Ultrastructural Observations on the Effect of 4-Hydroxyanisole on Normal Human Melanocytes in Tissue Culture

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Riley's classic 1970 experiment showing a specific cytotoxic effect of 4-hydroxyanisole (4-OHA) on tissue-cultured melanocytes of black guinea pig ear skin was repeated on normal human melanocytes, and the results were examined by electron microscopy. In dispersed tissue culture, no specific toxic effect on human melanocytes was observed following equally timed exposures to similar (10^{-3} M) or even higher (10^{-2} M) concentrations of the drug; plasma membrane, nucleus, and cytoplasmic organelles, including melanosomes were unaffected. The same applied to melanocytes of whole epidermis exposed for up to 24 hr to the same concentrations of 4-OHA in culture medium. Melanocytes of PUVA-treated skin similarly exposed for up to 24 hr to 10^{-2} and 10^{-3} M 4-OHA, likewise exhibited no evident morphological damage at the ultrastructural level. The discrepancy of results between guinea pig and man could have a variety of explanations, one of which could be due to a possible relatively low level of active tyrosinase in the human melanocytes (Riley believes the cytotoxic effect of 4-OHA to be due to the fact that it acts as a substrate for tyrosinase, toxic intermediates being liberated as a result). However, the lack of effect on the PUVA-activated melanocytes indicates that this cannot be the entire explanation.

There is considerable current interest in the possible therapeutic application of melanocytotoxic chemicals to the treatment of melanoma, and tissue culture of melanocytes provides an obvious tool for investigating and monitoring their effects. 4-Hydroxyanisole (4-OHA) is reported as specifically damaging to normal guinea pig melanocytes [1,2], and Harding-Passey mouse melanoma cells [3] in tissue culture. This effect is thought to be due to the production from oxidation of 4-OHA by the enzyme tyrosinase, of toxic intermediates, possibly free radicals, which damage membranes, and affect the RNA and protein synthesising mechanisms within the cell. In none of the studies mentioned were the results monitored by electron microscopy, which can provide more precise details of the location and nature of subcellular damage than light microscopic techniques. Bleehen [4] however, has published an electron micrograph purporting to illustrate "damage to outer membranes of melanosomes" and "considerable disruption of subcellular architecture" of part of a cultured Harding-Passey melanoma cell due to exposure to 4-OHA. It appears to us that despite this, the ultrastructural evidence on the effects of 4-OHA must still be regarded as insufficient, and even unconvincing, and that the matter merits further investigation, particularly with reference to the human melanocyte. It was decided to commence with observations on the normal human melanocyte in tissue culture in order to provide a base line of reference for projected future studies on melanoma, and for comparison with results of previous [5] and continuing investigations on the effects of dicarboxylic acids. Such a comparison is of interest because, whereas 4-OHA acts as a substrate for tyrosinase [1] dicarboxylic acids are inhibitors of the enzyme [6]. We have, in effect, repeated Riley's [1] experiments, using human instead of guinea pig melanocytes, and with electron microscopic assessment of the appearance of the treated cells. Apart from observations on melanocytes, possible effects of 4-OHA on keratinocytes in culture were also looked for.

MATERIALS AND METHODS

Dispersed tissue culture

Human skin was obtained from either reduction mammoplasties or abdominolipectomies. Skin samples were freed from fatty tissue and stretched over a skin-stretching device, and thin slices were then cut free-hand using a Down's dermatome containing a razor blade. The skin slices were then floated, dermis side down, on 0.3% trypsin in Dulbecco's saline. These were incubated at 37°C for 10-15 min, until separation of the epidermis and dermis was complete. The epidermis was then transferred to growth medium and shaken or gently scraped to release epidermal cells. The tissue culture medium consisted of Eagle's suspension medium with Earle's salts containing HEPES buffer, glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 20% fetal calf serum. Media and chemicals were obtained from Grand Island Biological Co., Flow Labs., ad Sigma.

Cells were seeded in vented Petri-dishes on collagen gels (method of W. Jones, Imperial Cancer Research Fund, personal communication) and maintained at 37°C in a humidified atmosphere of 5% CO₂, 95% air.

After growth for 5 days, 4-hydroxyanisole (4-methoxy-xyphenol—BDH Chemicals Ltd.) was dissolved in sterile water, filter sterilized, and added to the media to give final concentrations of between 1 and 5 x 10^{-7} to 10^{-8} M for periods of 20 min, 45 min, and 24 hr. Appropriate control cultures for each concentration and time were maintained.

Organ Culture

Small pieces of skin removed at upper dermal level, were cut with a razor blade and incubated in culture medium with concentrations of 10^{-1} M, 10^{-2} M, and 10^{-3} M 4-OHA for periods of 1, 5, and 24 hr. These specimens were obtained from normal skin, from unaffected forearm skin of a female psoriatic patient under PUVA treatment for 2 yr, and from unaffected forearm skin of a male psoriatic who had been exposed for the first time to PUVA 72 hr previously.

Preparation for Electron Microscopy

After exposure to 4-OHA the growth medium was removed from the plates, and the cultures were briefly rinsed in Dulbecco's saline. Fixation was carried out in 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4, for 30 min at room temperature, washed thoroughly in buffer and postfixed in Palade's osmium for 20 min at room temperature. The cells were dehydrated in ethanol and embedded in Araldite. The cell cultures and the collagen substrate were processed together. Control cultures were processed similarly.

For organ culture, the treated skin samples were fixed in Karnovsky's fixative at pH 7.4 in 0.1 M cacodylate buffer for 2 hr at room temperature. After rinsing in buffer, the skin was postfixed in Palade's osmium for 1 hr at room temperature. Subsequent dehydration and embedding was as described above.

Thin sections, stained with uranyl acetate and lead citrate, were examined in either a JEOL-100CX or a Philips 300 electron microscope.
RESULTS

Riley [1] reported maximum toxic effects of 4-OHA on "young" cultures (4-10 days) at concentrations of $5 \times 10^{-3} \text{M}$ and $1 \times 10^{-3} \text{M}$ for 30 min, and the present results relate largely to similar times and concentrations, as well as to the higher concentrations of $10^{-2} \text{M}$ and $10^{-1} \text{M}$ which were not used by him. Any specific damage to melanocytes should be evident at these concentrations.

Light Microscopy

None of the extreme toxic effects of 4-OHA described by Riley [1] on melanocytes of guinea pig ear skin were observed on human melanocytes of the present dispersed cultures. Even with concentrations as high as $10^{-2} \text{M}$ 4-OHA, and exposure to the drug for 45 min, no cytoplasmic blebbing nor disruption of cells was observed. At most, some retardation of dendritic processes, and surface wrinkling might be said to have occurred but these effects were in no way striking.

Electron Microscopy

**Dispersed cultures:** Cultures, both control and experimental, consisted of a mixture of keratinocytes and melanocytes, with the melanocytes showing a preferential tendency to be located close to the collagen substrate though they were also frequently seen entirely surrounded by keratinocytes. The appearance of control melanocytes is illustrated in Fig. 1. Cytoplasmic lipid droplets are frequently observed in control melanocytes and they often may be localized to a particular region of the cell, a fact which must be borne in mind when assessing the significance of their presence or absence, especially when comparisons with experimental material are being made. With the present material, serial, or closely interrupted sections through the same cell were frequently observed, so that a realistic estimate of the concentration of lipid droplets per cell could be made. Melanocytes exposed to $10^{-3} \text{M}$ 4-OHA (the highest concentration employed by Riley) exhibited no features which could be regarded as degenerative or the result of a toxic effect of the drug (Fig 2). The plasma membrane was always intact, the nucleus normal in appearance, and nothing abnormal was observed in the general cytoplasm, or with regard to individual cytoplasmic organelles, including melanosomes. The same was true of melanocytes exposed to the higher concentration of $10^{-2} \text{M}$ 4-OHA.

Keratinocytes likewise exhibited no morphological changes which could be attributed to a toxic effect of 4-OHA. As with melanocytes, their appearance was similar to keratinocytes of control cultures.

**Organ cultures:** Normal skin, not heavily pigmented, exposed to $10^{-1} \text{M}$ 4-OHA for 1, 5, and 24 hr. exhibited extensive general damage to both melanocytes and keratinocytes, as might be expected (Fig. 3). With a drug concentration of $10^{-2} \text{M}$, however such damage was much less severe, and when present was evident only at the edge of sections, where it could be attributed in part or whole to damage due to original cutting of the skin into pieces; where damaged melanocytes were observed, adjacent keratinocytes were equally damaged. The great majority of cells of both types did not differ in any observable respect from controls and there was no tendency for melanocytes to shrink, or retract from adjacent keratinocytes, which feature is a sensitive indicator of reaction to toxic substances (Fig. 4). The skin of the female psoriatic under treatment with PUVA for 2 yr was heavily pigmented and control thin sections revealed a high concentration of melanosomes in both melano-
Fig 2. Melanocyte from 5-day culture exposed to $10^{-3}$ M 4-hydroxyanisole for 45 min. Plasma membrane, nucleus, and cytoplasmic organelles appear unaffected. *Bar* = 1 μm. (× 10,230)

Fig 3. Epidermis of normal abdominal skin exposed to $10^{-1}$ M 4-hydroxyanisole for 5 hr in tissue culture medium. There is extensive damage to both melanocytes (*M*) and keratinocytes (*K*). *Bar* = 4 μm. (× 3,000).

cytes and keratinocytes. As with the normal skin described above, no specific damage to melanocytes was observed following exposure to the same concentrations of 4-OHA for the same time (Fig 5).

Fig 4. Melanocyte, in epidermis of abdominal skin exposed to $10^{-2}$ M 4-hydroxyanisole for 5 hr in tissue culture medium. There is nothing positively abnormal about the appearance of this cell, and it has not shrunken or retracted away from adjacent keratinocytes. Note centriole. *Bar* = 1 μm. (× 8,000).

Melanocytes of the male psoriatic before exposure to PUVA were relatively inactive (Fig 6) but control melanocytes 72 hr following exposure showed a significant increase in number of melanosomes of all stages and the keratinocytes also contained significantly greater numbers of fully melanized melanosomes. These evidently activated melanocytes showed no sign of spe-
Sept. 1981

EFFECT OF 4-HYDROXYANISOLE ON HUMAN MELANOCYTES

295

specific toxic damage following exposure to $10^{-2}$ M, or $10^{-3}$ M 4-OHA for up to 24 hr (Fig 7).

DISCUSSION

This study has failed to demonstrate a specific toxic effect of 4-hydroxyanisole (4-OHA) on normal human melanocytes in tissue culture, both dispersed and organ culture. Ultrastructural examination of the cells revealed that the cell membrane, nucleus, general cytoplasmic organelles, and melanosomes, were unaffected by concentrations of the drug even higher than those which Riley [1] reported, on the basis of light microscopic observations, as being severely cytotoxic to normal guinea pig ear melanocytes.

This discrepancy of results can have a number of explanations. It may be the result of a species difference between man and the guinea pig, though the basic structure of the cell, and the general process of melanogenesis, is, as far as is known, identical in the 2 instances. Riley [1,2] has presented evidence that 4-OHA is preferentially taken into the cytoplasm of the guinea-pig melanocyte to act as a substrate for tyrosinase, and believes that its effects are due to the resulting production of toxic intermediate products, and "damage to cell membranes". If this be so, the apparent lack of effect on the human melanocyte might be due to the fact that for some reason 4-OHA is unable to traverse the plasma membrane and enter the cell. According to Riley's [1] view, the presence of active tyrosinase within the cell is essential for 4-OHA to exert its toxic effect, and he presented evidence to show that in the guinea pig, the degree of effect was related to tyrosinase activity. The human melanocytes derived from the present mammoplasties and abdominal lipectomies certainly came from skin areas not extensively exposed to sunlight, and it could be said that their tyrosinase activity was minimal, and that, indeed, the lack of effect of 4-OHA on them confirms Riley's [1] views as to its mode of action on guinea pig melanocytes. However, they exhibited reasonable melanogenic activity in culture (see Figs 1,2), and one might have expected some effect with the higher dosage of $10^{-2}$ M 4-OHA. The more highly active melanocytes of the 2 patients being treated with PUVA likewise showed no specific damage. This could suggest that human tyrosinase, for whatever reason, does not react in the same way with 4-OHA as does the guinea pig enzyme, or that it needs to be present in particularly high levels before doing so, and hence producing a toxic effect. These possibilities could be tested either by stimulating cultured melanocytes by increasing the levels of cyclic-AMP in the cells [7], or by adding MSH or tyrosinase to the

FIG 5. Melanocyte in epidermis of arm skin of PUVA treated (2 yr) psoriatic which was exposed to $10^{-3}$ M 4-hydroxyanisole in culture medium for 24 hr. The cell appears unaffected. Note well-developed Golgi membranes, Go. Bar = 1 μm. (× 8,600).

FIG 6. Melanocyte in epidermis of unaffected arm skin of psoriatic prior to exposure to PUVA. The cell has a very inactive appearance, with few melanosomes or other cytoplasmic organelles. Bar = 1 μm. (× 8,500).

FIG 7. Unaffected arm epidermis of PUVA treated (72 hr previously) psoriatic after 24 hr exposure to $10^{-3}$ M 4-hydroxyanisole in culture medium. The keratinocytes contain many melanosomes and the melanocyte (M) is evidently more active than the pre-PUVA treated cell in Fig 6, and evidently unaffected by the drug. Bar = 3 μm. (× 3,600).
culture medium; Dewey, Butcher, and Galpine [3] have shown that added tyrosinase has a marked effect on the sensitivity of Harding-Passey melanoma cells to 4-OHA. It is proposed to carry out such experiments, but the possibility remains that some factor other than level of tyrosinase may be concerned.

What implications, if any, have the present results got for the possibility of treating human melanoma with 4-OHA? Bleehen [4] reported, but did not illustrate, a cytotoxic effect of 4-OHA on human melanoma cells in primary culture. It may be that this resulted from the presence of high levels of active tyrosinase within the cells, and that the matter is as simple as this. If 4-OHA is ever to be used systemically, or by perfusion, for the treatment of human melanoma, it would be important to know whether or not it would have a toxic effect on normal melanocytes. The present investigation has shown that it has no apparent specific effect on normal cells in tissue culture, but whether or not the same would apply in the intact body is another matter. Extrapolation of results, especially negative results, from tissue culture to a therapeutic situation in the living, always requires extreme caution, and particularly so in the present instance, where what has come to be regarded as a classic experiment on the guinea pig [1] has failed to be confirmed for man.

REFERENCES