The Activity of Pyridine Nucleotide-Cytochrome c Reductases, Cytochrome Oxidase, and Diaphorase in Epidermis in Various Stages of Malignant Transformation

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In a recent publication Reynafarje and Potter found an interesting difference between normal liver and hepatoma cells (14). These investigators showed that the activity of DPN1-TPN1 transhydrogenase and TPNH-cytochrome c reductase was absent or present only in negligible amounts in homogenates and cell fractions of the Novikoff hepatoma, whereas in liver DPN-TPN transhydrogenase was found limited to the mitochondrial membranes, and TPNH-cytochrome c reductase was located in the microsomes and mitochondria fractions. It would be interesting to study changes of a similar nature in the epidermis of mice undergoing carcinogenesis, since the following stages of malignant transformation can be examined: normal untreated epidermis, early and late hyperplastic epidermis, papillomas, and squamous-cell carcinomas (2). This report deals with the determination of the activity of TPNH-cytochrome c reductase, DPNH-cytochrome c reductase, cytochrome oxidase, and diaphorase in the above-mentioned stages of epidermal carcinogenesis.

MATERIALS AND METHODS

Swiss mice, 3–4 months of age, were used for the isolation of normal epidermis and for the preparation of hyperplastic epidermis, papillomas, and squamous-cell carcinomas (2). The latter three stages were induced following the application of a 0.3 per cent MC solution in benzene with a camel’s-hair brush No. 4 to the entire shaved back of mice 3 times weekly. Immediately after removal, normal and hyperplastic epidermis (1), papillomas, and carcinomas were placed into small glass homogenizers (Ten Broeck) surrounded by crushed ice. Connective and necrotic tissues were removed as completely as possible from the carcinomas. A small piece of each carcinoma was saved for histological examination. The tissues were then homogenized by hand in a few (2–5) ml of cold water. For some of the cytochrome oxidase determinations the tissues were homogenized directly in 0.25, 0.5, and 0.75 per cent sodium deoxycholate solutions (8). Portions of each homogenate were removed for total nitrogen determinations by the Kjeldahl method. The remainder of each homogenate was spun at 1800 r.p.m. for 10 minutes, and the enzyme activity and total nitrogen of the supernatant fractions were determined. Some of the supernatant fractions employed for cytochrome oxidase activity assay were diluted with sodium deoxycholate solution.

Reaction mixtures and assay procedures employed were as follows:

For cytochrome oxidase activity, a total volume of 3.0 cc contained 0.2 ml of 0.17 M sodium phosphate buffer, pH 7.4; 0.4 ml 1.7 × 10^-4 M reduced cytochrome c; and 0.01–0.1 cc of sample in water or in sodium deoxycholate. The volume was adjusted to 3.0 cc with H2O or sodium deoxycholate to make a 0.75 per cent solution. The cytochrome oxidase activity was measured in the water- and sodium deoxycholate-cleared preparations (8) by the macro procedure of Cooperstein and Lazarow (4) by measuring the change in optical density at 550 μm every 15 seconds in a Beckman model DU spectrophotometer.

For the assay of TPNH- and DPNH-cytochrome c reductase activity, 3.0 ml of reaction mixture containing the following materials in the

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concentrations indicated were used: 0.027 M nicotinamide; $2 \times 10^{-4}$ M NaCN; 0.033 M phosphate buffer, pH 7.4; 1 mg. cytochrome c; 213 µg. TPNH and 0.1–0.4 ml. sample; 300 µg. DPNH and 0.03–0.3 ml. sample; H₂O to make final volume of 3.0 ml. The activity of these enzymes was measured from the rate of reduction of cytochrome c at 550 mg every 15 seconds in the model DU Beckman spectrophotometer according to the procedures of Hogeboom (9) and Hogeboom and Schneider (10, 11) as employed by De Duve et al. (6). The reduction of cytochrome c upon the addition of DPNH or TPNH was used as measurement of the activity of DPNH- and TPNH-cytochrome c reductases, respectively. The amount of

### RESULTS AND DISCUSSION

The results on the cytochrome oxidase activity of epidermis and its various MC-induced transformations are given in Table 1. The specific cytochrome oxidase activity for both the homogenate and supernatant fraction was determined on a nitrogen basis, since there is an increase in the water content (2) and a decrease in the viscosity of epidermal cells undergoing carcinogenesis (5). It is possible that the increased softness of this tissue resulting from these changes facilitates the disruption of the cytochrome oxidase complex from the epidermal cells giving rise to the observed increase in its activity in the carcinomas. Normal and MC-treated epidermis and papillomas had comparable

<table>
<thead>
<tr>
<th>No. of applications of 0.5 per cent MC</th>
<th>Specific cytochrome oxidase activity of homogenates Na deoxycholate</th>
<th>Specific cytochrome oxidase activity of supernatants Na deoxycholate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (6)</td>
<td>0.9 ± 0.3</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>B (2)</td>
<td>2.7 ± 0.2</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>C (4)</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.7</td>
</tr>
<tr>
<td>D (5)</td>
<td>3.7 ± 1.0</td>
<td>4.2 ± 1.0</td>
</tr>
<tr>
<td>E (1)</td>
<td>0.9 ± 0.2</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>F (3)</td>
<td>1.1 ± 0.5</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>G (1)</td>
<td>1.2 ± 0.4</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>H (2)</td>
<td>1.5 ± 0.5</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>I (4)</td>
<td>1.4 ± 0.5</td>
<td>4.2 ± 1.0</td>
</tr>
<tr>
<td>J (3)</td>
<td>1.2 ± 0.5</td>
<td>4.7 ± 1.0</td>
</tr>
<tr>
<td>K (1)</td>
<td>1.1 ± 0.5</td>
<td>5.3 ± 1.7</td>
</tr>
<tr>
<td>L (3)</td>
<td>1.2 ± 0.5</td>
<td>6.7 ± 1.7</td>
</tr>
<tr>
<td>M (1)</td>
<td>1.0 ± 0.1</td>
<td>7.1 ± 1.7</td>
</tr>
<tr>
<td>N (2)</td>
<td>1.1 ± 0.1</td>
<td>8.4 ± 1.0</td>
</tr>
</tbody>
</table>

* Number of samples analyzed indicated in parentheses; epidermis removed 5 days after last MC application.

† Δ log (ferrocytochrome c)/min/mg nitrogen (4).

‡ Papillomas were highly keratinized.

A, B, C carcinomas were highly differentiated.

reduced cytochrome c oxidized by cytochrome oxidase and the amount of cytochrome c reduced by DPNH- and TPNH-cytochrome c reductases were calculated from the procedure of Cooperstein et al. (4).

For diaphorase activity the reaction mixture contained in a volume of 3.0 ml. the following: 0.033 M phosphate buffer, pH 7.4; $2 \times 10^{-4}$ M NaCN; 0.027 M nicotinamide; 300 µg. DPNH; 15.2 µg. 2,6-dichlorobenzenoneindophenol; 0.03–0.2 cc. of sample; and water to make 3.0 ml. The reduction in optical density at 600 mg of the dye was measured every 15 seconds in the model DU Beckman spectrophotometer.

Activity measurements of the enzymes were usually determined on at least two different tissue concentrations.

TPNH, DPNH, and cytochrome c (heart) were purchased from the Sigma Chemical Company.

cytochrome oxidase activities whether determined in water or 0.75 per cent sodium deoxycholate and whether expressed on a homogenate or supernatant nitrogen basis. The increase in cytochrome oxidase activity of the carcinomas, as compared with normal and MC-treated epidermis, is in contrast to the general finding that most tumors have less of this enzyme than do their tissues of origin (7).

The cytochrome oxidase activity of normal and MC-treated epidermis homogenized directly in sodium deoxycholate showed no consistent difference (Table 2). However, the cytochrome oxidase activity of late hyperplastic epidermis was nearly twice that of early hyperplastic epidermis, as determined by Warburgy manometry (8).

The specific activity of TPNH-cytochrome c reductase was rather low and fairly constant in hyperplastic epidermis and in several carcinomas.
TABLE 2

<table>
<thead>
<tr>
<th>Specific cytochrome oxidase activity of homogenates in Na deoxycholate</th>
<th>Specific cytochrome oxidase activity of supernatants in Na deoxycholate</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. applications of cent MC</td>
<td>(per cent)</td>
</tr>
<tr>
<td>0.75</td>
<td>0.2</td>
</tr>
<tr>
<td>0</td>
<td>2.1</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
<td>4.1</td>
</tr>
<tr>
<td>22</td>
<td>1.8</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
</tr>
<tr>
<td>22</td>
<td>1.8</td>
</tr>
<tr>
<td>50</td>
<td>2.1</td>
</tr>
<tr>
<td>90</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*For each concentration of Na deoxycholate the epidermis of five normal mice (epidermis removed 5 days after last MC application) were homogenized for analysis.

† Δ log (ferrocytochrome c)/min/mg nitrogen (4).

TABLE 3

<table>
<thead>
<tr>
<th>No. applications of</th>
<th>Specific activity† of TPNH-cytochrome c reductase in homogenate and supernatant</th>
<th>Specific activity† of DPNH-cytochrome c reductase in homogenate and supernatant</th>
<th>Specific activity† of diaphorase in homogenate and supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0(6)</td>
<td>Trace only</td>
<td>Trace only</td>
<td>Trace only</td>
</tr>
<tr>
<td>3(2)</td>
<td>0.06 ± 0.05</td>
<td>0.13 ± 0.04</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>6(2)</td>
<td>0.06 ± 0.05</td>
<td>0.19 ± 0.04</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>12(5)</td>
<td>0.08 ± 0.01</td>
<td>0.25 ± 0.05</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>18(5)</td>
<td>0.09 ± 0.01</td>
<td>0.27 ± 0.04</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>22(2)</td>
<td>0.09 ± 0.01</td>
<td>0.16 ± 0.02</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>25(2)</td>
<td>0.06 ± 0.01</td>
<td>0.19 ± 0.04</td>
<td>0.31 ± 0.05</td>
</tr>
<tr>
<td>30(2)</td>
<td>0.06 ± 0.01</td>
<td>0.19 ± 0.04</td>
<td>0.30 ± 0.05</td>
</tr>
<tr>
<td>Papillomas</td>
<td>0.45 ± 0.20</td>
<td>0.12 ± 0.02</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Papillomas</td>
<td>0.02 ± 0.00</td>
<td>0.03 ± 0.05</td>
<td>0.03 ± 0.05</td>
</tr>
<tr>
<td>A Carcinomas</td>
<td>0.06 ± 0.05</td>
<td>0.13 ± 0.05</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td>B Carcinomas</td>
<td>0.06 ± 0.05</td>
<td>0.13 ± 0.05</td>
<td>0.7 ± 0.05</td>
</tr>
</tbody>
</table>

*Number of samples analyzed indicated in parentheses; epidermis removed 5 days after last MC application.

† Δ log (ferrocytochrome c)/min/mg nitrogen (4).

‡ Δ log (reduced 2,6-dichlorobenzenoindophenol)/min/mg nitrogen.

The activity of DPNH-cytochrome c reductase of normal epidermis was about twice that of hyperplastic epidermis, papillomas, and some of the carcinomas (group B) whether the specific activity was expressed on a homogenate or supernatant basis (Table 3). One group (A) of highly differentiated carcinomas had a DPNH-cytochrome c reductase activity that was about the same as for normal epidermis. Reynafarje et al. showed that rat liver microsomes contained nearly 5 times the activity of DPNH-cytochrome c reductase per gm. of protein as did these particulates from the Novikoff hepatoma on the same basis of reference (14). Hogeboom and Schneider, on the other hand, showed that the specific activity of this enzyme in the mitochondria and microsomes of hepatoma 98/15 was several times greater than that of these particulates from normal mouse liver (11). Lenta and Riehl found that homogenates of mouse liver and hepatoma 98/15 had about the same DPNH-cytochrome c reductase activity and that the activity of this enzyme in the hepatoma was much greater than that measured in Sarcoma 37 and a mammary adenocarcinoma (12). Studies by Reif et al. demonstrated that homogenates of heart, liver, and kidney contained 2–4 times the DPNH-cytochrome c reductase activity of spleen, the Flexner-Jobling, Jensen, Walker, or Ehrlich tumors (13).

Diaphorase activity showed no significant change in the various stages of epidermis undergoing carcinogenesis (Table 3). Lenta and Riehl also found that the diaphorase activity of hepatoma 98/15 was only slightly less than that of normal mouse liver (12).

The results obtained on the activity of the enzymes studied above in the carcinogenesis of epidermis indicate that additional work on a variety of normal tissues and tumors is needed before a generalization can be made. This view has also been expressed by Reynafarje and Potter (14).

**SUMMARY**

1. Pyridine nucleotide-cytochrome c reductases, cytochrome oxidase, and diaphorase have...
been estimated in homogenates prepared from normal mouse epidermis and in this tissue in various stages of malignant transformation.

2. TPNH-cytochrome c reductase activity was absent or present in traces in normal mouse epidermis; low activities of similar magnitude were found in hyperplastic epidermis, papillomas, and carcinomas.

3. Normal mouse epidermis and about three-fourths of the squamous-cell carcinomas examined contained about twice as much DPNH-cytochrome c reductase activity as did hyperplastic epidermis and papillomas.

4. The diaphorase activity of normal and hyperplastic epidermis, papillomas, and carcinomas was about the same.

5. The cytochrome oxidase activity of the majority of squamous-cell carcinomas examined was greater than that found in normal and hyperplastic epidermis or in papillomas.

REFERENCES


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