Altered Biochemical Properties of Mitochondria in Mouse Mammary Epithelial Cells during Primary Culture

MICHAEL T. WHITE 1 AND S. NANDI
Cancer Research Laboratory and Department of Zoology, University of California, Berkeley, California 94720

ABSTRACT Various biochemical properties of mitochondria isolated from primary monolayer cultures of mammary epithelial cells from mid-pregnant or hormonally stimulated mice were examined daily for seven or eight days. When compared with mitochondria from mammary glands of mid-pregnant animals, the specific activities of several mitochondrial enzymes were greatly reduced in cells after seven days in culture. There was a 5- to 6-fold reduction in the specific activities of cytochrome oxidase, succinate dehydrogenase and α glycerophosphate oxidase while malate dehydrogenase and adenylate kinase activities were 2- to 3-fold lower. The reduction in mitochondrial enzyme activities was gradual and related to the length of time the cells were in culture. Progressive changes were also seen in the electrophoresis profiles of mitochondrial proteins in SDS-urea polyacrylamide gels. Mitochondria isolated from 1-, 2-, 3- and 8-day cell cultures showed a continuous reduction in the relative amounts of several mitochondrial polypeptides in the gel profiles. Addition of 35S-methionine to cell cultures for short and long periods indicated that mitochondrial protein synthesis continued throughout the 8-day culture period. However, the synthesis of several particular polypeptides was reduced progressively during the culture period. These studies indicate that mouse mammary epithelial cells have a lowered capacity for respiratory metabolism as a result of specific mitochondrial alterations which might be associated with the general loss of differentiated morphology by those cells during monolayer culture.

Normal and neoplastic mammalian cells in culture commonly show high rates of aerobic glycolysis as measured by lactate production (Gregg et al., '68; Gregg, '72). Undoubtedly, many factors, such as medium composition, culture pH, growth rate and cell density influence the level of aerobic lactate production. In cultures of normal mouse lymphocytes, Wang et al. ('76) directly linked glycolysis with cell proliferation. Glycolysis and respiration often tend to vary inversely (Paul, '65). Bissell et al. ('72) examined the relationship between lactate and CO₂ production in sparse, fast growing and dense, slow growing cultures of chick fibroblasts. They concluded that population density and not the growth rate determined the relative amounts of glycolysis and respiration in these cultures. Most studies on glucose metabolism in mammalian cell culture have dealt with fibroblasts, lymphocytes, established cell lines and transformed cells. Comparatively little is known about the metabolic properties of epithelial cells in primary culture.

In culture, epithelial cells from the mammary glands of mid-late pregnant mice rapidly lose many of the cytological features which characterize the developed glands in situ (Pickett et al., '75). These cells also showed high levels of lactate production which were seemingly independent of cell density and proliferation (White and Nandi, '77). In this paper, we examined the respiratory ability of mouse mammary epithelial cells as a function of time and loss of differentiated morphology during culture. We focused on the mitochondria of these cells and analyzed the specific activities of several key respiratory enzymes, the total protein composition and the patterns of new protein synthesis in mitochondria of...
cells after various periods in culture. These studies indicate that mouse mammary epithelial cells have a lowered capacity for respiratory metabolism as a result of specific mitochondrial alterations which might be associated with the general loss of differentiated functions by these cells during monolayer culture.

MATERIALS AND METHODS

Cell culture

Mammary gland tissue was excised aseptically from midpregnant BALB/cCrg1 mice or animals hormonally stimulated for five weeks with subcutaneously implanted pellets of 17\(\beta\) estradiol, deoxycorticosterone acetate as described previously (White et al., '77). Minced tissue was suspended in 0.1% collagenase (Type I1, 145 units/mg, Worthington Biochemical, Freehold, New Jersey) in Hank's balanced salt solution (BSS) (Gibco, Santa Clara, California) and rotated at moderate speed in a shaking 37°C water bath for 90 minutes. After centrifugation at 180 \(\times\) g for five minutes, the supernatant was discarded and the cell pellet was resuspended in 0.05% pronase (B grade, Calbiochem, La Jolla, California) and agitated for 30 minutes at 37°C. The suspension was centrifuged at 180 \(\times\) g for five minutes, the supernatant was discarded and the cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal calf serum (Pacific Biologicals, Richmond, California) and filtered through single, then through double layered 150-\(\mu\) mesh Nytex cloth (Tetko, Elmsford, New York). The cells were washed twice with Hank's BSS, scraped from the dish with a teflon spatula and centrifuged at 180 \(\times\) g for five minutes. The cell pellet was resuspended in a small volume (less than 5 ml) of cold sucrose medium (SM) (0.25 M sucrose, 2 mM EDTA and 50 mM Tris-HCl, pH 7.4) containing 1% (w/v) fraction V bovine serum albumin (BSA) (Pentex, Miles Labs., Elkhart, Indiana). The cells were disrupted in a 7-ml glass dounce (100 strokes, pestle A Bellco Glass, Vineland, New Jersey). The homogenate volume was adjusted to 25 ml with BSA-SM and centrifuged at 500 \(\times\) g for ten minutes at 5°C. The supernatant was recentrifuged at 500 \(\times\) g for ten minutes and 8,700 \(\times\) g for fifteen minutes to obtain a crude mitochondrial pellet. This fraction was resuspended in 25 ml BSA-SM and centrifuged at 8,700 \(\times\) g for 15 minutes. The partially purified mitochondrial pellet was resuspended in a small volume of BSA-SM, layered onto a 15-ml linear sucrose gradient (1.03-1.71 M sucrose in 2 mM EDTA, 25 mM Tris- HCl, pH 7.4), and centrifuged to equilibrium (2 hours at 25,000 rpm in a Beckman SW 27.1 rotor) at 5°C. The mitochondrial band was removed, resuspended in SM at a final sucrose concentration of 0.33 M and centrifuged at 10,000 \(\times\) g for 20 minutes at 5°C. The purified mitochondrial pellet was resuspended in a small volume of SM and assayed for enzymatic activity or treated for analysis of polypeptides by gel electrophoresis.

Enzyme assays

We assayed cytochrome oxidase spectrophotometrically by following the oxidation of reduced cytochrome C (equine heart Type VI, Sigma Chemical Co., St. Louis, Missouri) as described by Smith ('55). Malate dehydrogenase was assayed by the method of Ochoa ('55), adenylate kinase was assayed according to Schnaitman and Greenawalt ('68) and NADH oxidase was assayed spectrophotometrically observing the oxidation of NADH using cytochrome C as terminal electron acceptor (Green and Ziegler, '63). The test for monoamine oxidase followed the method of Wurtman and Axelrod ('63) with \(^{14}\)C-tryptamine (47 mCi/mmol, New England Nuclear, Boston, Massachusetts) as substrate. We assayed \(\alpha\) glycerophosphate oxidase (the mitochondrial form of \(\alpha\) glycerophosphate dehydrogenase) and succinate dehydrogenase as described by O'Brien and Gethmann ('73), which spectrophotometrically follows the reduction of p-iodonitrotetrazolium violet. All
enzymes were assayed for maximum activity which included whenever noted mitochondrial disruption by treatment with the nonionic detergent Lubrol WX (0.2 mg/mg mitochondrial protein, ICI America, Charlotte, North Carolina) before enzyme assay. This was of particular importance when the substrates of certain assays (ex. NADH in NADH oxidase) were not able to penetrate intact mitochondria.

Protein was determined by the method of Lowry et al. ('51) with BSA as a standard.

Polyacrylamide gel electrophoresis

The dissociation and electrophoresis of mitochondrial proteins were based on the method described by Maizel et al. ('68). Samples of sucrose gradient purified mitochondria (~50 µg protein each) were dissolved in urea, SDS and β mercaptoethanol (5 M, 1.0% and 0.2% respectively) by heating at 95°C for five minutes and layered onto gels after cooling. The gels, set in either plexiglass tubes (6 mm I.D.) or between glass plates as slabs 0.75 mm thick were composed of 10% acrylamide and 0.27% bisacrylamide (BioRad Labs., Richmond, California) in 4 M urea, 0.01 M EDTA, 0.1% SDS, 0.1% N,N,N',N'-tetramethylethylenediamine and 0.05 M Na phosphate buffer (pH 7.2). Polymerization was catalyzed with 0.5% ammonium persulphate. Tube gels were electrophoresed at 5 milliamperes (MA) per gel and slab gels at 12 MA/gel for 22 hours at room temperature in 0.05 M Na phosphate buffer (pH 7.2) containing 0.1% SDS and 0.01 M EDTA. The gels were fixed, stained with Coomassie blue and destained by the method of Smith et al. ('69). The gels were photographed and the negatives scanned in a densitometer. Polypeptide molecular weights were determined by coelectrophoretic standardization using murine mammary tumor virus (MuMTV) polypeptides (Dickson and Skehel, '74). In all cases where mitochondrial polypeptide gel profiles were compared, the samples were coelectrophoresed in the same chamber and approximately equal amount of protein was loaded into each gel.

Mitochondrial protein synthesis

Radioactively labelled mitochondrial proteins were obtained by growing mammary gland cells in culture for various periods in nutrient medium (DMEM + 10% FCS + 1 + PS) containing 1 µCi/ml 35S methionine (250 Ci/m mole, New England Nuclear). The labelling periods were as follows: the first 18 hours after plating; from the second to the third day of culture and from the seventh to the eighth day of culture. In every case, the cells were harvested 18 to 24 hours after the 35S-methionine was added to the medium. Prior to harvest, the cells were washed with Hank's BSS. Mitochondria were isolated and purified on sucrose gradients and mitochondrial polypeptides analyzed by electrophoresis on slab gels. Autoradiograms of the stained gels were made using Kodak Blue X-ray film (Eastman Kodak, Rochester, New York).

RESULTS

Initial experiments to determine organelle density, intactness and contamination by other cell components involved localization of the mitochondrial band in a linear sucrose gradient after centrifugation to equilibrium. The gradient profile of mitochondria isolated from mammary gland cells grown in culture for seven days is shown in figure 1. The activities of inner mitochondrial membrane cytochrome oxidase, outer mitochondrial membrane monoamine oxidase, total protein and density along the gradient are shown. Both mitochondrial enzymes were found in a uniform band with a mean density of 1.183 g/ml at 0°C. The major protein band was coincident with both bands of enzyme activity which indicated that the mitochondria were relatively homogeneous in size and were not disrupted during isolation. More than 80% of the cytochrome oxidase and 75% of the monoamine oxidase activities applied to the gradient were recovered in fractions 6 to 9, well away from other contaminating protein in the gradient. No lysozyme activity was detected in the mitochondrial fractions. Sucrose gradient purification resulted in a 2- to 3-fold increase in mitochondrial enzyme specific activity compared to the organelles obtained by differential centrifugation. Mitochondria isolated from the mammary glands of midpregnant or 5-week hormone stimulated animals had similar characteristics on a sucrose gradient forming a uniform band at a density of 1.182 g/ml at 0°C. By these criteria, mitochondria isolated from mammary glands or 7-day cultures of mammary gland cells were indistinguishable.

The specific activities of seven enzymes chosen for their diverse mitochondrial location and their key roles in oxidative metabolism were compared in gradient purified mito-
Fig. 1 Sucrose gradient profile of mitochondria isolated from mouse mammary epithelial cells after seven days in monolayer culture. The gradient and mitochondria were prepared and the fractions were assayed for protein, refractive index, cytochrome oxidase and monoamine oxidase activities as described in MATERIALS AND METHODS.

The comparison of mitochondrial properties was extended to structural and functional proteins analyzed by electrophoresis on SDS-urea polyacrylamide gels. Figure 2 shows the electrophoresis profiles of gradient purified mitochondria from the mammary glands of hormonally stimulated (5 weeks) animals and mammary cells after seven days in culture. Significant differences in the relative amounts of many mitochondrial polypeptides were evident. Polypeptide 2 (84,000 daltons) was highly reduced in the mammary cell mitochondria as were the relative proportions of polypeptides 3:4, 5:6:7, 8:9:10 and 11:12. These changes in proportion indicated a de-
### TABLE 1

**Enzymatic activities of sucrose gradient-purified mitochondria isolated in media containing 1% BSA**

<table>
<thead>
<tr>
<th>Enzymatic Activity</th>
<th>Mammary gland</th>
<th>Mammary gland cells</th>
<th>Mammary gland cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytochrome oxidase</strong></td>
<td>1365±93 (7)</td>
<td>234±26 (7)</td>
<td>5.83</td>
</tr>
<tr>
<td>Monoamine oxidase (tryptamine-14C)</td>
<td>310±27 (6)</td>
<td>247±20 (7)</td>
<td>1.26</td>
</tr>
<tr>
<td>α-glycerophosphate oxidase</td>
<td>14.1±.4 (4)</td>
<td>2.54±.040 (7)</td>
<td>5.56</td>
</tr>
<tr>
<td><strong>Succinate dehydrogenase</strong></td>
<td>7.4±.15 (4)</td>
<td>1.31±.024 (7)</td>
<td>5.65</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>1640±161 (6)</td>
<td>636±134 (7)</td>
<td>2.60</td>
</tr>
<tr>
<td><strong>NADH oxidase</strong></td>
<td>180±17 (4)</td>
<td>269±31 (7)</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>Adenylate kinase</strong></td>
<td>348±31 (5)</td>
<td>162±9 (7)</td>
<td>2.15</td>
</tr>
</tbody>
</table>

*Mean ± SE; numbers in parentheses = number of individual experiments.

**Pretreated with Lubrol WX.**

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**Fig. 2** Densitometer scans of photographs of Coomassie blue stained mitochondrial polypeptides electrophoresed in tube gels. Mitochondria were isolated from hormonally stimulated mouse mammary glands or seven day monolayer cultures of these mammary gland cells. Mitochondrial isolation, purification and electrophoresis were as described in MATERIALS AND METHODS. Fifty micrograms "Lowry" protein/sample were co-electrophoresed.
crease in the amount of several of these polypeptides in the mitochondria from mammary gland cells in culture.

After seven days in culture mammary epithelial cells have mitochondria with highly diminished activities of two key electron transport enzymes, several other enzymes and reduced amounts of several undefined structural/functional proteins. It is possible that these differences were the result of the method of gland dissociation and/or a selection of a specific cell type(s) during cell culture. To examine this possibility, mitochondria were isolated from mammary glands after the following treatment: undissociated glands; cells dissociated from whole glands plated and cultured for 30 minutes, 1 day, 2 days, 3 days and 7 days. Table 2 summarizes the enzyme activities determined in these experiments. There was no significant difference in the specific activities of monoamine oxidase, cytochrome oxidase, α-glycerophosphate oxidase and succinate dehydrogenase in mitochondria isolated from undigested glands and cells from enzymatically dissociated glands plated for 30 minutes. After one day in culture monoamine oxidase activity was unchanged while cytochrome oxidase activity dropped 3-fold, and α-glycerophosphate oxidase and succinate dehydrogenase were reduced nearly 2-fold. The same level of reduced activity is maintained in cells cultured for two days. By the third day, cytochrome oxidase activity was 4-fold lower while α-glycerophosphate oxidase and succinate dehydrogenase were greater than 2-fold lower. By day 7, the activity of these three enzymes was 6-fold lower while monoamine oxidase activity remained unchanged. The reduction in mitochondrial enzyme activities was gradual and related to the length of time the cells were in culture, not the method of gland dissociation. The procedure of gland dissociation results in an almost pure (>95%) population of parenchymal cells. These cells have mitochondrial properties identical to those organelles isolated from intact glands but after just 24 hours in culture, the mitochondrial properties of these cells have changed.

The gel electrophoresis profiles of mitochondrial proteins also revealed progressive changes related to the length of time these cells were cultured (fig. 3). Polypeptide 2 was present in mitochondria from cells cultured for one day but was nearly absent in cells cultured two to eight days (fig. 3). The change in the relative proportions of polypeptides 5:6:7 occurred after two days in culture as did the changes in proportion of polypeptides 8:9:10 (fig. 3). There was a gradual reduction in the amount of polypeptides 21 and 22 beginning on day 2 and continuing through day 8 (fig. 3). The change in the relative amounts of polypeptides 11:12 seen in comparisons of mammary gland and 7-day cultures of mammary gland cells (fig. 2) was evident after 24 hours of culture but became more prominent on succeeding days (fig. 3).

The changes in enzyme activities and protein components of mitochondria during cell culture were not the result of mitochondrial disaggregation. The ultrastructure of mitochondria seen in thin section electron micrographs of cells after various periods of culture appeared normal and mitochondrial disaggregation was not evident (White et al., '77; and unpublished observation).

Mammary epithelial cells continued to synthesize new mitochondria and mitochondrial proteins during monolayer culture as evidenced by labelling studies and analysis of mitochondrial protein synthesis by autoradiography of SDS-urea polyacrylamide gels.

**TABLE 2**

<table>
<thead>
<tr>
<th>Enzyme activity in mitochondria purified on sucrose gradients</th>
<th>Mammary gland</th>
<th>Mammary gland cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days in culture</td>
<td>Dissociated</td>
<td>1 2 3 7</td>
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</table>

| Monoamine oxidase | 0.84 | 1.20 | 1.25 | 1.39 | 1.16 |
| Cytochrome oxidase | 1.21 | 2.98 | 2.90 | 4.42 | 5.87 |
| a-glycerophosphate oxidase | 1.02 | 1.79 | 2.31 | 2.51 | 5.84 |
| Succinate dehydrogenase | 0.97 | 1.71 | 1.92 | 1.91 | 5.79 |

* The average of three separate experiments; mammary glands were from mice hormonally stimulated for five weeks. In each case, mammary gland and mammary gland cell mitochondria were compared, using mitochondria isolated, purified and assayed on the same day.
Figure 3 shows densitometer scans of autoradiographs of mitochondrial proteins from cells labelled with $^{35}$S methionine for various periods during culture. All of the major mitochondrial polypeptides were synthesized during the first 18 hours of culture. Cells labelled from the second to the third and the seventh to the eighth day of culture also synthesized most of the mitochondrial polypeptides (fig. 4). The pattern of newly synthesized proteins mimicked the gradual changes in relative proportion of the various polypeptides seen in figure 3. Most striking was the change in synthesis of polypeptide 8 (56,000 daltons). This polypeptide was made in significant amounts during the first 18 hours of culture, but synthesis was reduced from days 2 to 3 and very highly reduced from days 7 to 8 (fig. 4). This corresponded to the nearly complete absence of this polypeptide after day 1 as seen in stained gel profiles (fig. 3).

It is significant to note that mitochondrial protein synthesis continued in mammary cells in culture and the gradual reduction in cer-
Certain mitochondrial proteins appeared to result from the decreased net synthesis of these components.

**DISCUSSION**

Following enzymatic dissociation of the mouse mammary gland, parenchymal cells can be separated from fat cells, myoepithelial cells and the majority of fibroblasts by differential centrifugation. When plated at medium to high densities, the parenchymal cells settle, attach, proliferate and form a continuous sheet with a polygonal morphology within 24 to 48 hours. The strict criteria for the epithelial nature of these cells are the nonrandom attachment of the cells such that the basolateral surface attaches to the substratum and the apical surface faces up with numerous microvilli projecting into the me-
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Dium and the occluding tight junctions that connect adjacent cells (Pickett et al., '75). If cells are derived from mid-late pregnant mammary glands, accommodation to the crowded conditions of cell culture appears to reduce the ductal and lobulo/alveolar cells to roughly similar states of cytoplasmic development so that internal distinguishing features tend to disappear. Cells in these primary cultures never approach the differentiated state of secretory mammary epithelium (Pickett et al., '75). Ultrastructural evidence suggests that loss of differentiated functions in mammary epithelial cells begins when cells are seeded onto plastic or glass substrates in tissue culture (Emerman and Pitelka, '76).

Our results suggest that the mitochondria of mouse mammary epithelial cells undergo structural and functional changes in response to cell culture. It is tempting to speculate that these mitochondrial alterations reflect a loss of differentiated function in these organelles concomitant with the general loss of developmental morphology of these mammary cells.

There is compelling evidence for mitochondrial differentiation in the mouse mammary gland during pregnancy and lactation in vivo. Total mitochondrial protein and succinate oxidase and cytochrome oxidase activities increased gradually during pregnancy with a 2- to 3-fold increase occurring during early lactation (Jones and Rosano, '72). These changes were accompanied by a corresponding increase in the buoyant density of isolated mitochondria due to an expansion of inner membrane and matrix components (Rosano and Jones, '76). This expansion was indicated by a 3- to 4-fold increase in cytochrome oxidase activity and was confirmed in electron micrographic studies (Rosano et al., '76). The major phase of mitochondrial development occurs shortly after parturition during a period subsequent to epithelial cell proliferation in the gland (Jones and Rosano, '72, '76). These studies indicate that mitochondrial differentiation in the developing mouse mammary gland is geared to increased oxidative capacity of these cells during milk production and secretion.

Our results indicate that mitochondria show a markedly decreased oxidative capacity in mammary epithelial cells which have lost differentiated features as a result of cell culture. The specific activity of outer mitochondrial membrane monoamine oxidase did not change in mammary gland cells during culture. This is consistent with the observations of Rosano and Jones ('76) who found that monoamine oxidase activity changed very little compared to cytochrome oxidase and other enzymes in mitochondria isolated from the mammary glands of virgin, pregnant or lactating mice. Their interpretation was that the outer mitochondrial membrane was stable during mammary gland development. By analogy, it is possible that the outer mitochondrial membrane does not change during loss of differentiated functions in culture. This stability is not seen in the inner membrane and matrix components. Cell culture results in a 6-fold reduction in the specific activities of cytochrome oxidase, succinate dehydrogenase and α-glycerophosphate oxidase and a 2-fold reduction in matrix malate dehydrogenase.

The metabolic implications of the lowered specific activities of these enzymes is a severe limitation on the utilization of mitochondrial oxidative pathways with electron transport impaired at succinate dehydrogenase and cytochrome oxidase. The reduction in α-glycerophosphate oxidase and malate dehydrogenase limits two primary shuttle mechanisms whereby NADH produced in glycolysis can be transferred into and oxidized by mitochondrial electron transport. These cells are dependent upon glycolysis for energy production and lactate accumulation to regenerate NAD⁺.

In addition to enzymatic changes, quantitative and qualitative changes were seen in various protein components of the cultured cell mitochondrion. These alterations occurred gradually over several days and were not the result of mitochondrial disintegration but were due to modulation in the synthesis of mitochondrial proteins during cell culture. Assigning a structural or enzymatic function to the various mitochondrial polypeptides identified by gel electrophoresis would make it possible to determine which mitochondrial functions were altered during cell culture.

Epithelial cells from developing mouse mammary glands respond to monolayer culture by a loss of differentiated morphology. They no longer respond to the hormones which elicit differentiation and secretion in vivo. Recently, Emerman and Pitelka ('76) reported that mouse mammary epithelial cells cultured on floating collagen gels respond to exogenous hormones and differentiate into secretory cells. It would be of interest to examine the mitochondria of cells grown in this way to see if the changes we have observed could be
reversed. The ability to manipulate cellular and mitochondrial differentiation in cell culture would facilitate the understanding of the fundamental mechanisms of cellular differentiation in vivo.

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LITERATURE CITED


