrosine, and valine) over time by 100 μg LOX is shown in Fig. 1. In all but one case, maximum depletion of the LNAAs was observed at 2–4 h after LOX administration. Substantially similar results were obtained with LOX doses of 200 μg (not shown).

Production and SpA purification of anti-LOX antibodies in rabbit and goat

Anti-LOX antisera from rabbit after the third immunization and goat after the second immunization were submitted to SpA : Sepharose 4B chromatography. Antibody purification for both species was verified by high-pressure liquid chromatography. Recoveries of 2.58 mg/ml goat anti-LOX and 5.18 mg/ml of rabbit anti-LOX were determined by the method of Lowry et al. [11].

In vitro inhibition of LOX activity with anti-LOX antisera

The PeroXOquant H_2O_2 detection assay was used to assess LOX inhibition by unpurified mouse, rabbit, and goat anti-LOX antisera and SpA-purified rabbit and goat anti-LOX antisera. Unpurified sera from mouse (after the primary immunization), rabbit (after the third immunization), and goat (after the second immunization) demonstrated maximal LOX inhibitions of 52%, 62%, and 29%, respectively, when antisera were minimally diluted to 1 : 25. Maximum LOX inhibition achieved in vitro was 30% and 70% when 30 ng LOX was reacted with the SpA-purified goat and rabbit antisera, respectively. Fig. 2 shows the percentage LOX inhibition achieved with various concentrations of SpA-purified rabbit anti-LOX.

In vivo inhibition of LOX activity with anti-LOX antisera

LOX activity in vivo over time was measured in plasma samples from three mice that received LOX alone by

Table 1 Enhancement of melphalan cytotoxicity with LOX and anti-LOX antibodies. LOX (100 μg) was given i.v. followed by SpA-purified goat anti-LOX antibodies (0.2 ml at 2.53 mg/ml) given i.p. to athymic mice. Melphalan was dosed at 71 mg/m^2 or 35.5 mg/m^2, which represents 100% or 50%, respectively, of the LD_{10} (*LOX* L-amino acid oxidase, *Ab* antibody, *SpA* staphylococcal protein A). The increase in survival of all the treated groups compared with the vehicle-treated groups was significant ($P < 0.05$). *$P < 0.05$ compared with group treated with melphalan alone

Experiment number	Treatment group	Time of LOX dose (h)	Time of Ab dose (h)	Time of melphalan dose (h)	Median survival (days)	% increase in survival[a]
1	0.5 melphalan			6	31.5	162.5
	0.5 melphalan + Ab		3	6	27.5	129.2
	0.5 melphalan + LOX	0		6	29	141.7
	0.5 melphalan + Ab + LOX	0	3	6	33	175.0
	Vehicle				12	
2	0.5 melphalan				28.5	137.5
	0.5 melphalan + Ab + LOX	0	3	4	32.5	170.8
	0.5 melphalan + Ab + LOX	0	3	6	32	166.7
	Vehicle				12	
	0.5 melphalan				27.5	111.5
	1.0 melphalan				30	130.8
	0.5 melphalan + Ab + LOX	0	3	4	28	115.4
	0.5 melphalan + Ab + LOX	0	3	5	28	115.4
	0.5 melphalan + Ab + LOX	0	3	6	31.5	142.3*
	0.5 melphalan + Ab + LOX	0	3	8	28	115.4
	0.5 melphalan + Ab + LOX	0	3	9	27	107.6
	Vehicle				13	
4	0.5 melphalan				27	68.8
	1.0 melphalan				30	87.5
	0.5 melphalan + Ab + LOX	0	3	6	30	87.5*
	Vehicle				16	

[a] Defined as (*median survival of treated animals*) − (*median survival of control animals*) median survival of control animals

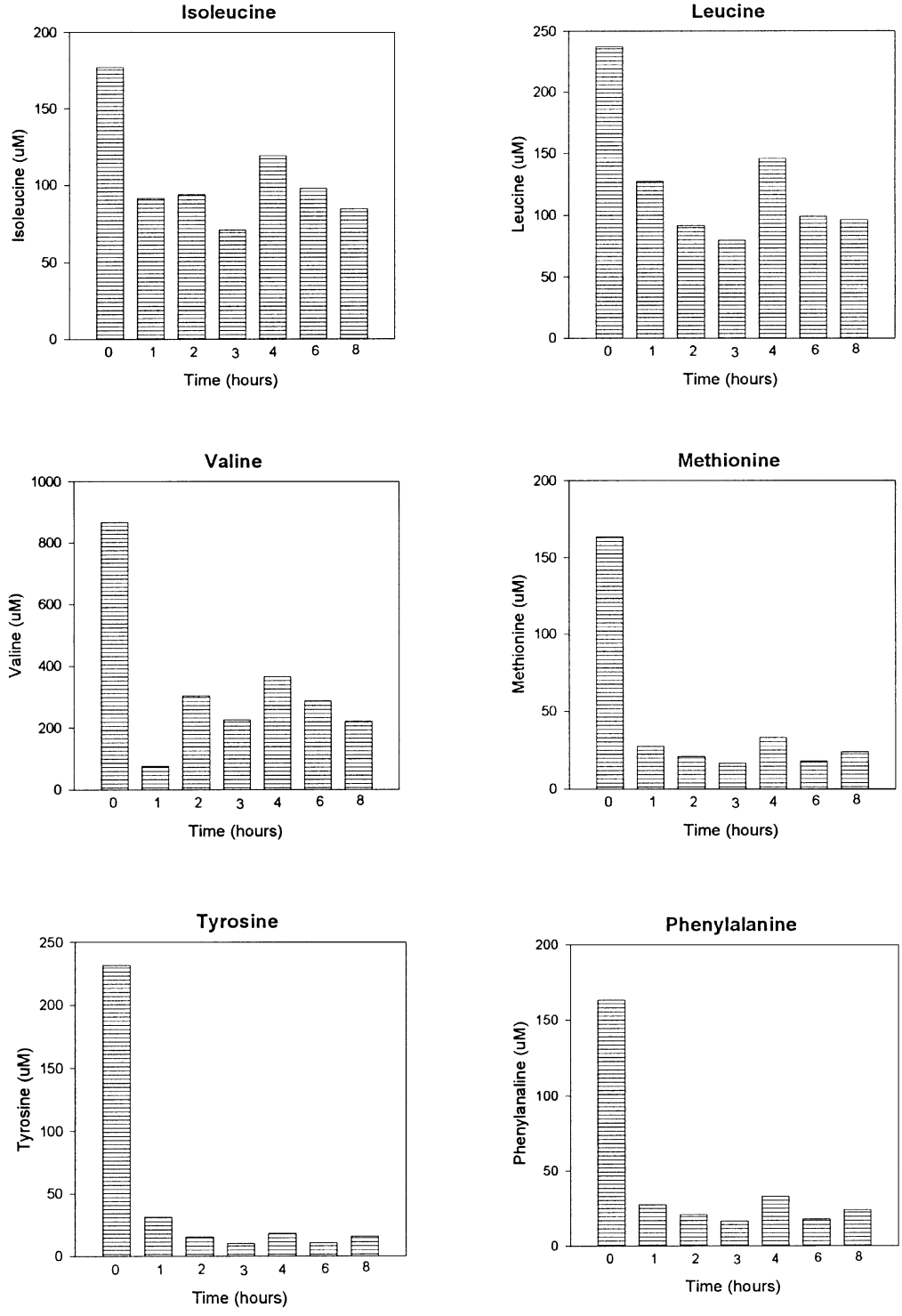

Fig. 1 Time course of depletion of LNAAs (isoleucine, leucine, methionine, phenylalanine, tyrosine, and valine) by LOX doses of either 0 μg or 100 μg. Groups consisting of three BALB/c athymic mice were given a tail vein injection i.v. at t = 0 h. Mice were serially bled at various time-points after LOX injection, and plasma amino acid levels were determined as described in Materials and methods

i.v. injection and from three mice that received SpA-purified rabbit anti-LOX serum by i.p. injection 4 h after receiving i.v. LOX. The PeroXOquant H_2O_2 detection assay demonstrated gradual decreases in LOX activity over time in both groups, with a substantially greater decrease in LOX activity in those animals receiving rabbit anti-LOX. As shown in Fig. 3, blood samples for three mice, taken 6 h after giving anti-LOX

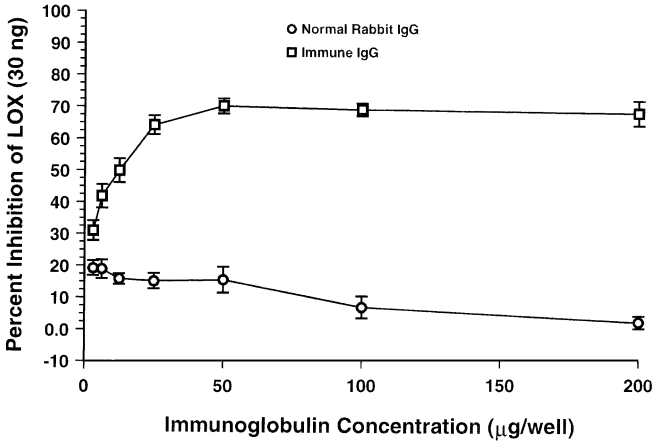

Fig. 2 In vitro inhibition of LOX activity with SpA-purified rabbit anti-LOX antiserum. LOX activity was measured as described in Materials and methods. Normal rabbit IgG is shown as a control

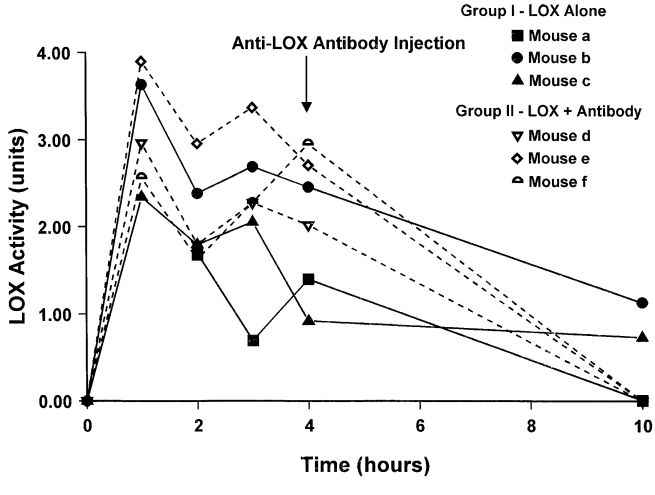

Fig. 3 In vivo inhibition of LOX activity with anti-LOX antiserum. LOX activity in vivo over time was measured in plasma samples using the PeroXOquant H_2O_2 assay. Three mice received LOX (100 μg) alone by i.v. injection, and three mice received i.v. LOX (100 μg) followed at 4 h by SpA-purified rabbit anti-LOX serum i.p. (1.0 ml at 5 mg/ml)

serum, showed decreases in LOX activity of 100%, 89%, and 100% from peak LOX levels, whereas samples from three mice not given anti-LOX serum showed decreases of 80%, 59%, and 52% from peak LOX levels. These differences were statistically significant ($P < 0.01$).

Enhancement of melphalan cytotoxicity using LOX and anti-LOX antibodies

As demonstrated in Table 1, the addition of LOX and anti-LOX antibodies to melphalan therapy increased the antineoplastic activity of melphalan at 50% of the LD_{10} in three separate in vivo treatments of intracranial D54 MG glioma. In two cases, the activity was equal to or greater than that achieved by melphalan at 100% of the LD_{10}. The dosing regimen that consistently achieved this effect was 100 mg LOX given at $t = 0$ h followed at $t = 3$ h by anti-LOX antibodies, followed 3 h later at $t = 6$ h with melphalan. No enhancement of antitumor activity was observed when LOX and anti-LOX antibodies were added to the melphalan treatment given at 100% of the LD_{10} (data not shown). No increased weight loss or mortality secondary to LOX/anti-LOX antibodies was seen in excess of that produced by melphalan alone.

Discussion

Chemotherapeutic modalities that selectively target malignant cells by exploiting characteristics unique to the neoplastic process or its location offer the potential of a major increase in the therapeutic index. The competitive nature of the facilitated transport system of LNAAs at the BBB provides the opportunity to increase the transport of amino acid-derived drugs, such as the phenylalanine derivative melphalan, across the BBB, thus potentially increasing the therapeutic effect. Melphalan, an antineoplastic agent active against a spectrum of extraneural malignancies such as rhabdomyosarcoma, ovarian carcinoma, and neuroblastoma, has also demonstrated impressive activity against CNS malignancies such as medulloblastoma [5, 10, 18, 20]. Nevertheless, the cytotoxic potential of melphalan against brain tumors may be limited by its poor rate of entry into the brain [7]. A diet-induced, plasma amino acid-depleted state, while slightly enhancing melphalan uptake across the BBB, is limited in its usefulness as a method of amino acid modulation. Endogenous protein stores replete plasma amino acid levels before substantial LNAA depletion is achieved. LOX produces rapid and profound amino acid depletion (present studies; [24]) and thus serves as a more viable option to enhance melphalan uptake. Unfortunately, LOX also demonstrates similar oxidative metabolism to melphalan [19].

The goal of this investigation was to produce an anti-LOX antiserum that would provide a means to limit the duration of LOX activity in vivo. With the ability to halt LOX activity promptly, one may maximally deplete LNAAs and then inhibit residual LOX activity prior to treatment with melphalan. Previous studies [22] have suggested that a rabbit anti-LOX antiserum can produce a 20% reversible inactivation of LOX activity. We produced anti-LOX antisera in three species – mice, rabbit, and goat – that demonstrated substantial inhibition of LOX activity in vitro – 52%, 62%, and 29%, respectively – as measured by the

PeroXOquant H_2O_2 detection system. SpA-purified goat and rabbit antisera achieved 30% and 70% inhibition, respectively, of LOX activity in vitro. These results reflect the activity-neutralizing nature of the anti-LOX antisera.

Once LOX inhibition was demonstrated in vitro, we attempted to establish optimal parameters for in vivo dosing. We first defined the optimal dose of LOX to be 100 μg given i.v. through the tail vein followed by antiserum 3–4 h later. This schedule reflects the minimum dosage of LOX that achieved maximal LNAA depletion in the majority of cases. These findings suggest a LOX/anti-LOX dosing regimen of 100 μg LOX at t = 0 h followed 3–4 h later by anti-LOX. SpA-purified rabbit anti-LOX given in this fashion resulted in significant ($P < 0.05$) reduction of LOX activity in vivo. At 6 h after anti-LOX serum administration, LOX activity was reduced by an average of 64% in those animals receiving LOX alone, as compared with an average 97% reduction in those animals also receiving anti-LOX antibodies.

In the final stage of this investigation, we pretreated nude mice bearing intracranial D-54 MG glioma xenografts with LOX, followed by anti-LOX before giving melphalan. The addition of LOX and anti-LOX antibodies to melphalan therapy increased the cytotoxic effect of melphalan at 50% of the LD_{10}, equaling or exceeding the efficacy of melphalan at 100% of the LD_{10}, in two separate in vivo treatments of intracranial D54 MG glioma. The dosing regimen that consistently achieved this effect was 100 μg LOX given at t = 0 h, anti-LOX antibodies given at t = 3 h, and melphalan given at t = 6 h despite greater LOX depletion at a later time after administration of anti-LOX antibodies. This may reflect the repletion of amino acids at later time-points. Interestingly, no enhancement of cytotoxicity was observed when LOX and anti-LOX antibodies were added to the melphalan treatment at 100% of the LD_{10}. This suggests that the marked permeability of D-54 MG glioma to melphalan [4] may mask an augmenting effect of LOX and anti-LOX antibodies and may warrant a similar investigation using less melphalan-permeable tumor lines. Nevertheless, the achievement of the full-dose effect of an alkylating agent with half the dose is a significant observation.

The goal of this study was to produce an anti-LOX antiserum that would facilitate LOX-mediated enhancement of intracranial melphalan delivery. Our findings of greater survival of mice treated with melphalan after a course of LOX and anti-LOX as compared with those treated with melphalan and melphalan plus LOX alone has implications for the role of this anti-LOX antibody in the treatment of CNS tumors. Development of a monoclonal anti-LOX antibody specific for the active site of the enzyme is under way, with the intent of improving the effectiveness of the LOX/anti-LOX approach. Ultimately, development of a genetically engineered antibody that achieves maximal inhibition of LOX activity with minimal immunogenicity is expected to establish this treatment modality as an effective clinical option.

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