DNA Damage and Selective Toxicity of Dopa and Ascorbate:Copper in Human Melanoma Cells

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ABSTRACT

Six of eight human melanoma lines showed increased sensitivity to killing by dopa and by ascorbate:copper compared with two fibroblast strains and four other human cell lines of nonmelanoma origin. Catechol, epinephrine, and α-methyldopa, but not 5,6-dihydroxyindole, exhibited a similar degree of selectivity. Toxicity was greatly reduced when brief exposure times or high cell densities were used. Depending upon culture conditions, melanoma cells accumulated more [3H]dopa- and [14C]ascorbate-derived isotopic label within the first five min than fibroblasts, but after one hr this difference was less marked. The catalase activity in melanoma cells was not less than that in fibroblasts. Using two independent methods to determine each type of damage, dopa and ascorbate:copper were found to induce DNA breaks in both cell types but not DNA repair synthesis or DNA interstrand cross-links. More DNA breaks were found in melanoma cells (two lines) than in fibroblasts. Semiconservative DNA synthesis was inhibited immediately, recovered within six hr, and in melanoma cells, was again inhibited after 24 hr. RNA synthesis was inhibited less than DNA synthesis. Human cell lines with differential sensitivity to γ-radiation, ultraviolet light, cross-linking agents, or monofunctional alkylating agents exhibited normal survival levels when treated with dopa or ascorbate:copper.

INTRODUCTION

Most attempts to develop chemotherapeutic agents specific for melanoma have involved exploitation of various stages of melanin biosynthesis: inhibition of enzymes (6, 23, 26, 32) and phenylalanine and tyrosine deprivation (6); or paradoxically, use of agents which are likely to enhance melanin production (23, 24). Dopa, for example, was reported (23, 24, 31, 33) to be selectively toxic in mouse and human melanoma cells compared with mouse L-cells and human fibroblasts, an effect in which the tyrosinase inhibitor 5,6-dihydroxyindole appeared to be an intermediate (24). Melanocyte-stimulating hormone either inhibits (23) or enhances (11, 27) the growth of melanoma cells. The combination of ascorbate and cupric ions has also been found to be melanoma selective, supposedly due to preferential uptake of ascorbate and/or copper followed by DNA damage (3). Apart from the question of melanoma selectivity, many reports have suggested that ascorbate and dopa, with or without copper, degrade DNA in vitro and are active against tumor cells in general (2, 4, 12, 17, 35, 36).

We have now extended the observations of melanoma selectivity and DNA damage in a variety of human cell types and have identified several properties of these systems which could affect their use in humans.

RESULTS

Cell Survival. The dose-survival responses were plotted as shown in Chart 1 because a semilogarithmic relationship was generally found and because the presence of DNA damage (see below) suggested analogy with the survival curves obtained using alkylating agents (10) and UV (20). To allow for occasional nonlinearity, comparisons of survival are presented using the $D_{50}$ value (Table 1). However, $D_{50}$ (dose required to reduce survival by a factor of 0.37 on the linear part of the survival curve) values (not shown), indicative of the slope of the survival curve, showed the same qualitative result. By these criteria, 6 of 8 allogeneic melanoma lines were more sensitive to killing by dopa than 4 other nonmelanoma cell lines and 2 fibroblast strains (Chart 1; Table 1). With the exception of the dopa-sensitive line MM 127, the order of sensitivity to ascorbate:copper was the same as for dopa (Table 1). The colony-counting method for determination of cell survival gave essentially the same results (Table 1). The pattern of sensitivity among the melanoma lines did not correlate with differences in other properties reported previously, such as growth rate, chromosome number, degree of melanization, or resistance to other drugs (18, 25). Melanoma sublines resistant to killing by monofunctional agents

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MATERIALS AND METHODS

The DJM, ST, and GM 2250 human lymphoblastoid cell lines were provided by Dr. P. Imray of this institute. The human melanoma sublines MM253c1-3D and MM253c1-40G were derived by repeated treatment of the parent line (MM253c1; Ref. 19) with microsome-activated 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide or microsome-activated cyclophosphamide, respectively. The human fibroblast strains MBP (female) and PGP (male) were derived from skin biopsies of adults. The origin of the other cell types has been described (10, 20, 21, 25).

Cells were cultured in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Commonwealth Serum Laboratories, Melbourne, Australia) containing 10% fetal calf serum, 100 IU penicillin per ml, 100 μg streptomycin per ml, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Assays for Mycoplasma (21) were negative. Cell lines were used 24 hr after passage. Unless otherwise stated, cell survival was determined by a modified clonal assay (10), involving treatment of $2 \times 10^5$ cells/16-mm Linbro well and pulse labeling of colonies with $[3H]$thymidine 6 to 7 days after treatment. The colony-counting method was carried out using duplicate 60-mm plates seeded with $2 \times 10^5$ cells 24 hr before treatment and stained with Giemsa for visual counting of colonies (more than 50 cells/colony) 7 to 14 days after treatment. No correction for initial cell multiplicity (10) was required.

5,6-Dihydroxyindole was synthesized by the method of Axelrod and Lerner (1). Stock solutions of CuSO4 were prepared in water. The other compounds were dissolved in complete medium, and the pH was returned to 7.2 if required. All solutions were used immediately.
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(MM253c1-3D) or cross-linking agents (MM253c1-4CG) exhibited the same D50 with dopa or ascorbate:copper as did the control cell line (MM253c1). Lymphoblastoid cell lines sensitive to γ-radiation (ST) or UV (GM 2250) were somewhat more resistant to the above agents than the control line (DJM).

At drug levels of 5 to 10 times the D50, cells became rounded and detached from the surface after 2 hr of treatment. This contrasted with the ability of the same cells to remain attached for long periods after treatment with supralethal doses of γ-rays and, to a lesser extent, of alkylating agents and UV. When the treatment was limited to 2 hr, much higher drug levels were required to achieve the same degree of cell killing (Chart 1, C and D), but the differential between MM96 and PGP was maintained.

Preliminary studies showed that the toxicity of these compounds decreased when initial cell densities above 10^5/16-mm well were used. To investigate this effect and to compare cell survival using an independent method, growth was followed at 2 different cell densities for periods of up to 17 days (Chart 2). The melanoma line MM96 again showed enhanced sensitivity to dopa and to ascorbate:copper, as judged by earlier inhibition of growth and by the lower drug level required to do so. In both cell types, a 10-fold increase in cell density greatly reduced toxicity, with respect to both the timing and degree of growth inhibition.

The selectivity of dopa for melanoma cells applied to other

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**Chart 1.** Time and dose responses of survival of human cells treated with dopa or ascorbate:copper. A, dopa (continuous treatment); O, PGP fibroblasts; □, MM96 melanoma cells; ◇, MM253c1. B, ascorbate:5 μM copper (continuous treatment); O, PGP; □, MM96; ◇, MM253c1; ascorbate alone; O, PGP; ■, MM96. C, dopa (2-hr treatment); symbols as in A. D, ascorbate:10 μM CuSO4 (2-hr treatment); symbols as in B.

**Chart 2.** Growth of cells treated with dopa or ascorbate:copper. Cultures seeded at 2 different cell densities (10^4 or 10^5/60-mm plate) were treated with the drug initially and whenever the medium was changed (every 3 to 4 days). When confluent, cultures were reseeded at 10^4 or 10^5 cells/plate and the treatments were continued. Growth (number of cells per plate) is expressed as a percentage of control cultures harvested at the same time. A, 10^4 PGP fibroblasts/plate treated with 20 (△), 50 (○), or 200 [ ■] μM dopa and 10^4 MM96 cells/plate treated with 20 (△), 50 (○), or 200 [ ■] μM dopa. B, symbols as in A except that the initial cell density was 10^5/plate. C, 10^4 PGP fibroblasts/plate treated with a mixture of 5 μM CuSO4 and 50 μM ascorbate (△), 200 μM ascorbate (○), or 500 μM ascorbate (■) and 10^4 MM96 cells/plate treated with a mixture of 5 μM CuSO4 and 50 μM ascorbate (△), 200 μM ascorbate (○), or 500 μM ascorbate (■). D, symbols as in C except that the initial cell density was 10^5/plate.

**Table 1.** Toxicity of dopa and ascorbate:copper in human cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>D50 (μM)</th>
<th>Ascorbate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fibroblasts</strong></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>MBP</td>
<td>40 ± 2^c</td>
<td>45 ± 10</td>
</tr>
<tr>
<td>PGP</td>
<td>30 ± 8</td>
<td>10 ± 3</td>
</tr>
<tr>
<td><strong>Melanoma</strong></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>MM96</td>
<td>5.8 ± 1</td>
<td>4.0 ± 1</td>
</tr>
<tr>
<td>MM127</td>
<td>8.2 ± 0.9</td>
<td>54 ± 12</td>
</tr>
<tr>
<td>MM138</td>
<td>2.3 ± 0.3</td>
<td>2.5</td>
</tr>
<tr>
<td>MM170</td>
<td>6.5 ± 4</td>
<td>2.0</td>
</tr>
<tr>
<td>MM200</td>
<td>9.5 ± 2.5</td>
<td>3.5 ± 1.5</td>
</tr>
<tr>
<td>MM214</td>
<td>26 ± 8</td>
<td>35</td>
</tr>
<tr>
<td>MM229</td>
<td>4.0 ± 2</td>
<td>16</td>
</tr>
<tr>
<td>MM253c1</td>
<td>19 ± 6</td>
<td>50 ± 10</td>
</tr>
<tr>
<td>MM253c1-3D</td>
<td>17</td>
<td>57</td>
</tr>
<tr>
<td>MM253c1-4CG</td>
<td>15</td>
<td>43</td>
</tr>
<tr>
<td><strong>Lymphoblastoid</strong></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>DJM (normal donor)</td>
<td>29 ± 10</td>
<td>38 ± 12</td>
</tr>
<tr>
<td>ST (ataxia telangiectasia)</td>
<td>96 ± 2</td>
<td>100</td>
</tr>
<tr>
<td>GM 2250 (xeroderma pigmentosum)</td>
<td>104</td>
<td>130</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>HeLa-S3</td>
<td>100</td>
<td>40</td>
</tr>
</tbody>
</table>

^a Treatments were continuous.
^b CuSO4 (5 μM) added.
^c Mean ± S.E.
^d Numbers in parentheses, number of experiments.
^e D50 = 100 μM, determined by colony count.
^f D50 = 7.5 μM, determined by colony count.
o-dihydroxy compounds, with the exception of 5,6-dihydroxindole (Table 2). Of the 4 melanoma lines tested, the MM138 cells were the most sensitive; the dopa-resistant MM253c1 line was also quite susceptible to killing by these agents.

Attempts to determine the toxicities of ascorbate and copper separately were only partly successful. Ascorbate alone was much less toxic than the combination, with some selectivity for melanoma cells (Chart 4). Copper alone, however, gave variable $D_{50}$ values for MM96 (5 to 100 $\mu M$), depending possibly on small variations in initial cell density and on the particular batch of fetal calf serum. To maintain reproducible levels of toxicity therefore, only the combination was used in the following studies.

Catalase Activity. The suggestion that tumor cells may be susceptible to ascorbate killing because of catalase deficiency (15) prompted a study of this enzyme in some of the cell types used. Melanoma cells were found to have an equal or higher catalase activity per cell compared with fibroblasts, whether released by hypotonic, freezing, or detergent treatment (Table 3). Small cells such as MM96 and MM127 had a much higher catalase activity than fibroblasts compared on a volume basis. The catalase inhibitor 3-amino-1,2,4-triazole (2) was equally effective for inhibition of the enzyme in melanoma cells and fibroblasts.

Uptake of Dopa and Ascorbate. The rate of drug uptake was studied in PGP and MM96 at a single concentration (100 $\mu M$) and using a large excess of medium to avoid significant depletion of the extracellular drug level. MM96 cells attached to a plastic surface accumulated 3-fold more $[14C]$ascorbate than PGP after 5 min but by 15 min apparently lost most of this activity (Chart 3A). Although further washing of the monolayers had little qualitative effect on these results, parallel experiments were also carried out using cell suspensions for which the washing of aliquots on filters was more rapid and reproducible. The same difference in drug transport was found, even though the intracellular levels of $[14C]$ascorbate were lower for both cell types (Chart 3C).

Attached cells accumulated dopa less readily than ascorbate, with no significant difference between PGP and MM96 (Chart 3B). In suspension, however, MM96 took up almost 2-fold more dopa than PGP within 5 min and maintained this difference for the 1-hr period studied. Cell monolayers kept at 0° for 15 min accumulated the same level of dopa or ascorbate as cells at 37° (Chart 3). This was also the case for suspensions of MM96. PGP fibroblasts, on the other hand, exhibited very slow uptake of either drug when kept in suspension at 0°. The same difference in drug transport was found, even though the intracellular levels of $[14C]$ascorbate were lower for both cell types (Chart 3C).

DNA and RNA Synthesis. In these and subsequent experiments, high drug doses were used to achieve toxicity for MM96 because high cell densities and brief treatment times were needed to follow the biochemical changes. DNA synthesis was inhibited almost immediately, to an extent which correlated approximately with the degree of toxicity expected (Chart 4A). Recovery from dopa treatment occurred in both cell types within 6 hr, but by 24 hr, DNA synthesis in MM96 cells was again inhibited. PGP fibroblasts, but not MM96, recovered slowly from ascorbate:copper treatment. Because of the diffi-
Chart 5. DNA and RNA synthesis. Cultures seeded 24 hr previously \((5 \times 10^4\) cells/16-mm Linbro well) were treated for 2 hr. At various times after initiation of treatment, duplicates were pulsed for 45 min with [2-\(^{14}\)C]thymidine \((0.5 \mu\text{Ci/ml; 25 Ci/mmol})\) mixed with \([5-\text{H}]\)uridine \((10 \mu\text{Ci/ml; 42 Ci/mmol})\). The cells were detached with trypsin and washed onto glass fiber discs with water prior to liquid scintillation counting. The results are expressed as a percentage of controls pulsed at the same time. A, DNA synthesis; C, PGP fibroblasts treated with dopa \((3 \text{ mM})\); 2, PGP fibroblasts treated with ascorbate \((200 \mu\text{mM})/\text{CuSO}_4 \((10 \mu\text{mM})\); E, MM96 treated with dopa \((3 \text{ mM})\); 3, MM96 treated with ascorbate \((200 \mu\text{mM})/\text{CuSO}_4 \((10 \mu\text{mM})\). B, RNA synthesis; same symbols as A. Points, means of duplicates. All standard deviations were less than 10%.

As an independent test for DNA interstrand cross-linking, DNA samples were also subjected to a renaturation assay (Table 4). Cells treated with melphalan, an interstrand cross-linking agent \((19)\), showed high levels of renaturation whereas dopa and ascorbate: copper treatment resulted in slightly lower renaturation levels than the controls. Simultaneous treatment with dopa and melphalan gave a renaturation level only slightly less than that from melphalan alone. In these assays, the DNA was first heated at pH \(10.5\) to fix cross-links involving purines.

DNA repair synthesis \((9, 19)\) could not be detected following dopa or ascorbate: copper treatment, as judged by autoradiography \(<10\) grains/ lightly labeled nucleus) or by increased incorporation of [\(^{3}\text{H}\)]thymidine in the presence of hydroxyurea \(<10\%\) above controls). UV \((10 \text{ J/sq m})\), a long-patch repair agent \((19)\) used to demonstrate the efficiency of these techniques in both cell types, induced 30 to 100 grains/ lightly labeled nucleus and 150 to 200\% enhancement of [\(^{3}\text{H}\)]thymidine incorporation.
A urine sample was taken and the patient was started on a high-protein diet and hydration. The patient did not respond to initial treatment, and the condition worsened. The patient's condition deteriorated further, and the patient died 10 days after admission.

The patient's death is being investigated further, and the family has been informed of the results. The patient's family has requested that the case be reviewed by an independent expert in critical care medicine.
cate that melanization per se may not be central to that action of these drugs. Genetic and hormonal control of the factors (22) regulating postdopa stages of melanization offers a plausible although as yet untested explanation for the above differences and for variation between melanoma cell lines used in the present study. Other catechols are also effective (33, 34) and in this work appeared to be more selective than dopa, raising the possibility of obtaining more effective derivatives. It should be noted, however, that selectivity for melanoma as distinct from other tumors has not yet been demonstrated for dopa or ascorbate in vivo.

This study found 2 properties of the dopa and ascorbate: copper systems which may limit therapeutic approaches: loss of toxicity using high cell densities or short treatment times. In addition, administration of dopa to melanoma patients may be inadvisable (29), and experimental studies using guinea pigs showed that tumor growth was inhibited by an ascorbate-deficient diet but not by normal or elevated ascorbate levels (14). Redox systems of even higher selectivity may therefore be required for successful application of the phenomenon in humans.

REFERENCES

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