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Cytotoxicity of 4-Hydroxyanisole and Tyrosinase Activity in Variant Cell Lines of B16 Melanoma

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Abstract—*The melanocytotoxic effects of 4-hydroxyanisole (4-OHA) are thought to depend upon its conversion to toxic oxidation products by the enzyme tyrosinase. In this study, the cytotoxicity of 4-OHA was examined in different B16 melanoma cell lines that show varying levels of tyrosinase and after stimulation by melanocyte-stimulating hormone (MSH) and all-trans-retinoic acid (RA). 4-OHA decreased cell survival of three melanotic and one amelanotic cell line in culture, but the effect was unrelated to their tyrosinase activity or the subcellular localization of the enzyme. Although stimulation of tyrosinase activity with RA enhanced the cytotoxicity of 4-OHA, no similar enhancement occurred with α-MSH. It appears that there is no relationship between the cytotoxic effects of 4-OHA and intracellular tyrosinase and the enhancement of its cytotoxicity by RA may well be related to the antiproliferative effects of the retinoid.*

INTRODUCTION

CERTAIN phenolic compounds are known to have cytotoxic actions on pigment cells and this has led to interest in their use as therapeutic agents in malignant melanoma. One of the most active of these phenols is 4-hydroxyanisole (4-OHA) and numerous studies have shown that it has cytotoxic effects on melanocytes [1–3] and melanoma cells [4–7].

The cytotoxicity of these phenols depends upon their conversion to toxic oxidation products and in melanoma cells this is thought to be brought about by tyrosinase [8, 9]. More recently, there have been suggestions that other oxidative enzyme systems, notably those present in mitochondria, may bring about the conversion of phenols to toxic products [10–12].

In order to re-examine the importance of tyrosinase for the melanocytotoxicity of 4-OHA, we have measured its cytotoxic effects in variant cell lines of the B16 murine melanoma that express different levels of tyrosinase activity.

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MATERIALS AND METHODS

Cell lines and culture conditions

The melanotic B16-F1, B16-F10 and B16-BL6 variant cell lines of the B16 melanoma were used. In a few experiments an amelanotic cell line, B16-F10DD, kindly donated by Dr. D.C. Bennett, was also used. The cultures were maintained at 37°C in Eagle's minimal essential medium with Earles Salts, supplemented with vitamins, non-essential amino acids, 10% foetal calf serum and containing penicillin (5000 IU/ml) and streptomycin (5000 μg/ml) and 0.2% sodium bicarbonate in an atmosphere of 5% CO_2 in air.

Cells were plated in this medium (1×10^6 cells/75 cm^2 flasks) which was replaced on days 1 and 3. The cells were harvested on day 4 using 0.02% EDTA and 2.5% trypsin (9:1) for counting and the measurement of tyrosinase activity.

Drugs

4-Hydroxyanisole was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). It was stored in the dark at a concentration of 10 mmol/l at −20°C and immediately prior to use was diluted in culture medium. Diethyldithiocarbamate (DDTC) was obtained from Sigma Ltd and was dissolved in culture medium immediately prior to use. α-MSH was a gift from Ciba-Geigy Ltd, Basle, Switzerland.

The peptide was dissolved in culture medium immediately prior to use. All-*trans*-retinoic acid was a gift from Dr. C. Redfern. It was stored in the dark under N_2 at −20°C. Before each experiment, it was dissolved in ethanol and diluted in culture medium.

Drugs were added on day 0 and at times of refeeding. In the experiment with all-*trans*-retinoic acid control cultures received the same final ethanol concentration as that of the treated cultures. The ethanol concentration never exceeded 1%.

Cell survival

The numbers of cells were counted at the end of each experiment and percentage cells remaining calculated as $T/C \times 100$ where T and C are the numbers of cells in test and control cultures respectively.

Tyrosinase assay

Tyrosinase activity was measured by a modification of the method of Pomerantz [13] which measures the amount of ^3H_2O released during the conversion of [^{3}H]tyrosine to L-dopa. Following harvesting, the cells were stored at −70°C. When required, the cells were reconstituted in 0.1 M phosphate buffer (pH 7.4) and sonicated.

Subcellular localization of tyrosinase

Cells (3×10^6) were sonicated in 2 ml 0.1 M phosphate buffer for 10 s. One millilitre was taken for assay and the remaining aliquot was centrifuged at 1500 ***g*** for 10 min. The pellet (nuclear and membrane protein) was retained for assay and the supernatant was centrifuged at 13,400 ***g*** for 20 min. The supernatant (soluble fraction) was retained for assay and the pellet washed in 1 ml 0.1 M phosphate buffer and centrifuged at 13,400 ***g*** for 10 min. The washings and the pellet (particulate fraction) were taken for assay.

Samples of the different fractions were also taken for electron microscopy.

RESULTS

Tyrosinase activity

All four cell lines displayed tyrosinase activity after 4 days in culture. The F1 cells contained the highest level of activity and the amelanotic F10DD cells the least (Table 1). In the three melanotic cell lines, around 10% of the tyrosinase activity was found to be localized in the particulate fraction and presumably represented activity that was associated with the melanosomes. Electron microscopy confirmed that intact melanosomes were present in the particulate and not in the soluble or nuclear fractions. The proportion of tyrosinase activity in the particulate fraction was lower (3.0%) in the amelanotic F10DD cells.

The tyrosinase inhibitor diethyldithiocarbamate failed to inhibit tyrosinase activity in the three melanotic cell lines when present at a concentration of 10^{-7} M (Table 1). Higher concentrations (10^{-6} and 10^{-5} M) were not used because it was shown in preliminary experiments that the inhibitor totally prevented cell growth at these concentrations.

α-MSH (10^{-7} M) increased tyrosinase activity in the three melanotic cell lines (Table 1). There was, however, little change in the subcellular localization of tyrosinase after α-MSH (Table 1).

All-*trans*-retinoic acid (10^{-7} M) also increased tyrosinase activity and, as with α-MSH, the greatest effect occurred in the F1 cells (Table 1). The effect of the retinoid on subcellular localization of tyrosinase was studied on only two occasions and was confined to the F10 cells. As with α-MSH, RA produced little change in the subcellular distribution of tyrosinase (Table 1).

4-Hydroxyanisole had no effect on tyrosinase activity.

Cell survival

Similar numbers of cells were present for the four lines after 4 days in culture. The effects of 4-OHA are shown in Fig. 1. Low concentrations (10^{-6} and 10^{-5} M) of 4-OHA had little effect on cell numbers but in the presence of 10^{-4} M 4-OHA, cell survival was decreased to around 14 and 18% in the F1 and F10 cells respectively and to around 9% in the BL6 cells. At the highest concentration of 4-OHA (10^{-3} M), cell survival was approx. 2% in all three cell lines. The F10DD cells were exposed to only one concentration of 4-OHA (10^{-4} M) and at this concentration cell survival was reduced to around 20% (Table 2).

The tyrosinase inhibitor DDTC had no effect on the growth of the four cell lines at concentrations of 10^{-7} and 10^{-8} M and failed to alter the cytotoxic effect of 4-OHA (Table 2).

α-MSH decreased cell growth in the three melanotic cell lines but it failed to enhance the cytotoxicity of 4-OHA (Fig. 1, Table 2).

All-*trans*-retinoic acid (10^{-7} M) also decreased cell growth (Table 2). Unlike α-MSH, however, RA significantly enhanced the cytotoxic effects of 4-OHA in the F1 and the F10 cells, although the effect was just short of significance in the case of the BL6 cells (Table 2).

DISCUSSION

The present results confirm previous reports that 4-OHA has cytotoxic effects on melanoma cells. It has been known for some time that phenols and other hydroxylated benzene derivatives are cytotoxic and at least two mechanisms of action have been proposed [14, 15]. One suggestion is that these compounds are oxidized to corresponding

Table 1. Tyrosinase activity and its subcellular localization in different B16 melanoma cell lines and the effect of diethyldithiocarbamate (DDTC) (10^{-7} M), α-MSH (10^{-7} M and all-trans-retinoic acid (RA) (10^{-7} M)

Cell line	Tyrosinase activity (dpm × 10^{-3}/10^6 cells/h)		Subcellular localization of tyrosinase (% of total) Nuclear/membrane	Soluble	Particulate
BL6	37.6 ± 6.5	(12)	1.2 ± 0.5	85.4 ± 5.0	9.3 ± 3.9
F1	75.7 ± 17.3	(12)	1.7 ± 0.6	88.3 ± 2.3	8.8 ± 0.5
F10	42.6 ± 8.9	(12)	1.3 ± 0.3	86.5 ± 1.9	10.8 ± 1.4
F10DD	6.8 ± 1.6	(4)	1.9	92.5	3.0
After DDTC					
B16	30.9 ± 7.6	(3)	—	—	—
F1	54.7 ± 8.0	(3)	—	—	—
F10	40.3 ± 2.8	(3)	—	—	—
After α-MSH					
BL6	128.4 ± 28	(3)	2.2 ± 1.2	88.6 ± 5.7	7.5 ± 4.8
F1	203.7 ± 22	(3)	6.2 ± 2.6	79.1 ± 7.0	11.4 ± 3.0
F10	150.1 ± 26	(3)	3.8 ± 2.2	88.1 ± 3.5	6.0 ± 1.3
After RA					
BL6	69.0 ± 8.2	(5)	—	—	—
F1	96.6 ± 10.8	(5)	—	—	—
F10	53.9 ± 12.2	(5)	4.8	78.4	7.6

The number of experiments is given in parentheses and the results are expressed as means ± S.E.M. Where no S.E.M. is given, only two experiments were carried out.

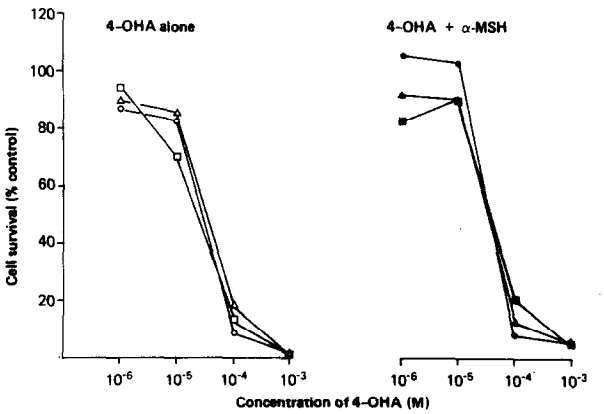

Fig. 1. Effect of varying concentrations of 4-hydroxyanisole (4-OHA), either alone or in the presence of α-MSH (10^{-7} M) on cell survival of three variant cell lines of B16 melanoma. The results are expressed as a percentage of cell survival in the control cultures that contained either no additions or α-MSH alone. The values are the means of 3–5 experiments and the S.E.M. were similar in range to those in Table 2. o—o *BL6;* □—□ *F1;* △—△ *F10.*

benzoquinones and these produce cell damage by inactivating enzymes involved in DNA replication. On the other hand, it has been proposed that cytotoxicity depends upon the formation of superoxide and hydroxyl radicals. It has also been suggested that in melanoma cells these oxidations are mediated by tyrosinase (see [16]). A further suggestion has been made recently that the S-phase toxicity [17] may be related to the inhibition by 4-OHA of ribonucleotide reductase [18].

In the present study, we found no relationship between the cytotoxicity of 4-OHA and tyrosinase activity in B16 melanoma. Thus, of the different cell lines examined, it was the BL6 cells that showed the lowest survival in the presence of 4-OHA, yet it was the F1 cells that showed the highest level of tyrosinase. Moreover, the amelanotic F10DD cells were also susceptible to the cytotoxic action of 4-OHA, despite their low level of tyrosinase activity.

In order to become active in the cell, it is necessary for the tyrosinase to bind to the melanosome [19]. We therefore wondered whether the cells showed differences in their levels of bound tyrosinase. However, this was not the case, since the subcellular localization of tyrosinase was found to be similar in the different cell lines.

The cytotoxicity of 4-OHA was also unaffected by the presence of the tyrosinase inhibitor DDTC. In the present study, however, there was very little reduction in tyrosinase activity at concentrations of 10^{-7} and 10^{-8} M and at higher concentrations the inhibitor completely abolished cell growth.

It was therefore decided to examine the effect of agents that stimulated tyrosinase activity. MSH peptides are well known stimulators of tyrosinase and, as a result of this action, will inhibit the growth of melanoma cells [20, 21]. Both effects were seen in this study but when the growth inhibition was taken into account, α-MSH failed to potentiate the cytotoxic effects of 4-OHA despite the increase in tyrosinase activity. In a recent study, Pawelek and Murray [22] found that β-MSH increased the cytotoxicity of dopa phosphates in S91 Cloudman melanoma cells.

Retinoids have also been shown to stimulate

Table 2. Cell survival of different B16 melanoma cell lines in the presence of 4-OHA (10^{-4} M) and the effect of diethyldithiocarbamate (DDTC) (10^{-7} M), α-MSH (10^{-7} M) and all-trans-retinoic acid (RA) (10^{-7} M)

	BL6		F1		F10		F10DD	
	Cell No. ($\times 10^6$)	%	Cell No. ($\times 10^6$)	%	Cell No. ($\times 10^6$)	%	Cell No. ($\times 10^6$)	%
No additions	21.7 ± 3.6	—	24.0 ± 1.5	—	22.8 ± 4.9	—	19.0 ± 2.4	—
4-OHA	2.9 ± 0.3**	14.6 ± 3.8	4.9 ± 1.1**	19.5 ± 6.3	2.8 ± 0.9*	12.7 ± 0.6	3.8 ± 0.7	20.1 ± 2.9
DDTC	21.6 ± 3.1	—	22.3 ± 2.5	—	21.6 ± 3.5	—	12.8 ± 1.9	—
DDTC + 4-OHA	3.1 ± 0.5	16.2 ± 3.8	4.7 ± 1.3	21.2 ± 8.3	2.4 ± 1.1	10.7 ± 2.1	2.3 ± 0.5	18.6 ± 3.7
No additions	22.6 ± 1.7	—	21.9 ± 0.9	—	24.2 ± 1.2	—	—	—
4-OHA	2.1 ± 0.9****	9.0 ± 3.9	2.4 ± 0.6****	13.0 ± 2.5	4.2 ± 1.0****	18.0 ± 4.9	—	—
α-MSH	13.2 ± 1.8*	—	12.3 ± 1.0****	—	16.2 ± 1.0***	—	—	—
α-MSH + 4-OHA	1.3 ± 0.5	9.3 ± 2.3	2.3 ± 0.6	20.6 ± 3.4	2.2 ± 0.9	13.2 ± 5.5	—	—
No additions	20.5 ± 2.2	—	20.6 ± 1.0	—	21.0 ± 2.1	—	—	—
4-OHA	5.1 ± 2.7***	22.7 ± 8.7	4.7 ± 0.8****	23.2 ± 2.7	2.7 ± 0.7****	12.8 ± 2.4	—	—
RA	6.0 ± 1.2****	—	3.7 ± 0.5****	—	7.9 ± 1.3***	—	—	—
RA + 4-OHA	0.25 ± 0.03	6.1 ± 0.6	0.62 ± 0.3††	16.5 ± 5.4	0.45 ± 1.7†	6.4 ± 1.7	—	—

The results are the means ± S.E.M. of at least three experiments. The percentage survival values after 4-OHA were calculated as the percentage of cell survival in the No Additions group or the DDTC, α-MSH and RA groups where appropriate.

P values: * <0.05, ** <0.01, *** <0.005, **** <0.001 compared with No Additions group or 4-OHA group (†).

tyrosinase activity [23] and this was confirmed in the present study. Unlike α-MSH all-*trans*-retinoic acid also enhanced the cytotoxic effects of 4-OHA in the B16 cells. However, this could well be related to its growth inhibiting effects, which, unlike those of MSH, are independent of tyrosinase [24].

The present results therefore suggest that the cytotoxicity of 4-OHA is not dependent upon tyrosinase. This is consistent with the view of Picardo *et al.* [12] that catechol toxicity is due to the oxygen radicals that are formed extracellularly as the catechols undergo decomposition. This could explain why the toxicity of such compounds is not exclusive to melanoma cells. Wick [25] has suggested that the reduced forms of the quinols are also active and will affect DNA synthesis by altering the redox conditions of the cell. The cytotoxicity of the phenols and related compounds could therefore be mediated in a number of ways which, even in melanoma cells, may not be dependent upon tyrosinase.

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