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THE MECHANISM OF TOXICITY OF 5-*S*-CYSTEINYLDOPA TO TUMOUR CELLS

HYDROGEN PEROXIDE AS A MEDIATOR OF CYTOTOXICITY

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Abstract—5-*S*-Cysteinyldopa, a melanin precursor, has been shown to possess selective toxicity to tumour cells *in vitro* and *in vivo*. The mechanism of cytotoxicity of the catechol was studied in comparison with L-dopa and 5-*S*-cysteaminyldopamine. Growth inhibition of human neuroblastoma cell line of YT-nu by 5-*S*-cysteinyldopa was completely depressed by addition of catalase. Superoxide dismutase and five drugs thought to scavenge hydroxyl radicals or quench singlet oxygen had little effect on the cytotoxicity. Hydrogen peroxide itself was also cytotoxic at low concns. These results indicated that hydrogen peroxide was a mediator of the cytotoxicity of 5-*S*-cysteinyldopa. It is suggested that reaction of the catechol with cellular superoxide radicals contributes to the production of hydrogen peroxide in addition to autoxidation. Catalase reduced the cytotoxicity of L-dopa by half, while it had no inhibitory effect on the strong cytotoxicity of 5-*S*-cysteaminyldopamine.

We have been studying the antitumour activity of 5-*S*-cysteinyldopa and related compounds [1, 2]. This catecholic amino acid is an intermediate in the metabolic pathway from L-dopa to the red-brown pigment, phaeomelanin [3]. We have previously reported that 5-*S*-cysteinyldopa exhibits cytotoxicity against a variety of human cell lines including melanomas and neuroblastomas, while it does not affect Chinese hamster fibroblast Don-6 and mouse fibroblast L929 *in vitro* [1]- 5-*S*-Cysteinyldopa also possesses antitumour activity against murine L1210 leukaemia and B-16 melanoma *in vivo* with no untoward effects on the host [1].

It has been suggested that the mechanism of action of catechols may either involve oxidation to *o*-benzoquinone with subsequent sulfhydryl scavenging and inhibition of enzymes essential for DNA synthesis or involve autoxidation to form cytotoxic active oxygens such as H_2O_2, superoxide radical ($O_2^{\cdot}$), and hydroxyl radical ($\cdot OH$) [4]. Singlet oxygen may also be a cause of cytotoxicity through lipid peroxidation [5].

In this study we attempted to elucidate the mechanism of selective cytotoxicity of 5-*S*-cysteinyldopa in comparison with L-dopa and 5-*S*-cysteaminyldopamine. L-Dopa shows selective cytotoxicity but was less effective than 5-*S*-cysteinyldopa [1]. Although 5-*S*-cysteaminyldopamine has a structure analogous to 5-*S*-cysteinyldopa, it exhibited strong, non-selective cytotoxicity [2]. Experiments were designed to find out if cells can be protected from the cytotoxicity of the catechols by addition of enzymes and scavengers which decompose active oxygens. We found out that the addition of catalase could depress completely the cytotoxicity of 5-*S*-cysteinyldopa, indicating H_2O_2 to be the mediator of cytotoxicity.

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MATERIALS AND METHODS

Chemicals. 5-*S*-Cysteinyldopa and 5-*S*-cysteaminyldopamine were synthesized in our laboratory [2]. L-Dopa and superoxide dismutase (SOD) were purchased from Sigma Chemical Co. (St. Louis, MO), and catalase from Boehringer Mannheim GmbH (Mannheim, F.R.G.). D-Mannitol, sodium benzoate and thiourea were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan) and 2,5-dimethylfuran and 1,4-diazabicyclo[2,2,2]octane (DABCO) from Aldrich Chemical Co. (Milwaukee, WI).

Cells. The cell line of human neuroblastoma YT-nu was a gift from Professor T. Suzuki, Department of Pathology, School of Medicine, Niigata University. Single-cell suspensions in RPMI 1640 medium, supplemented with 10% foetal calf serum, 1000 units/ml penicillin, and 100 μg/ml streptomycin, were cultured at 37° under 95% air–5% CO_2.

Effects of enzymes and scavengers decomposing active oxygens on cytotoxicity of the catechols. The methods for this experiment were essentially similar to those previously described by us [1]. Single-cell suspensions in RPMI 1640 medium were inoculated in 60-mm Falcon Petri dishes, and cells were allowed to attach for 24 hr prior to exposure to a catechol. After the cells were washed, 1 ml of a Hanks' balanced salt solution containing a catechol and an enzyme or a scavenger was added and then the cultures were incubated at 37° for 1 hr. Cells were

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harvested by trypsinization with 0.25% trypsin–EDTA and counted in a Model Z Coulter counter. Results are expressed as the percentage of growth inhibition according to the formula [(No. of control cells − No. of treated cells)/No. of control cells] × 100 by comparison with parallel control cultures that were manipulated similarly except that they did not have a catechol and an enzyme or a scavenger added.

Acute toxicity of 5-S-*cysteaminyldopamine in mice.* Young C57BL/6 × DBA/2 F_1 male mice (10 mice per group) weighing about 20 g were used. The compound was given as 1 ml of a 0.9% NaCl solution i.p. to three groups of mice (250, 500 and 1000 mg/kg). Animals were observed for 30 days.

RESULTS

Effects of enzymes and scavengers decomposing active oxygens on cytotoxicity of the catechols

Catalase and SOD are enzymes that dismutate H_2O_2 and $O_2^{\cdot}$, respectively. D-Mannitol [6], benzoate [7] and thiourea [6, 7] are scavengers of ·OH. DABCO and dimethylfuran are thought to quench singlet oxygen [5]. Table 1 summarizes the effects of these chemicals on the cytotoxicity of 5-*S*-cysteinyldopa, L-dopa and 5-*S*-cysteaminyldopamine.

5-*S*-Cysteinyldopa at 1 mM inhibited the growth of YT-nu cells by 50%. This cytotoxicity was completely depressed by addition of 100 μg/ml of catalase. The growth inhibitory effect of 6 mM L-dopa was partially depressed by addition of catalase; the 44% inhibition was reduced to 26%. On the other hand, catalase was not effective at all against the strong cytotoxicity of 5-*S*-cysteaminyldopamine. SOD and other drugs except thiourea had little effect on the cytotoxicity of these catechols. Although thiourea was fairly effective in depressing the cytotoxicity, the effect may be attributed to its ability to decompose H_2O_2; reaction of 1 mM H_2O_2 with 100 mM thiourea was found to proceed rapidly with a half-life of less than 1 min. A weak inhibitory effect was observed with dimethylfuran, but the result seemed to be ambiguous because of the high toxicity of the compound itself. These findings indicated that the cytotoxicity of 5-*S*-cysteinyldopa was mediated not by $O_2^{\cdot}$, ·OH or singlet oxygen, but by H_2O_2. Furthermore, since catalase, a large protein, cannot penetrate the cell membrane, it is suggested that H_2O_2 is formed from 5-*S*-cysteinyldopa either intracellularly and excreted, or at the cell surface, and its cytotoxic effect exerted extracellularly [8].

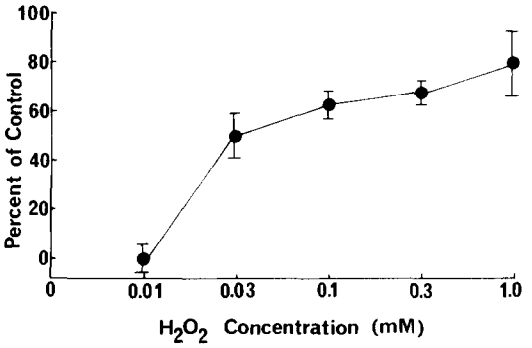

Fig. 1. Cytotoxicity of H_2O_2 on YT-nu cells. Conditions for the experiment were similar to those described for catechols (see Materials and Methods). Values and bars represent means ± S.E. for three separate experiments.

Cytotoxicity of H_2O_2 in vitro

Since H_2O_2 was found to be responsible for the cytotoxicity of 5-*S*-cysteinyldopa, the cytotoxicity of H_2O_2 itself was then examined. As shown in Fig. 1, 0.03 mM H_2O_2 caused growth inhibition of the cells by 50% which was equivalent to the effect of 1 mM 5-*S*-cysteinyldopa. At higher concns H_2O_2 exhibited a higher cytotoxicity and nearly 85% growth inhibition was observed at 1 mM.

H_2O_2 *formation upon autoxidation of the catechols*

Fig. 2 shows the time course of H_2O_2 production upon autoxidation of the catechols in a buffer of pH 7.4. 5-*S*-Cysteinyldopa and L-dopa, which are selectively toxic to tumour cells, produced H_2O_2 at slower rates, while 5-*S*-cysteaminyldopamine, non-selectively toxic to cells, produced H_2O_2 very rapidly. The amounts of H_2O_2 accumulated during the 1-hr incu-

Table 1. Effects of enzymes and scavengers decomposing active oxygens on the cytotoxicity of catechols*

Experiment	Reagent only	Catechol (concn) 5-*S*-Cysteinyldopa (1 mM)	L-dopa (6 mM)	5-*S*-Cysteaminyldopamine (1 mM)
Catechol only		50 ± 3	44 ± 2	64 ± 2
Reagent added (concn)				
Catalase (100 μg/ml)	6 ± 1	3 ± 3	26 ± 1	66 ± 1
SOD (100 μg/ml)	4 ± 2	50 ± 3	49 ± 1	67 ± 1
Thiourea (100 mM)	−9 ± 5	8 ± 2	21 ± 1	64 ± 5
Mannitol (100 mM)	−10 ± 1	53 ± 5	49 ± 2	N.D.†
Sodium benzoate (100 mM)	18 ± 2	48 ± 6	N.D.	N.D.
DABCO (100 mM)	23 ± 2	50 ± 1	N.D.	N.D.
Dimethylfuran (100 mM)	42 ± 1	40 ± 2	N.D.	N.D.

* Results are expressed as percentages of growth inhibition according to the formula [(No. of control cells − No. of treated cells)/No. of control cells] × 100 by comparison with parallel control cultures (means ± S.E. for three separate experiments).

† Not determined.

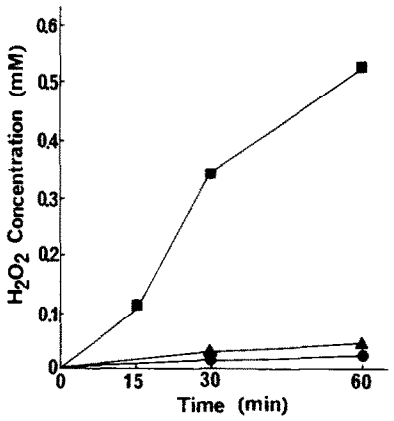

Fig. 2. H_2O_2 production upon autoxidation of the catechols. A solution of 1 mM 5-S-cysteinyldopa (●), 6 mM L-dopa (▲) or 1 mM 5-S-cysteaminyldopamine (■) in 0.05 M sodium phosphate buffer, pH 7.4, was incubated at 37°. At different times 0.2-ml aliquots were taken and mixed with 1.8 ml of 0.5 M HCl to terminate the oxidation. The amount of H_2O_2 formed was determined by the method of Hildebrandt and Roots [14] with minor modifications. Values represent means of two separate experiments.

bation of 1 mM 5-S-cysteinyldopa, 6 mM L-dopa and 1 mM 5-S-cysteaminyldopamine were 0.015, 0.037 and 0.52 mM, respectively. Although 1 mM 5-S-cysteinyldopa is more cytotoxic to tumour cells than 6 mM L-dopa [1], the amounts of H_2O_2 formed upon autoxidation are reversed. Thus, mechanisms other than autoxidation may contribute to the H_2O_2 production from 5-S-cysteinyldopa when in contact with tumour cells. The strong, non-selective toxicity of 5-S-cysteaminyldopamine might be related to the rapid production of H_2O_2. Addition of 100 μg/ml catalase to the medium containing 1 mM H_2O_2 greatly reduced the cytotoxicity of H_2O_2; the growth inhibition was decreased to 20%. Thus, the failure of catalase to protect the cells from the cytotoxicity of 5-S-cysteaminyldopamine seemed to indicate that the cytotoxic action of H_2O_2 produced from the catechol was exerted intracellularly. The possibility that this catechol exerts its toxicity through a different mechanism could not be ruled out.

Acute toxicity of the catechols in mice

We have previously reported that mice given 5-S-cysteinyldopa i.p. at a dose of 1000 mg/kg for 12 consecutive days showed no signs of systemic toxicity [1]. On the other hand, the acute i.p. dose of 5-S-cysteaminyldopamine lethal to 50% of mice was found to be 550 mg/kg, as calculated by the method of Litchfield and Wilcoxon [9].

DISCUSSION

The present study shows that the cytotoxicity of 5-S-cysteinyldopa is mediated by H_2O_2 formed from the catechol. This finding excludes the possibility that the cytotoxicity results from the sulfhydryl scavenging action [1, 4] of the *o*-benzoquinone formed from 5-S-cysteinyldopa. Peterkofsky and Prather [8] have reported that reducing agents such as ascorbic acid and glutathione are cytotoxic to cultured fibroblast cells by virtue of H_2O_2 formation. Catechols are also potent reducing agents.

Graham *et al.* [4] have shown that the rate of autoxidation of catechols correlates with their cytotoxicity. In this study, a clear correlation was found between the rate of H_2O_2 production from the catechols (rate of autoxidation) and their toxicity *in vivo* as well as *in vitro*. 5-S-Cysteinyldopa, which produces H_2O_2 at a much slower rate, is selectively toxic to tumour cells *in vitro* and exhibits essentially no toxicity *in vivo* [1]. On the other hand, 5-S-cysteaminyldopamine, which produces H_2O_2 very rapidly, is strongly toxic to both human neuroblastoma and Chinese hamster fibroblast cells [2], and exhibits a strong toxicity in mice.

It still remains unknown why 5-S-cysteinyldopa is selectively toxic to tumour cells. The reduced level of SOD and the resulting increase of the $O_2^{\cdot}$ concn are unique biochemical features of tumour cells [10]. Furthermore, we have recently shown that 5-S-cysteinyldopa is capable of reacting rapidly with $O_2^{\cdot}$ [11]. It thus seems possible that, when in contact with tumour cells, 5-S-cysteinyldopa reacts with cellular $O_2^{\cdot}$ to form H_2O_2 which in turn attacks the cell membrane.

Wick [12] has reported that dopa analogues such as dopa methyl ester are effective antitumour agents against B-16 melanoma, C1300 neuroblastoma, and L1210 and P388 leukaemias. The effects of these drugs on melanoma cells might be ascribed to the specific enzyme, tyrosinase [13]. However, the mechanisms of action on the other types of cells were less clear. The present results on L-dopa seem to suggest that the cytotoxicity of these drugs also results to some extent from the H_2O_2 production.

Finally, the finding that thiourea decomposes H_2O_2 at a high rate points to the need for caution in using this compound as a scavenger of ·OH.

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