Effect of Dicarboxylic Acids (C₆ and C₉) on Human Choroidal Melanoma in Cell Culture

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In cell culture, azelaic acid (C₉) has been shown to have an antiproliferative and cytotoxic effect on human and murine malignant cutaneous melanocytes. Normal melanocytes are unaffected, as are normal choroidal melanocytes. Here, effects on cell kinetics and ultrastructure of cells of a human choroidal melanoma line have been studied. Cells were exposed to single doses of disodium salts of azelaic (C₂Na) and adipic (C₆Na) acids at concentrations of 10⁻² M and 5 × 10⁻² M for 48 hr. C₂Na at 5 × 10⁻³ M had a significant effect on proliferation at 24 and 48 hr and this was not reversible on removal of diacid. At 5 × 10⁻² M for 24 hr, C₂Na had no effect and at 5 × 10⁻² M for 48 hr had an effect which was marginally significant, but reversible. Swelling and disruption of mitochondria was seen in cells exposed to C₂Na at 5 × 10⁻² M for 1 hr and longer, but even at 10⁻¹ M, cells exposed to C₂Na were minimally affected. The results could encourage further investigations of the feasibility of azelaic acid therapy for uveal and ocular adnexal melanoma.

Azelaic acid (C₉ dicarboxylic acid), topically applied, has a beneficial clinical effect on cutaneous hyperpigmentary disorders involving hyperactive and abnormal melanocytes,¹⁻⁴ and in cell culture at concentrations greater than 10⁻³ M, exerts an antiproliferative and cytotoxic effect on malignant melanocytes of human and murine origin which is time- and dose-dependent.⁵⁻⁸ Similar effects have been demonstrated for other tumoral cell lines.⁵⁻⁹ Experiments with isolated rat liver mitochondria have shown that azelaic acid inhibits respiration, and with submitochondrial particles that it competitively inhibits NADH-dehydrogenase, succinic dehydrogenase, and other oxido-reductases, including tyrosinase.¹⁰

In general, normal cells have been reported to be unaffected by exposure to azelaic acid in culture,⁸⁻¹¹ and Hu et al¹² have shown that neither iridial nor choroidal melanocytes of adult rhesus and cynomolgus macaques are damaged by exposure to azelaic acid in culture at concentrations toxic to B16 mouse melanoma cells. In the context of the above reports, it is obviously of interest to determine if azelaic acid has any effect on malignant choroidal melanocytes, and here we report results on a human choroidal melanoma cell line.

Fig. 1. Histograms to show the effect of C₂Na and C₀Na at concentrations of 10⁻² M and 5 × 10⁻² M over a 2-day period on the mean total growth of a human choroidal melanoma cell line expressed as a percentage of the initial cell count on day 0. *P = <0.001.
Fig. 2. Survey micrograph of cells of control choroidal melanoma culture. Melanosomes size and content varies from cell to cell. X1950. Bar = 1 μm.

Materials and Methods

Cell Cultures

Cells of human choroidal melanoma line OM 431, kindly provided by Professor D. Albert of Boston, were plated in 35 mm Petri dishes at approximately 1.8 × 10^5 cell/Petri dish. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The cells were grown in 3 ml of RPMI 1640 L. 2Na with 20 mm Hepes buffer (Gibco, Uxbridge, Middlesex, UK), supplemented with 10% fetal calf serum (heat-inactivated and mycoplasma-screened, from Gibco), 2 mM glutamine (Gibco), 100 IU/ml penicillin (Glaxo, Greenford, Middlesex, UK), 10 μg/ml streptomycin (Evans, Dunstable, UK) and 0.25 μg/ml fungizone (Gibco). After 24 hr incubation the medium was replaced with 3 ml of medium containing the appropriate drug concentrations.

Dicarboxylic Acids

The dicarboxylic acids used were adipic acid (C₆) and azelaic acid (C₇), added to the culture medium as disodium salts (C₆2Na and C₇2Na) to give final concentrations of 10⁻² M, 5 × 10⁻² M and 10⁻¹ M. Adipic acid has been shown to have little or no effect on tumoral cells at concentrations at which they are affected by azelaic acid, and its use therefore serves as an additional control for any effects due to the latter. The pH and osmolarity of all the media with added diacids were within physiological range.

Cell Counts

Petri dishes were inoculated with approximately 1.8 × 10^5 cells/dish and grown for an initial period of 24 hr. Cell counts were then done on randomly se-
Fig. 3. Cell of control culture for comparison with experimentals (Figs. 4–7). Few melanosomes were present in this section of the cell. X5878. Bar = 1 μm.

lected dishes to establish consistency of cell numbers per dish, and also to determine the initial cell count prior to drug exposure.

The culture medium was then replaced with medium containing C₂Na₂ and C₆Na₂ at concentrations of 10⁻² M and 5 × 10⁻² M, and incubation was continued. Attached cells were harvested after 2, 6, 24 and 48 hr of exposure to the diacid salts, and counted in a Coulter counter. Counts of parallel control cultures were also done. Reversibility of the drug effect after 48 hr drug exposure was investigated by replacing the diacid media with diacid-free media at 48 hr, and incubating for a further 48 hr before counting cells.

Throughout, duplicate counts were done for each Petri dish, and the whole experiment was performed in triplicate.

Viability Tests

Cells which detached after drug exposure, and those which remained attached to the Petri dish were separately tested for viability using the Trypan blue exclusion test. These cells were also incubated for a further 48 hr with diacid-free media and counted to investigate the reversibility of drug effect.

Analysis of Counts

Histograms (Fig. 1) were prepared to show the mean total growth of the choroidal melanoma line expressed as a percentage of the initial cell count prior to the addition of diacids. The raw data of counts were analyzed by students' t-test, with the addition of Bessel's correction to produce P-values for each count done as compared to parallel control figures.
Electron Microscopy

Confluent cultures of cells were exposed to each of the diacid salts at concentrations of $10^{-2}$ M, $5 \times 10^{-2}$ M and $10^{-1}$ M, for 1 and 6 hr. After incubation, the cells were washed in PBS and fixed for 5 min in 2.5% glutaraldehyde in 0.2 M cacodylate buffer, postfixed in 2% aqueous osmium tetroxide, and dehydrated in graded ethanols. At 70% ethanol, they were gently scraped off the Petri dish and spun down to a pellet. After complete dehydration, the pellet was embedded in Araldite resin, and thin sections, stained with uranyl acetate and lead citrate, were examined by electron microscopy.

Results

Cell Counts

Figure 1 shows the course of cell growth during 48 hr of exposure to the diacids as a percentage of initial cell counts before adding the drugs, in comparison to untreated control cultures.

Cells exposed to $C_6$ and $C_9$ at a concentration of $10^{-2}$ M behaved similarly to the controls over this period. A continuous increase in cell number to 120-140% of the initial counts after 24 hr and 175-200% at 48 hr was observed.

With $C_6$ at $5 \times 10^{-2}$ M, cells stayed at the same level of around 100% of initial count throughout the period. The number of cells exposed to $C_9$ at $5 \times 10^{-2}$ M fell significantly within 24 hr to 75% and to 33% at 48 hr. The reversibility of the diacid effect was tested on parallel cultures after 48 hr. After replacement of medium, with diacid-free medium, cells previously exposed to $C_6$ at $5 \times 10^{-2}$ M recovered and more than doubled their original number within 4 days. With $C_9$ at $5 \times 10^{-2}$ M, cells did not recover, but stayed at 30-40% of the initial counts over the period treated.
Fig. 5. Cell of culture exposed to $5 \times 10^{-2}$ M C92Na for 6 hr. Note gross swelling of mitochondria with disruption of internal cristae. Compare with Figures 6 and 7. X5878. Bar = 1 μm.

Electron Microscopy

Figure 2 is a survey micrograph of a control culture and Figure 3 an individual control cell for comparison with cells from experimental cultures (Figs. 4–7). The round, electron-dense organelles in these and other figures are melanosomes, the content of which varied considerably from cell to cell. Mitochondria, also particularly electron-dense in these cells, are smaller and more irregular in shape. Cells of cultures exposed to both diacids (C6 and C9) at $10^{-2}$ M for 1 hr were indistinguishable from controls. With C9 at $10^{-2}$ M for 6 hr a degree of mitochondrial swelling was apparent, and this was much more evident at $5 \times 10^{-2}$ M for 1 hr (Fig. 4), while at $5 \times 10^{-2}$ M for 6 hr, mitochondria were grossly swollen, with disruption of cristae, and loss of internal substance (Fig. 5).

With C6, by contrast, mitochondrial swelling and disruption were not apparent at $5 \times 10^{-2}$ M for 6 hr (Fig. 6), and even at $10^{-1}$ M the changes were minimal (Fig. 7).

Discussion

This study has shown that human choroidal melanoma cells in culture exhibit a similar susceptibility to the antiproliferative and antimitochondrial effects of azelaic acid, as do human and murine melanoma cells5–8 and cells of other tumoral lines.6,9 In a previous experiment,7 a significant effect of azelaic acid (C9) on human melanoma cells was first demonstrable at $5 \times 10^{-2}$ M for 24 hr, and the same result was found here; in both instances, exposure to adipic acid (C6) at the same concentration and time had no significant effect. At $5 \times 10^{-2}$ M for 48 hr a difference in effect was also apparent, the numbers of cells exposed to C9 being reduced to 20% of control, and those
exposed to C₆, to 60%. These observations underline the greater effect of C₉, and confirm biochemical experiments showing that antienzymatic activities of dicarboxylic acids against mitochondrial oxidoreductases are related to increasing chain length from C₆ to C₁₃.¹⁰

Murine melanoma cells in culture would appear to be more susceptible to C₉ than the two lines of human melanoma cells referred to above. Thus, proliferation of Harding Passey and Cloudman S91 cells was significantly affected after exposure to 10⁻² M for 24 hr. Geier et al.¹¹ have also demonstrated a significant difference in dose-effect ratio as between two human melanoma lines, and this possibility of variation in susceptibility should be borne in mind when comparing results from different centers employing different cell lines, and different periods of exposure.

The current results naturally raise the question of the possible use of azelaic acid as a chemotherapeutic agent for the treatment of uveal and ocular adnexal melanoma in vivo. Here, of course, topical application, which has proved successful with cutaneous lesions, is not in question. In our earlier investigations we combined oral administration with topical application and this led Willshaw and Rubinstein¹⁴ to try the effect of 12 g orally per day for 12 weeks on cases of choroidal melanoma. That this regimen had no effect became understandable following results of a study on the metabolism of azelaic acid.¹⁵ This showed that after oral administration, independent of dosage, 60% is excreted unchanged in the urine within 12 hr; serum concentration peaks between 2 and 3 hr after administration, reaching negligible levels after 8 hr. Clearly, with oral administration,
achieving a sufficient concentration of diacid and maintaining it over a period, at whatever lesional site, is a major problem. Direct intraocular injection would appear not to be feasible. We have administered the di-sodium salt of azelaic acid continuously by intravenous infusion for more than a week, and by intra-arterial injection, to desperate terminal cases of disseminated melanoma without any deleterious local or systemic effects. Nüssgen et al.16 have shown that intravenous injection of a 0.32 M solution of C₂₂Na into mice xenotransplanted with human melanoma was followed by a clear reduction of the mitotic index (M1) and of the autoradiographic ³H-thymidine labelling index within the tumor. The latter effect was greatest in the perivascular regions of tumoral vessels. It is possible, taking anatomical factors into consideration, that comparable systemic administration might be applied with good effect to human ocular melanoma for longer periods, and repeated after interruption. With this ultimate possibility in mind, the development and testing of modified, more active, and longer-acting analogues of the diacids are also being considered. An alternative approach to the problem of achieving adequate exposure of the intraocular melanoma to azelaic acid might be to conjugate the acid with a monoclonal antibody specific for such cells. Although still in an experimental phase, such modes of drug delivery, using the antibody as a homing device, are attracting considerable attention17,18 and may be feasible provided conjugation were chemically possible, while maintaining activity of the diacid.
Key words: azelaic acid, choroidal melanoma, cell culture

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References