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HISTOLOGICAL LOCALIZATION OF 4-S-CYSTEINYLPHENOL IN MELANOMA-BEARING MICE

BY

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ABSTRACT

4-S-cysteinylphenol (4-S-CP), the S-homologue of tyrosine, has been recently synthesized as a selective chemotherapeutic agent against malignant melanoma and has been shown to be a specific substrate for tyrosinase *in vitro*. *In vivo* incorporation of 4-S-CP into the B16 and Harding Passey (HP) melanomas and the systemic organs have been evaluated by the autoradiographic method. The distribution of the silver grains indicated that 4-S-CP was selectively incorporated into both the B16 and HP melanomas. 4-S-CP was excreted mainly from the kidneys and there was an accumulation of 4-S-CP in the reticulo-endothelial system. These results seemed to contribute to the utilization of 4-S-CP and other related compounds as chemotherapeutic agents against malignant melanoma.

INTRODUCTION

The melanocytes and melanoma cells possess a special enzyme, tyrosinase, which catalyzes the oxidation of tyrosine and L-3, 4-dihydroxyphenylalanine (L-dopa) to melanin. Using this enzymatic activity, a number of studies concerned with selective toxicity of melanin precursors and their analogues against malignant melanoma cells have been reported (Fujita *et al.* [1]; Graham *et al.* [2]; Ito *et al.* [3]; Wick *et al.* [4]; Yamada *et al.* [5]), since they are considered to act as substrates toward tyrosinase, generate cytotoxic products (Ito and Fujita [6]; Wick *et al.* [7]) and inhibit DNA polymerase (Wick [8]).

Ito *et al.* [3] have synthesized 4-S-cysteinylphenol (4-S-CP), the S-homologue of L-dopa, in an attempt to obtain anti-tumor agents. 4-S-CP is structurally similar to tyrosine, differing only in containing a sulfur atom (Fig. 1A). 4-S-CP is also considered to be oxidized to 4-S-cysteinylcatechol and 4-S-cysteinylhydroquinone (Fig. 1C) with tyrosinase and molecular oxygen, in the same way as tyrosine is oxidized to L-dopa and dopa-quinone (Fig. 1B). We confirmed that 4-S-CP is selectively incorporated into the melanotic human malignant melanoma cells *in vitro*, and the incorporation is inhibited by phenylthiourea, an inhibitor of tyrosinase (T. Nakamura *et al.* [9]).

In this study we examined *in vivo* the localization of 4-S-CP in the melanoma-bearing mice and discussed the mechanism of its absorption and excretion.

MATERIALS AND METHODS

4-S-CP was obtained from the reaction of phenol and L-cystine in boiling aqueous

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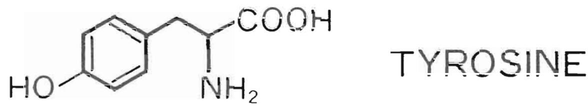

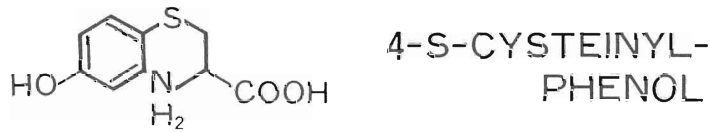

Fig. 1a.

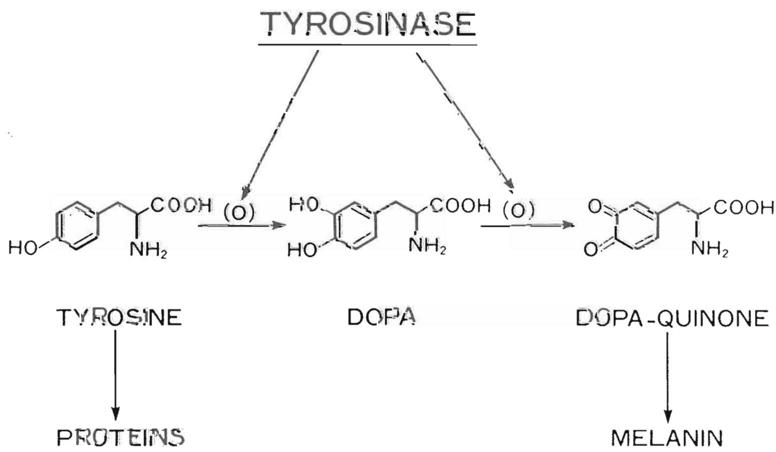

Fig. 1b

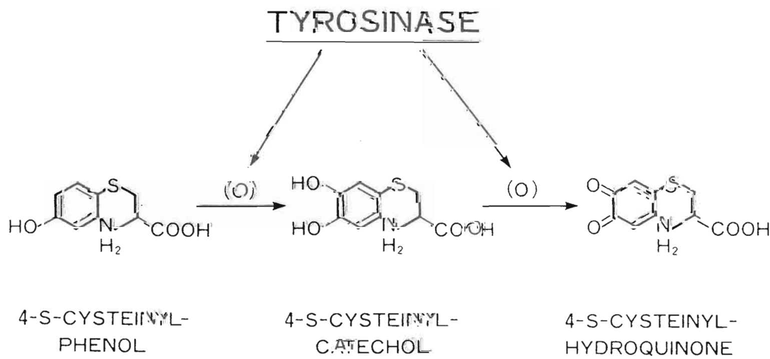

Fig. 1c.

Fig. 1. A: Structure of tyrosine and 4-S-CP B: Oxidation of tyrosine in presence of tyrosinase C: Oxidation of 4-S-CP in presence of tyrosinase.

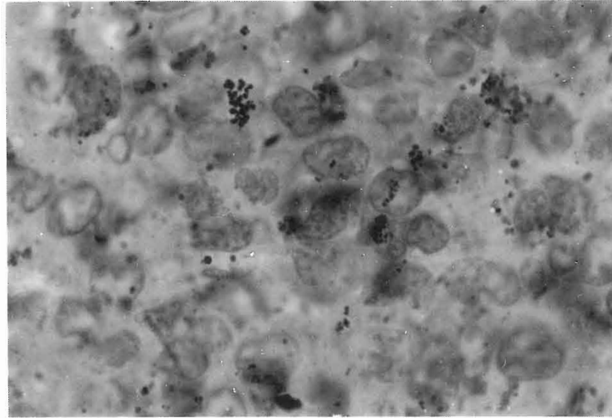

Fig. 2a

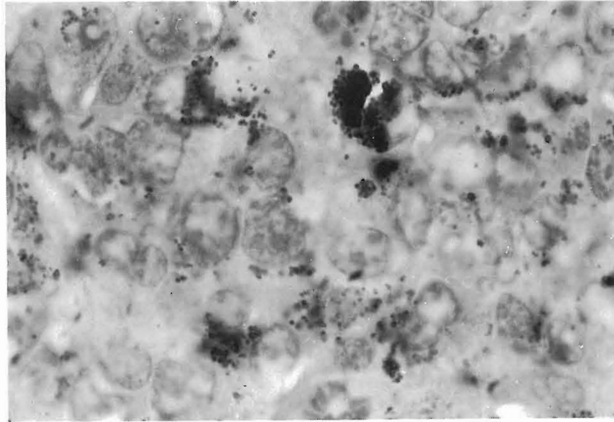

Fig. 2b.

Fig. 2. Microautoradiography Aggregation of the silver grains on B16 melanoma cells (A) and Harding Passey melanoma cells (B) (×1,600).

HBr and isolated from 2-S-cysteinylphenol by fractional crystallization by one of the authors (S.I.); the details on the synthesis of the agent have been reported elsewhere (Ito *et al.* [3]). Tritiated 4-S-CP (specific activity; 6.67 Ci/mmol) was produced by Amersham International Co. (Backinghamshire, England). The suspension of the B16 mouse melanoma cells in physiological saline (5×10^5 cells per 0.1 m*l*) was implanted into the left hind footpad of the C57BL/6J male mice aged 4 weeks. Harding Passey (HP) melanoma cells were similarly inoculated into the ddY male mice aged 4 weeks. On the 17th day after inoculation, 530 μCi of [^{3}H] 4-S-CP in physiological saline were injected intraperitoneally. The mice were killed 6, 12, 24, 48, 96 and 120 hours after injection. The melanoma, liver, kidneys, heart, skin, adrenals, brain, eyes and spleen were removed after the depletion of blood and fixed by 10% formaldehyde solution. After dehydration, thin sections were deparaffinized and coated with two-fold diluted Sakura NR-M_2 emulsion (Konishiroku

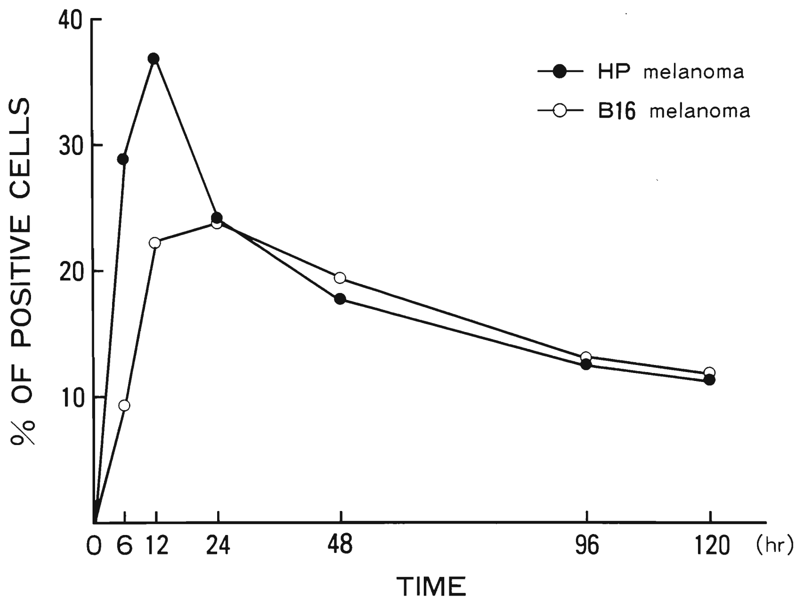

Fig. 3. Rate of grain-positive cells in malignant melanomas.

Photo Co., Tokyo) by the dipping method. They were dried and placed at 4°C in the dark for 3 weeks. Then the films for autoradiograph were developed in the Konidol-X developer (Konishiroku Photo Co.), fixed in Fujifix (Fuji Photo Film Co., Tokyo), rinsed with running water and then lightly stained with Giemsa and hematoxylin solution. On account of the radiological background, the cell with more than five silver grains was determined as being positive for 4-S-CP, and the rate of grain-positive cells was measured in each tissue.

Results

There was a relatively dense accumulation of the silver grains into the B16 (Fig. 2A) and HP (Fig. 2B) melanoma cells. Fig. 3 shows the rate of positive cells in the melanomas. Silver grains were observed in 37% of the HP melanoma cells 12 hours after injection and 23% of the B16 melanoma cells 24 hours after injection. The cells in the organs described in Fig. 4 showed a relatively high rate of incorporation. Twenty percent of the epithelial cells of the renal proximal tubuli were positive for the grains 6 hours after labeling, but this accumulation declined gradually. There was a peak of incorporation in the nerve cells of the brain at the 96 hours. The positive rate was 8% of the epidermal keratinocytes 48 hours after labeling. Hepatocytes showed a small amount of accumulation. Incorporation in the splenic reticuloendothelial system (RES) cells increased gradually. Only a few silver grains were recognized in the dermal melanocytes in both the adrenal cortex and medulla, myocardium and retinal pigment epithelium of the C57BL/6J mice.

Discussion

It is well-known that the special enzyme, tyrosinase, which mediates the conversion of tyrosine and L-dopa to melanin is restricted to the normal and malignant melanocytes. We have previously showed that human pigmented melanoma cells show a significantly greater incorporation of 4-S-CP in comparison with the amelano-

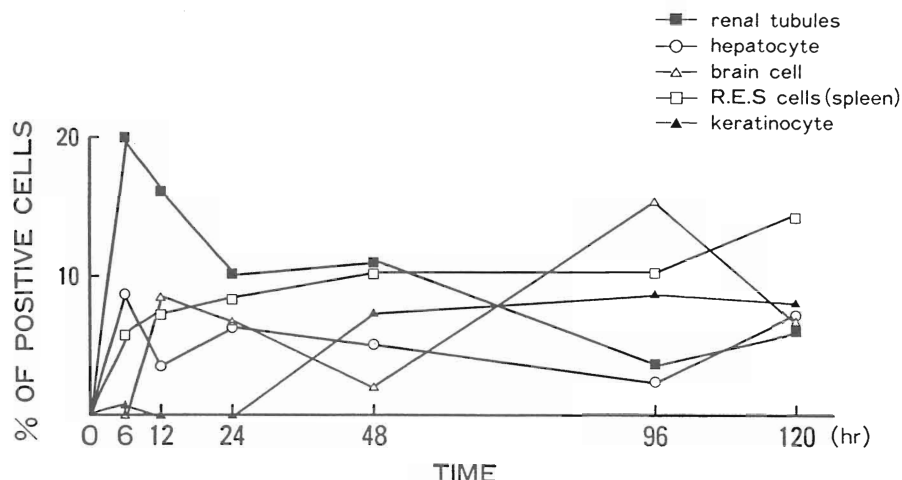

Fig. 4. Rate of grain-positive cells in liver, kidney, bone marrow, epidermis, brain and spleen in C57Bl/J6 mice.

tic melanoma cells and HeLa S_3 cells, which do not synthesize melanin, and clarified that this incorporation is inhibited under treatment with phenylthiourea, an inhibitor of tyrosinase (Nakamura *et al.* [9]). The present *in vivo* study revealed a relatively higher incorporation of 4-S-CP into the melanoma cells than the other tissues of the melanoma-bearing mice, and this result corresponds with the data of the *in vitro* experiment. The localization of 4-S-CP in the renal proximal tubuli suggested that the kidneys play a major role in the excretion of the agent.

At 120 hours after labeling, the rate of incorporation in the renal tubuli, hepatocytes, nerve cells, keratinocytes and bone marrow cells became almost equivalent, progressing into the plateau phase. On the contrary, the fact that the incorporation into the splenic RES cells increased at this period suggested that 4-S-CP could accumulate in the RES.

Incorporation of 4-S-CP into the HP melanoma showed a higher rate at 6 and 12 hours after labeling than into the B16 melanoma. The melanosome of the HP melanoma is morphologicaly different from that of B16 melanoma, and the former is related to pheomelanogenesis and the latter to eumelanogenesis (Jimbow *et al.* [10]). It is not considered that the morpholgical difference of the melanosomes is responsible for controlling the incorporation rate, since both pheomelanogenesis and eumelanogenesis pass through the same pathway from tyrosine to dopa-quinone (Jibmow *et al.* [11]), and 4-S-CP was thought to be coincident to tyrosine in this pathway. There is some possibility that the activity of tyrosinase and its turnover rate might play an important role in the incorporation of 4-S-CP rather than the kind of melanosome.

It is important to measure the absolute quantity of the incorporated 4-S-CP in each tissue because the exact radioactivity in the tissue can not be determined by the autoradiographic method. In the present study, however, the rate of incorporation into the melanoma cells was much higher than into the other tissues, and from the consideration of our previous study the incorporation is thought to be specific for the melanotic malignant melanoma.

Melanoma cells possess the enzyme,

tyrosinase, and a metabolic pathway for the conversion of tyrosine and L-dopa to melanin. Several tyrosine derivatives including 4-S-CP have displayed selective toxicity against the melanoma cells *in vitro* (Yamada *et al.* [5]), and hydroquinone has been demonstrated to have a selective toxicity against the melanocytes *in vivo* (Bleehan *et al.* [12]). This study informs in detail the *in vivo* localization of the 4-S-CP in the melanoma-bearing mice and will contribute to the application of this agent and other related compounds to the selective chemotherapeutic agents against malignant melanoma.

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