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DISTRIBUTION AND METABOLISM OF TRITIUM-LABELLED 5-*S*-CYSTEINYLDOPA IN MICE

SHOSUKE ITO, RYOJI TERADAIRA and KEISUKE FUJITA

Insitute for Comprehensive Medical Science, School of Medicine, Fujita-Gakuen University, Toyoake, Aichi 470-11, Japan

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Abstract—It has been shown that 5-*S*-cysteinyldopa is an intermediate in the pathway from L-dopa to phaeomelanins and also has antitumour activity. Urinary excretion, tissue distribution and metabolism of the tritium-labelled amino acid, injected intraperitoneally, were studied with normal and B-16 melanoma-bearing mice, and the results were compared with those using L-dopa. Nearly 80 per cent of the radioactivity of 5-*S*- cysteinyldopa injected was excreted within 24 hr in both groups. No tissues selectively incorporated the amino acid and the radioactivity ratio of melanoma : serum was 1.0. Urinary metabolites of 5-*S*-cysteinyldopa were separated by chromatography on Dowex 50W. In contrast to L-dopa, this catechol was not rapidly metabolised; normal mice excreted as such 41 per cent of the total activity applied and B-16 melanoma-bearing mice, 74 per cent. These results are consistent with the facts that 5-*S*-cysteinyldopa is essentially non-toxic to mice and that it is a good biochemical marker of melanoma.

In addition to L-3,4-dihydroxyphenylalanine (L-dopa), another catechol amino acid 5-*S*-cysteinyldopa (cys-dopa) has been detected in human and animal melanomas [1–3]. This unique amino acid arises in melanocytes by the addition of cysteine or glutathione to dopaquinone which is produced by the oxidation of L-dopa with tyrosinase [4, 5]. It has been demonstrated that detection of increased amounts of this catechol in urine may be an indication of melanoma metastasis [6].

Recently, Wick *et al.* have shown that L-dopa is selectively toxic to melanoma cells *in vitro* [7] and that its analogues possess antitumour activity against several experimental tumour systems [8–10]. We have then demonstrated that cys-dopa is approx. 10 times more toxic to a variety of cultured human tumour cells than is L-dopa and also exhibits antitumour activity against murine L1210 leukaemia and B-16 melanoma with no untoward effects on the host [11].

Because of its therapeutic use in the treatment of Parkinson's disease, the metabolism of L-dopa has been extensively studied [12]. However, information on the metabolic fate of cys-dopa has been limited except for a report on the isolation of *O*-methylated 5-*S*-cysteinyldopa from the urine of melanoma patients [13]. We therefore studied tissue distribution and metabolism of ^{3}H-labelled cys-dopa in normal and B-16 melanoma-bearing mice. The results were compared with those using ^{3}H-labelled L-dopa.

MATERIALS AND METHODS

Chemicals. L-Dopa and mushroom tyrosinase (grade III) were obtained from Sigma Chemical Co., St. Louis, Mo. Ro4-4602 (Benserazide hydrochloride), an inhibitor of dopa decarboxylase [14, 15], was a gift from Hoffman LaRoche Co., Tokyo Branch, Tokyo, Japan. L-[G-^{3}H]-Dopa (sp. act., 13.4 Ci/mmole) and DL-[2-^{14}C]-dopa (sp. act., 46.8 mCi/mmole) were obtained from New England Nuclear Co., Boston, Mass. ^{3}H-Labelled 5-*S*-cysteinyldopa ([^{3}H]-cys-dopa) was synthesised by the method of Ito and Prota [16] using mushroom tyrosinase. The reaction mixture contained: 1 mCi of L-[^{3}H]-dopa, 9.9 mg (50 μmoles) of carrier L-dopa, 12.1 mg (100 μmoles) of L-cysteine in 5.0 ml of 0.05 M sodium phosphate buffer, pH 6.8, and 1000 units of tyrosinase. While the specific activity of L-dopa used was 20 mCi/mmole, that of the product was 12.7 mCi/mmole. Thus, approx. one-third of the radioactivity was lost during the evaporation of the 2 M HCl eluate because of acid-catalysed exchange. The radioactive compounds were dissolved in physiological salines containing carrier amino acid.

Urinary excretion and tissue distribution of radioactivity. Male C57BL/6 × DBA/2 F_1 (BDF_1) mice of 4 weeks of age were purchased and bred for 1 week before implantation of melanoma cells. B-16 Melanoma was maintained by serial subcutaneous implantation of tumour cells following standard National Cancer Institute protocols [17]. When tumours had grown to approx. 1 cm in diameter, radioactive compound (either [^{3}H]-cys-dopa or L-[^{3}H]-dopa) was injected intraperitoneally at a dose of 5 mg/kg with 5 μCi per mouse. In the experiments with L-dopa, Ro4-4602 in saline was injected i.p. at a dose of 500 mg/kg per mouse 1 hr prior to L-dopa. In experiments of urinary excretion of radioactivity, mice were immediately housed separately in metabolic cages and bred for 7 days after injection. Urine specimens were collected in beakers containing 0.5 ml of 0.1 M HCl and 10 mg of sodium metabisulphate at predetermined times. Mice for studies of tissue distribution of radioactivity were killed 24 hr after the administration of radioactive compound by exposure to ether and blood letting. Selected tissues were taken and weighed, and approx. 100 mg of the

samples except adrenal (several mg) were transferred to scintillation vials containing 1.0 ml of Soluene-350 (Packard Instrument Co., Downers Grove, Ill.). The samples were heated at 37° for 24 hr, and then 15 ml of scintillation fluid (Scintisol 500, Wako Chemical Co., Osaka, Japan), 1.5 ml of 0.5 M HCl and 0.1 ml of 30% H_2O_2 were added. These samples were kept overnight at 4° in order to decrease chemiluminescence and were counted on a Packard 2650 scintillation counter.

Chromatography of 24-hr urine on Dowex 50W. Urine specimens collected during the 24 hr period after injection were combined and evaporated in a rotary evaporator, dissolved in 2 M HCl and centrifuged to remove insoluble materials. An aliquot of the supernatant containing either approx. 1 μCi (for cys-dopa) or 2 μCi (for L-dopa) together with carrier, 5 mg of either cys-dopa or L-dopa, was placed on a column (1.2 × 20 cm) of Dowex 50W-X2 (200–400 mesh H^+form). The column was eluted with 2 M HCl, and fractions of 10 ml were collected. Each fraction was counted on a liquid scintillation counter and analysed spectrophotometrically between 240 and 340 nm.

RESULTS

Tritium-labelled cys-dopa was synthesised from L-[^{3}H]-dopa and L-cysteine by oxidation with mushroom tyrosinase. This labelled compound was injected to normal and B-16 melanoma-bearing mice. Of the total radioactivity injected, 75–80 per cent was recovered in the urine within 24 hr after injection and additionally a few per cent during the 1–7 day period (Fig. 1a). There was little difference in the total recovery of radioactivity between normal and melanoma-bearing mice. Similar patterns of excretion of radioactivity were obtained after the injection of L-[^{3}H]-dopa, with somewhat lower recoveries (Fig. 1b).

Tissue distribution of radioactivity after the injection of radioactive compound was examined at 24 hr, since it has been reported that in B-16 melanoma-bearing mice, melanoma:serum radioactivity ratio after the injection of DL-[2-^{14}C]-dopa reached a maximum at 24 hr [18]. Fig. 2a shows the concentration of ^{3}H in various tissues in normal and melanoma-bearing mice after the injection of [^{3}H]-cys-dopa. No tissues selectively incorporated the amino acid in both groups. Melanoma tissue did not concentrate the radioactivity; the melanoma:serum radioactivity ratio was 1.0. The highest radioactivity concentration was found in kidney. This is presumably due to renal excretion of cys-dopa and its metabolites. Fig. 2b shows the tissue distribution of ^{3}H after the injection of L-[^{3}H]-dopa to mice pretreated with Ro4-4602. The specific radioactivity was maximal again in the kidney. No concentration of ^{3}H radioactivity was observed in the melanoma; the melanoma:serum ratio was 0.85. As the possibility remained that the low ratio was due to ^{3}H loss during the metabolism to melanins, an experiment with DL-[2-^{14}C]-dopa was carried out. The tumour:serum ratio rose only slightly to 1.4 (data not shown).

Metabolic fate of [^{3}H]-cys-dopa was studied on 24-hr urine specimens by chromatography on Dowex

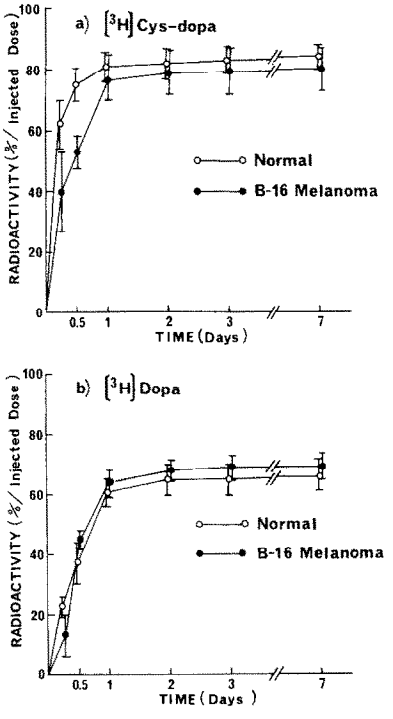

Fig. 1. Accumulative recovery of radioactivity in urine after the injection of either (a) [^{3}H]-cys-dopa or (b) L-[^{3}H]-dopa. Values represent mean ± S.E. for 4 mice per group.

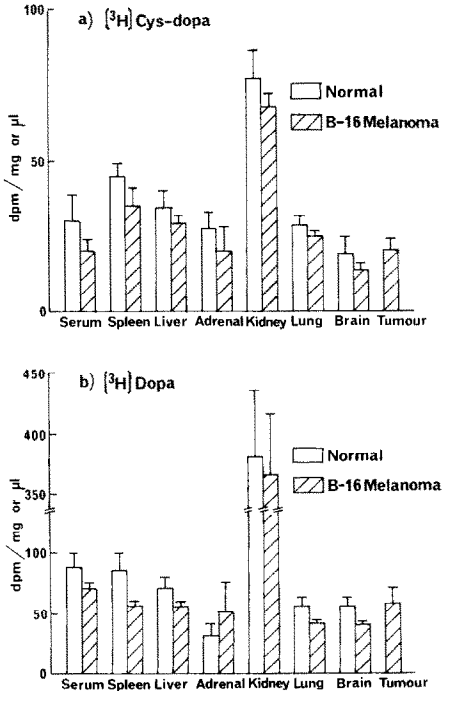

Fig. 2. Tissue distribution of radioactivity 24 hr after the injection of either (a) [^{3}H]-cys-dopa or (b) L-[^{3}H]-dopa. Values represent mean ± S.E. for 5 mice per group.

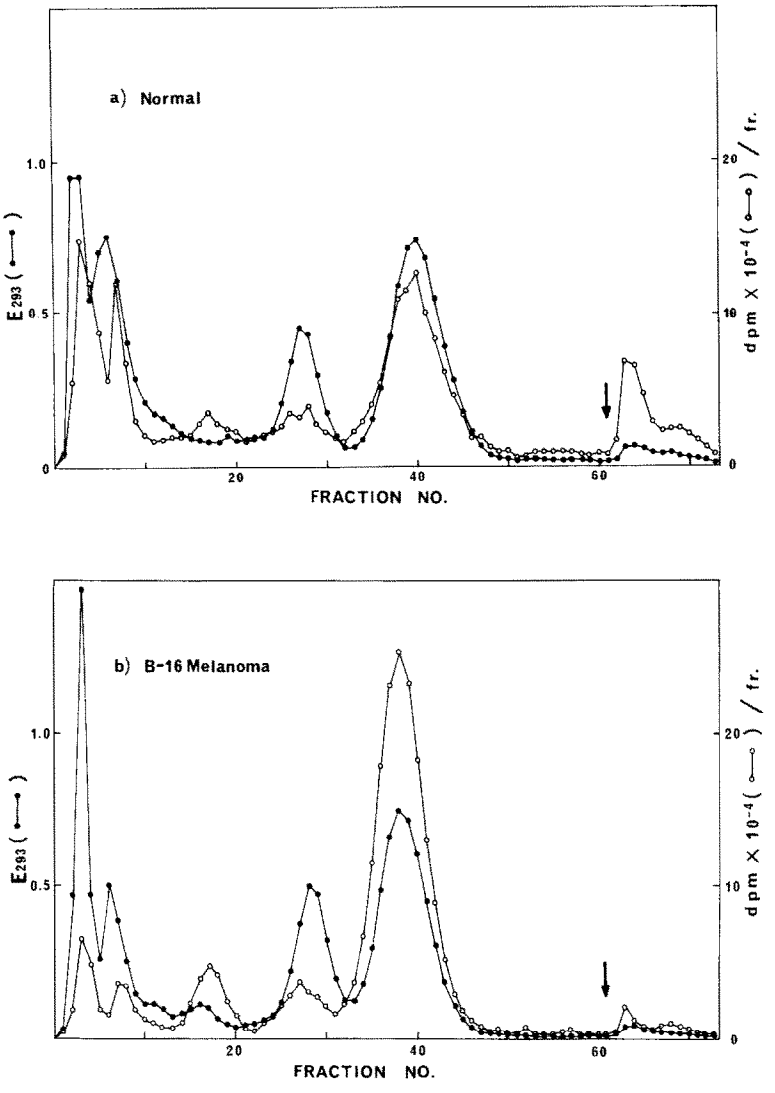

Fig. 3. Elution patterns of urinary metabolites of [^{3}H]-cys-dopa on Dowex 50W, (a) in normal mice and (b) in B-16 melanoma-bearing mice. Carrier cys-dopa (5 mg) was co-chromatographed for identification. The column was eluted with 2 M HCl until fraction 60 and then with 6 M HCl (indicated by the arrow).

50W. Elution patterns of radioactivity are depicted in Fig. 3. In chromatography of the urine of normal mice (Fig. 3a), a peak at fractions 34–48 corresponded to unchanged cys-dopa which accounted for 41 per cent of the total activity applied. A number of metabolites that are more acidic than cys-dopa were eluted prior to cys-dopa. Basic metabolites appearing in fractions 62–67 accounted for 11 per cent. 5-*S*-Cysteinyldopamine and *O*-methyl-5-*S*-cysteinyldopa [13] may appear in these fractions. The urine of melanoma-bearing mice exhibited a chromatographic pattern significantly different from that of normal mice (Fig. 3b); the recovery of cys-dopa was much higher, being 74 per cent of the total activity applied, and the amount of basic metabolites was negligible (2.5 %).

Chromatographic analyses of the urines of mice injected with L-[^{3}H]-dopa gave the following results: (1) Elution patterns were significantly different between normal and B-16 melanoma-bearing mice. (2) Cys-dopa, a possible metabolite of L-dopa, was not found in both groups. (3) A basic metabolite was found only in the urine of normal mice that accounted for 8.5 per cent of the total activity and was tentatively identified as 3-methoxytyramine (3-*O*-methyldopamine) by comparison of the elution position with an authentic sample.

DISCUSSION

Several metabolic pathways are possible for cys-dopa. In pigment-producing cells 5-*S*-cysteinyldopa (cys-dopa) and its isomer, 2-*S*-cysteinyldopa, are metabolised to phaeomelanins by oxidation, cyclisation and polymerisation [4]. The pigments have been isolated from feathers, hair and melanomas [4, 19]. Trichochromes, the simplest phaeomelanins with dimeric structure, have also been found in the

urine of patients with melanoma metastases [20]. In addition to this oxidative metabolism, cys-dopa may undergo metabolism known for L-dopa [12], such as *O*-methylation, decarboxylation and deamination. Agrup *et al.* isolated *O*-methylated 5-*S*-cysteinyldopa from the urine of melanoma patients [5].

Nearly 80 per cent of radioactivity of [^{3}H]-cys-dopa was excreted within 24 hr, and its incorporation into tissues was negligible, indicating that the oxidative metabolism was not significant in the tissues examined. This may partially account for the low toxicity of cys-dopa in mice [11].

The finding that the melanoma failed to concentrate the radioactivity of not only [^{3}H]-cys-dopa but also L-[^{3}H]-dopa was at first surprising. There have been several reports on the tissue distribution of radioactivity and its incorporation into melanoma following the administration of labelled dopa [15, 18, 21, 22]. As stated by Meier *et al.* [18], it seems that more than pigmentation is necessary for incorporation of radioactivity into the melanoma. It may be possible that in B-16 melamona the exogenous L-dopa administered behaves differently from the endogeneous L-dopa formed *in situ*.

In contrast to L-dopa, cys-dopa is not rapidly metabolised; a major radioactive compound in the urine was the unchanged cys-dopa. This indicates that cys-dopa is a poor substrate for catechol-*O*-methyltransferase, dopa decarboxylase and other catecholamine-metabolising enzymes. It is likely that this metabolic inertness of cys-dopa makes it a good biochemical indicator of melanoma [6]. It is interesting that normal mice metabolise cys-dopa to a much greater extent than B-16 melanoma-bearing mice. However, biochemical mechanisms that cause this difference remain for further studies.

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