Pyruvate Utilization, Phosphocholine and Adenosine Triphosphate (ATP) Are Markers of Human Breast Tumor Progression: A 31P- and 13C-Nuclear Magnetic Resonance (NMR) Spectroscopy Study

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Abstract

We have used 31P- and 13C-nuclear magnetic resonance spectroscopy to measure key metabolite levels and fluxes through enzymes regulating phospholipid and mitochondrial metabolism in normal human mammary epithelial cells. We have compared these values to those found in a progression series of breast cancer cell lines of varying metastatic potential established from a single patient. We find a 16–19-fold increase in phosphocholine content in two primary breast cancer cell lines (21PT and 21NT) and a 27-fold increase in phosphocholine content in the metastatic breast cancer cell line (21MT-2) compared with the normal breast epithelial cell strain 76N. Thus, phosphocholine may serve as a metabolic marker for the human breast cell progression state. A 30% decrease in ATP levels, a 83% decrease in phosphocreatine levels, along with a 2-fold increase in NAD* + NADH levels in 21PT, 21NT, and 21MT-2 cells compared to the normal breast cells further suggests impaired mitochondrial metabolism in the breast carcinoma cell lines. Consistent with this suggestion is our finding that the primary breast cancer cell lines (21PT and 21NT) and the metastatic breast cell line (21MT-2) showed a 50 and 89% relative reduction, respectively, in the flow of pyruvate utilized for mitochondrial energy generation compared to pyruvate utilized to replenish tricarboxylic acid cycle intermediates. These results demonstrate that diminished mitochondrial energy generation may be quantitatively related to the progression state of human breast cells.

Introduction

Breast cancer is a leading cause of cancer-related deaths of women (1), yet there is a paucity of information concerning the biochemical changes that occur during human breast tumorigenesis and progression. We have addressed these questions using a multidisciplinary approach that combines recent advances in the culture of normal and tumor-derived human breast cells (2, 3) and NMR spectroscopy to simultaneously quantify cellular metabolites in actively metabolizing cell systems. This allows us to measure directly the biochemical changes arising during breast cell progression. Although considerable effort has been devoted to determining the genetic events in breast cell carcinogenesis, the relationship of the changes to the biochemical phenotype of the cancer cell is essentially unknown. A more complete understanding of the biochemical phenotype of normal cells versus cancer cells may allow for a more focused study of important genetic changes leading to these biochemical changes. In addition, such biochemical alterations might be useful prognostic markers of breast cell progression and could allow one to predict response to therapy or modify cellular biochemistry to improve treatment efficacy.

To date, all NMR investigations have been carried out on breast cancer cell lines of metastatic origin (4–6); the successful long-term growth in culture of primary tumor-derived breast cells has been difficult to achieve. This problem was surmounted by Band and Sager (2) when they established three human breast cancer cell lines derived from primary and metastatic breast tumor specimens obtained from the same patient. The two primary breast cancer cell lines (21PT and 21NT) were derived from the primary tumor specimen in a patient with an infiltrating and intraductal carcinoma of the breast that was estrogen receptor and progesterone receptor negative. The third breast cancer cell line (21MT-2) was derived from a metastatic pleural effusion from the same patient. Of the two lines derived from primary tumors, the 21PT cells are probably at an earlier stage of progression than are the 21NT cells because the former are not tumorigenic in the nude mouse assay, contain about 20% near diploid cells, and appear more normal morphologically (3). The 21MT-2 cells of metastatic origin resemble 21NT cells in their growth factor requirements, morphology and elevated expression of erbB2 protein. Both the 21NT and 21MT-2 cells were tumorigenic in nude mice with the 21MT-2 cells producing a greater proportion of tumors with a much shorter latency of tumor formation compared with the 21NT cell line (2, 3).

In the present investigation, normal mammary epithelial cells (76N), primary mammary tumor cell lines (21PT, 21NT), and a metastatic cell line (21MT-2) were studied by 31P- and 13C-NMR spectroscopy. Changes in phospholipid content and mitochondrial function were measured by NMR spectroscopy and related directly to breast cell progression state. These results help elucidate the biochemical modifications linked to breast cell transformation and tumorigenesis, as well as have important potential clinical application in the noninvasive assessment of tumorigenesis, metastatic potential, and response to therapy of in vivo breast cancers.

Materials and Methods

Cell Cultures. Normal breast epithelial cell strain (76N) was derived from reduction mammaplasty tissue (2) and was provided by Dr. Ruth Sager (Dana-Farber Cancer Institute, Boston, MA). The tumor-derived human primary breast cell lines (21NT and 21PT) were derived from mastectomy samples from a patient with infiltrating ductal and intraductal carcinoma (3), whereas the metastatic cell line (21MT-2) was derived from a pleural effusion from the same patient (3). The 21PT, 21NT, and 21MT-2 cell lines were also provided by Dr. Ruth Sager. All cell lines were Mycoplasma negative. For experiments, the cells were grown in T-flasks (175 cm2) at 37°C in DMEM-1 medium in a humidified atmosphere of 6.5% CO2. When 75% confluent, the...
cells were harvested with 0.025% trypsin-0.01% EDTA solution. Trypsin digestion was stopped with soybean trypsin inhibitor, and the cells were used to seed the microcarriers (described below) or were diluted 1:4 and reincubated into new T-flasks.

The seeding process onto Collagen-coated Cytodex III beads (diameter, 170–220 μm), obtained from Pharmacia (Piscataway, NJ), has been described previously (7, 8). For the NMR perfusion experiments, 5 ml of beads with the attached cells were placed in a specially designed 20-mm diameter (volume, 10 ml) bioreactor (7) and continuously perfused with a oxygenated (40% O2 + 6.5% CO2) gas mixture, DFCI-1 medium (pH 7.3–7.4), at a rate of 4.4 ml/min. All cultures were placed in the NMR bioreactor 3 days before NMR measurements. At the time of NMR study, the cells were proliferating on the beads with a cell doubling time of 2.6 days for the 21MT-2 cells and 3.6 days for the 76N cells.

NMR Measurements. NMR measurements were performed at 8.4 Tesla using a spectrometer built at the Francis Bitter Magnet Laboratory (Massachusetts Institute of Technology, Cambridge, MA). The specially designed, large volume NMR cell bioreactor, used with a commercial broadband observable proton decouple probe for phosphorus (145.6 MHz) NMR, was described previously (7, 8). Baseline 31P-NMR spectra were recorded at 145.6 MHz by using a 90° pulse and a 10 s repetition delay. Time domain data were collected as 1000 points using a spectral sweep width of 10 KHz. Line broadening of 10 Hz was used for spectral processing. Using NMR1 software, we obtained peak areas relative to a methylphosphonic acid reference in a 1-mm sealed capillary.

For the 31P-NMR studies of cells grown on microcarriers, a series of baseline, fully relaxed spectra (20-min acquisition time) were obtained over a 24-h period. After this period, cell culture samples were taken for determination of cell number and cell viability.

Cell extracts were analyzed using a 10-mm commercial broadband observable proton decouple probe. Serial 31P-NMR spectra for extracts were typically acquired using a 45° pulse with a 2-s repetition delay. Time domain data were collected as 2000 data points by using a sweep width of 5 KHz. Line broadening of 1 Hz was used for spectral processing.

13C-NMR spectra of perchloric acid extracts were recorded at 90.4 MHz by applying a 60° pulse with a repetition time of 6 s. A WALTZ-16 proton decoupling sequence (9), using 4 W of radiofrequency power, was applied for 1.5 s during both the interpulse delay and data acquisition. Time domain data were collected as 4000 points by using a spectral sweep width of 24 KHz. Zero-filling to 8000 data points with a line broadening of 5 Hz was obtained for all 13C-NMR spectral processing. NMR1 software was used to obtain peak areas relative to a (13C) mannose reference placed in a 1.5-mm capillary tube. The probe was kept at 20°C by using a variable temperature system. Resonance assignments for the 13C-NMR spectra were made by comparing them to spectra of pure metabolites in the DFCI-1 medium, as well as from the spectra of published chemical shift data. The chemical shifts are expressed as ppm relative to tetramethylsilane by using as a secondary reference the δ(C1) resonance of NaHCO3 at 100.6 ppm. All spectral intensities used in the calculations were corrected for nuclear Overhauser and T2 effects.

Cell Extract Preparation. Cells in eight 175-cm² T-flasks at 75% confluence were used for each cell extract. After removal of media, the cells were rinsed with ice cold 0.9% NaCl and frozen in liquid nitrogen. The frozen cells were scraped into ice cold 7% (w/v) perchloric acid. The mixture was homogenized at 4°C with a Dounce homogenizer and centrifuged for 5 min at 5000 × g at 4°C. The clear supernatant was neutralized with 3 MKOH to pH 8.5 and centrifuged for 10 min at 5000 rpm. The supernatant was frozen, lyophilized, and stored at −80°C until use in NMR spectral analysis when it was reconstituted with 1.5 ml H2O containing 20% (v/v) D2O.

TCA Cycle Metabolism Experiments. Cell extracts for the 13C-NMR studies of glucose metabolism were prepared by growing each cell line in eight 175-cm² T-flasks with carbohydrate-free DFCT-1 medium containing unlabeled (13C) glucose added at 2 mg/ml (11 mm). At 75% confluence, the old medium was removed and the new carbohydrate-free DFCT-1 medium containing 11 mm (13C) glucose was added. After 20 h, the labeled media was removed, stored, and frozen at −80°C. The cell extracts were then prepared as described above. An estimate of the fraction of pyruvate entering the TCA cycle under glucose feeding was determined for each cell line based on the labeling pattern of glutamate. From these labeling patterns, quantitative estimates of PDH flux to PCarb flux were determined by using the 13C enrichment at glutamate C4 relative to the enrichments at glutamate C2 and C3 using the technique of Cohen (10).

Measurement of Intracellular pH and Intracellular Magnesium. The PCr to P i chemical shift was used to estimate the intracellular pH for the cells in vitro (11). We calculated the intracellular-free magnesium concentration by using the method described by Mosher et al. (12), which accounts for the influence of the pH on the chemical shift separation of α- and β-phosphorus resonances of ATP.

Results

Normal Breast Cells and Metastatic Breast Cancer Cells in Perfused Microcarrier Culture. The 31P-NMR spectra of normal breast cell strain 76N and the metastatic breast carcinoma cell line (21MT-2) both showed resonances from PE, PC, Ppc, ATP, NAD+ (NAD), and UDPG. The major differences in the 31P-NMR spectra of perfused normal breast cells and metastatic breast cancer cells on microcarriers are the increase in the intensity of the PC and GPC signals and the decrease in intensity of PCr signal in the metastatic breast cell line 21MT-2 compared to the spectra of 76N cells. For the 21MT-2 cells on perfused microcarrier beads, the intracellular pH was found to be 7.27 ± 0.06 with a corresponding intracellular-free magnesium of 0.285 ± 0.024 mM. The intracellular content of ATP and PCr was calculated from the area ratio of ATP and PCr to a known capillary standard (concentration = 0.1 mM)/number of cells in the bioreactor assuming an NMR-visible cell culture volume of 8 ml. From these experiments on perfused 21MT-2 cells, we estimate the ATP content/cell to be 8.4 ± 1.1 fmol/cell (n = 3) and the PC content/cell to be 7.4 ± 2.5 fmol/cell (n = 3).

Comparison of Phosphate Metabolite Levels of Intact Perfused Metastatic Breast Cancer Cells Grown on Microcarriers to Extracts Prepared from These Perfused Microcarrier Cultures. The 31P-NMR metabolites in mole percentage for a mean cell density of 1.8 × 10⁵ cells/ml of 21MT-2 cells perfused on microcarrier beads was quantitatively compared with the mole percentage of phosphate metabolites in perchloric acid extracts of 21MT-2 cells grown on microcarriers (see Table 1). The intact 21MT-2 cells grown on beads were found to have significantly higher levels of β-ADP and NAD+ + NADH compared to cell extracts prepared from 21MT-2 cells grown on microcarrier beads (see Table 1). The lower concentrations of ADP and NAD+ + NADH found in the intact cell

Table 1 Phosphate metabolite concentrations (mole percentage ± SD). intracellular pH, and intracellular magnesium concentration of 21MT-2 cells perfused on microcarrier beads (n = 3) were compared to perchloric acid extracts of 21MT-2 cells grown on microcarriers (n = 2) and to perchloric acid extracts of 21MT-2 prepared from six 75% confluent T-flasks (n = 2).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>21MT-2 cellsa (n = 3)</th>
<th>21MT-2 cellsb (n = 2)</th>
<th>21MT-2 cellsc (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>22.2 ± 2.7</td>
<td>12.7 ± 5</td>
<td>10.0 ± 1.9</td>
</tr>
<tr>
<td>PC</td>
<td>10.3 ± 3.4</td>
<td>10.0 ± 1.1</td>
<td>10.0 ± 1.5</td>
</tr>
<tr>
<td>GPE</td>
<td>6.8 ± 1.0</td>
<td>2.9 ± 2.8</td>
<td>2.2 ± 1.3</td>
</tr>
<tr>
<td>GPC</td>
<td>7.2 ± 2.4</td>
<td>3.2 ± 2.3</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>Pcr</td>
<td>0.8 ± 0.3</td>
<td>0.9 ± 0.06</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>β-ATP</td>
<td>11.8 ± 1.1</td>
<td>10.9 ± 0.4</td>
<td>13.3 ± 1.4</td>
</tr>
<tr>
<td>β-ADP</td>
<td>0.6 ± 0.3</td>
<td>3.1 ± 1.3</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>GDPG</td>
<td>3.2 ± 0.5</td>
<td>2.6 ± 2.5</td>
<td>3.0 ± 2.3</td>
</tr>
<tr>
<td>NAD+ + NADH</td>
<td>8.4 ± 1.1</td>
<td>13.6 ± 0.6</td>
<td>14.5 ± 3.7</td>
</tr>
<tr>
<td>pH</td>
<td>7.27 ± 0.06</td>
<td>NA²</td>
<td>NA²</td>
</tr>
<tr>
<td>Free mg²</td>
<td>0.285 ± 0.024</td>
<td>NA²</td>
<td>NA²</td>
</tr>
</tbody>
</table>

a 1.8 × 10⁵ cells/ml perfused on beads.

b Perchloric acid extracts of cells grown on microcarriers.

c Perchloric acid extracts of cells grown on T-flasks.

NA, not available.

1.8 × 10⁵ cells/ml perfused on beads.

Perchloric acid extracts of cells grown on microcarriers.

Perchloric acid extracts of cells grown on T-flasks.

Level significantly different (P < 0.05) when compared to intact 21MT-2 cells perfused on beads.

NA, not available.
preparation may be related to the large proportion of β-ADP and NAD+ + NADH, which is protein bound and, thus, NMR "invisible" in intact cells compared to cell extracts prepared from the microcarrier culture.

Comparison of Phosphate Metabolite Levels Measured of Metastatic Breast Cancer Cells Prepared from Perchloric Acid Extracts of Cells Grown on Perfused Microcarriers to Perchloric Acid Extracts of Cells Grown on T-flasks. The 31P-NMR metabolites in mole percentage for perchloric acid extracts prepared from 21MT-2 cells perfused on microcarrier beads was quantitatively compared with the mole percentage of phosphate metabolites in perchloric acid extracts of 21MT-2 on T-flasks as a monolayer (see Table 1). The extracts from 21MT-2 cells grown on beads were found to have no statistically significant differences in levels of PE, PC, GPE, GPC, PCT, β-ATP, β-ADP, UDPG, and NAD+ + NADH compared to 21MT-2 cells grown on T-flasks as a monolayer (see Table 1). Thus, due to the added resolution of NAD+ + NADH compared to 21MT-2 cells grown as a monolayer and the fact that data obtained from perchloric acid extracts as compared to intact cells and the fact that data obtained from perchloric acid extracts from monolayer culture in T-flasks at 75% confluence were not statistically different from extracts prepared from microcarrier culture, we used data from cell extracts prepared from monolayer T-flask culture for comparative studies between the four breast cell lines.

Comparison of Phosphorus-containing Metabolite Levels Measured from Cell Extracts of Normal Breast Cells and Breast Cell Lines Grown in T-flasks. Fig. 1 shows a comparison of the 31P-NMR spectra of perchloric acid extracts of normal breast cell strain (76N), primary breast cancer cell line (21NT and 21PT), and metastatic breast cell line (21MT-2) all grown in T-flasks. Table 2 summarizes the mole percentage of each phosphate metabolite in these extracts for the 76N, 21PT, 21NT, and 21MT-2 cells. The primary carcinoma cell line 21PT showed a higher fold increase in PE concentration (P = 0.0001), a 2.8-fold increase in GPC concentration (P = 0.0001), and a 1.7-fold increase in NAD+ + NADH concentration (P = 0.03) compared to the normal breast epithelial cell line 76N. The primary carcinoma cell line 21PT had a 92% decrease in PC concentration (P = 0.0001) and a 29% decrease in ATP concentration (P = 0.008) compared to the normal breast epithelial cell line 76N. There were no statistically significant differences in PE, GPE, UDPG, or β-ADP between the primary carcinoma cell line 21PT and the normal breast epithelial cell line (P > 0.05).

A quantitative comparison of 21PT and 21NT is shown in Table 2. The primary breast carcinoma cell line 21PT (tumorigenic in nude mice) had a 1.7-fold increase in PE concentration (P = 0.005), a 18% decrease in PC concentration (P = 0.03), and a 1.7-fold increase in ATP concentration (P = 0.008) compared to the normal breast epithelial cell line 76N. There were no statistically significant differences in PE, GPE, UDPG, or β-ADP between the primary carcinoma cell line 21PT and the normal breast epithelial cell line (P > 0.05).

In addition, Table 2 shows a quantitative comparison of phosphate metabolites between the primary tumorigenic cell line 21NT and the metastatic cell line 21MT-2. The metastatic breast cell line 21MT-2 showed a 1.7-fold increase in PE concentration (P = 0.002) and a 46% decrease in GPC concentration (P = 0.007) compared to the primary breast cell line 21NT.

The GPC:PC ratio (mean ± SD) for the 76N, 21PT, 21NT, and 21MT-2 cell lines was 6.64 ± 2.6, 0.85 ± 0.09, 1.17 ± 0.11, and 0.32 ± 0.16, respectively. Thus, the primary breast cell lines 21NT and 21MT-2 were found to have a marked 83% decrease in the GPC:PC ratio compared to the normal breast epithelial cell line 76N (P < 0.02). The metastatic breast cell line 21MT-2 had a 66% decrease in the GPC:PC ratio compared to the primary breast cell lines 21NT and 21PT (P = 0.001).

13C-NMR of Cell Extracts. 13C-NMR spectra of perchloric acid extracts prepared from 76N, 21NT, and 21MT-2 cells after a 20-h incubation at 37°C with 11 mm [1-13C]glucose showed the following resonances: [1-13C]glucose (94.9 and 94.5 ppm), [1-13C]lactate (20.8 ppm), [1-13C]galactose (17.3 ppm), [1-13C]glutamate (27.7 ppm), [1-13C]glutamate (34.2 ppm), and [1-13C]glutamate (55.4 ppm). An

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Table 2 Phosphate metabolite concentrations (mole percentage ± SD) in perchloric acid extracts prepared from six 75% confluent T-flasks of normal breast epithelial cell strain (76N) compared to primary breast cancer cell lines (21PT and 21NT) and metastatic breast cancer cells (21MT-2).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>76N cells</th>
<th>21PT cells</th>
<th>21NT cells</th>
<th>21MT-2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 3)</td>
<td>(n = 4)</td>
<td>(n = 3)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>PE</td>
<td>6.7 ± 1.9</td>
<td>7.5 ± 1.0</td>
<td>12.8 ± 0.4</td>
<td>11.4 ± 3.8</td>
</tr>
<tr>
<td>PC</td>
<td>0.4 ± 0.2</td>
<td>7.1 ± 0.9</td>
<td>5.8 ± 0.6</td>
<td>10.0 ± 1.1</td>
</tr>
<tr>
<td>GPE</td>
<td>2.2 ± 0.9</td>
<td>3.3 ± 0.8</td>
<td>3.0 ± 0.7</td>
<td>2.5 ± 1.9</td>
</tr>
<tr>
<td>GPC</td>
<td>2.1 ± 0.4</td>
<td>6.0 ± 0.6</td>
<td>6.8 ± 0.2</td>
<td>3.1 ± 1.4</td>
</tr>
<tr>
<td>PCT</td>
<td>2.8 ± 0.5</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.02</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>β-ATP</td>
<td>15.8 ± 1.4</td>
<td>11.3 ± 1.4</td>
<td>9.3 ± 0.4</td>
<td>9.5 ± 2.2</td>
</tr>
<tr>
<td>β-ADP</td>
<td>3.1 ± 1.6</td>
<td>3.0 ± 0.8</td>
<td>4.5 ± 1.0</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td>UDPG</td>
<td>4.0 ± 0.8</td>
<td>2.9 ± 1.3</td>
<td>1.5 ± 0.2</td>
<td>2.8 ± 2.5</td>
</tr>
</tbody>
</table>

a Normal breast.
b Primary breast cancer derived, nontumorigenic.
c Primary breast cancer derived, tumorigenic.
d Metastatic.
e Level significantly different (P < 0.05) when compared to 21PT cells.
f Level significantly different (P < 0.05) when compared to 76N cells.
g Level significantly different (P < 0.05) when compared to 21PT cells.

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Fig. 1. 31P-NMR spectra of perchloric acid extracts of 76N, 21PT, 21NT, and 21MT-2 cell lines grown on T-flasks: 45° pulse, repetition delay = 2 s, number of acquisitions = 10,240, line broadening of 1 Hz.
estimate of the pyruvate entering the TCA cycle via PCarb compared to that entering via PDH was determined from quantitation of glutamate enrichment at the C2 + C3 glutamate peaks compared to the amount of 13C label at the C4 glutamate resonance. [3-13C]-labeled pyruvate entering the TCA cycle via PCarb will label glutamate in both the C2 + C3 position, whereas [3-13C]-labeled pyruvate entering the TCA cycle via PDH will label glutamate at the C4 position. The calculated PCarb:PDH ratios for 76N, 21PT, 21NT, and 21MT-2 were 0.33 ± 0.08, 0.55 ± 0.08, 0.62 ± 0.05, and 2.89 ± 1.13, respectively. Thus, the PCarb route is estimated to be about 4-fold more active than the PDH route in the metastatic 21MT-2 cells than in the primary breast cancer 21NT cell line (P = 0.026). The PCarb route is estimated to be about 2-fold more active than the PDH route in the primary breast cancer cell lines (21PT and 21NT) compared to the normal breast cell line 76N (P = 0.005). The 13C enrichments of glutamate C1 and glutamate C5 were also monitored because substantial labeling at these sites would affect any analysis of the competing pathways. The lack of detectable label at C1, and C5 glutamate in these breast extracts suggests the absence of significant pyruvate recycling in the phosphoenolpyruvate cycle.

Discussion

31P-NMR spectroscopy was used to measure the concentrations of high-energy phosphate metabolites and phospholipids in normal breast epithelial cells (76N) and a series of three human breast carcinoma cell lines (21PT, 21NT, and 21MT-2) established from primary and metastatic breast tumor specimens obtained from the same patient. We found a 16–19-fold increase in PC concentrations in the two primary tumor lines (21NT and 21PT) compared with the normal breast epithelial cell line (76N). The metastatic breast cell line (21MT-2) was found to have a 27-fold increase in PC concentration compared with the normal breast epithelial cell line (76N). From our perfused microcarrier cell cultures of 21MT-2 cells with a 0.1-mm capillary standard in a 8-ml NMR bioreactor, we estimate the total PC content in the 21MT-2 cell line to be 7.4 fmol/cell. Thus, the PC content of normal breast (76N cells) is 0.27 fmol/cell. The metastatic breast cancer cell line T47D clone 11 grown on perfused spheroids was found to have a total PC content of 16 fmol/cell (13). In this perfused spheroid model, a significant fraction of dead cells may exist in the center of the spheroid, which might lead to higher levels of measured phospholipid compared to a culture of a uniform population of well-perfused viable cells. To our knowledge, there are no directly comparable data of PC content for normal breast epithelial cells nor for nonmetastatic human breast cancer cell lines. Elevated PC concentrations have been found in Ha-ras-transformed NIH3T3 cells (15.9 nmol/mg of protein) compared to NIH3T3 cell controls (5.8 nmol/mg of protein; Ref. 14).

This marked increase in PC concentration suggests either a marked increase in the rate of synthesis of PC or, alternatively, a marked decrease in PC utilization in the breast carcinoma cell lines compared with the normal breast (76N) cells. PC serves as the activated species in the synthesis pathway of phosphatidylcholine. Phosphatidylcholine, the most abundant phospholipid in mammalian cells, plays a major role in both membrane structure and signal transduction (15). Choline is phosphorylated by ATP via the cytosolic enzyme choline kinase to produce PC, which then reacts with CTP to form CDP-choline catalyzed by CTP:PC cytidylyltransferase (see Fig. 2). Activation of CTP:PC cytidylyltransferase in vivo by translocation from cytosol to a membrane fraction has been demonstrated in a variety of cell types (16, 17). The rate of this reaction governs the overall rate of phosphatidylcholine synthesis. The PC unit of CDP-choline is then transferred to a diacylglycerol to form phosphatidylcholine. The marked increase in PC levels for the primary breast carcinoma cells (21NT and 21PT) compared with normal breast (76N) cells would be best accounted for by a marked decrease in the CTP:PC cytidylyltransferase activity and/or a marked increase in choline kinase activity in the primary breast cancer cell lines compared to the normal breast epithelial cells. The metastatic breast cancer cell line 21MT-2 has a significantly higher concentration of PC than do the primary breast cancer cell lines (21NT and 21PT). This suggests that additional alterations in choline kinase and CTP:PC cytidylyltransferase activity occur with breast cancer cell progression. The cellular concentration of PC has been reported to be significantly elevated (2.4-fold) in Ha-ras-transformed NIH 3T3 cells compared to control NIH 3T3 cells (18). These high PC levels in the ras-transformed cells have been confirmed by other investigators and were shown to arise from increased choline kinase activity in the NIH 3T3 model system (14).

Phosphatidylcholine degradation is controlled in part by phospholipase A forming lysophosphatidylcholine. GPC is formed from lysophosphatidylcholine and, subsequently, may be further degraded to choline and glycerol-3-phosphate (see Fig. 2). The concentration of GPC may be important, as well, in the regulation of membrane phospholipid composition (19). GPC is formed from lysophosphatidylcholine via two separate reactions catalyzed by (a) lysophosphatidylcholine and (b) lysophospholipase A forming lysophosphatidylcholine. The first reaction controls the overall degradation of phosphatidylcholine.
phatidylcholine, and the second reaction is a way of changing the fatty acid concentration of phosphatidylcholine. Lysosomichitin concentration is in general kept under tight control because small increases in its concentration in membranes causes enhanced fluidity, altered activity of membrane associated enzymes, and altered cell surface recognition properties. The 3-fold elevation in GPC concentration in both the 21PT and 21NT cells compared to the 76N cells would serve to minimize a parallel increase in lysosomichitin in the face of increased production of phosphatidylcholine. The 2-fold decrease in GPC concentrations along with the 1.4-fold increase in PC concentrations in the metastatic cell line 21MT-2 compared to the primary tumor cell lines 21PT and 21NT would have the net effect of additionally increasing lysosomichitin concentrations and, thus, membrane fluidity in the 21MT-2 cell line compared to the 21PT and 21NT cell lines. Such membrane fluidity changes and cell surface recognition changes may be important factors influencing the metastatic potential of the 21MT-2 cells.

The GPC:PC ratio in the steady state is directly related to the net flux through CTP:PC cytidyltransferase to the net flux of GPC degradation back to choline. The GPC:PC ratio for the 76N cells is 6.6 ± 2.7 (n = 3) compared to 0.9 ± 0.1 (n = 4) and 1.2 ± 0.1 (n = 3) for 21PT cells and 21NT cells, respectively, and 0.32 ± 0.16 (n = 4) for 21MT-2 cells. Thus, there is a marked roughly 83% decrease in the GPC:PC ratio in the 21PT and 21NT cell lines compared with the normal epithelial cell lines 76N (P = 0.007 and 0.024, respectively). The metastatic breast cell line 21MT-2 was found to have an additional 66% decrease in the GPC:PC ratio (P = 0.001), suggesting a substantial decrease in the CTP:PC cytidyltransferase net flux and increase in the GPC glycerophosphohydrolase net flux in the metastatic breast cell line (21MT-2) compared with the primary breast cancer cell lines (21PT and 21NT). These results reveal that important changes in the kinetics of phosphatidylcholine synthesis and degradation occur with breast cancer cell progression and metastasis.

The 30% decrease in ATP levels along with the 2-fold increase in NAD\(^+\) + NADH levels in 21PT, 21NT, and 21MT-2 cells compared to the normal breast cells may suggest impaired mitochondrial metabolism in the breast carcinoma cell lines. The most malignant cell line of the series 21MT-2 had the lowest ATP levels and the highest NAD\(^+\) + NADH levels. Warburg (20, 21) first postulated that the respiration of tumors was disturbed and that this led to the persistence of aerobic glycolysis in tumors.

In normal breast tissue, treatment with estradiol increased creatine kinase activity in the carcinoma cell lines; or (b) a decreased capacity of the carcinoma cell lines to store excess ATP as PCr. Creatine kinase has been found to be responsive to estrogen in previous studies of normal and cancerous human mammary tissue. In normal breast tissue, treatment with estradiol increased creatine kinase-specific activity (23). The fact that these carcinoma cell lines are all estrogen receptor negative might explain the low levels of PCr found in the carcinoma cell lines despite being grown in media containing estradiol. The ratio of PCr:ATP in the estrogen receptor-negative 21MT-2 cell line perfused on microcarrier beads was 0.07 in the present study. Previous NMR studies of estrogen receptor-positive metastatic breast cell lines MCF-7 cells grown in basement membrane gel were found to have a PCr:ATP ratio of 0.2 (6) and T47D-clone 11 cells grown on beads were found to have a PCr:ATP ratio of 0.4 (24). These findings suggest that a functional estrogen receptor may induce creatine kinase enzyme activity in these metastatic breast cancer cell lines and this in turn may result in a higher PCr concentration normalized to ATP in the estrogen receptor-positive metastatic breast cell lines compared to the estrogen receptor-negative metastatic breast cell line 21MT-2.

In summary, the breast cancer cell lines in the present NMR study are all established from the same patient and provide a well-characterized tumor progression series from the nontumorigenic 21PT cell line to the highly tumorigenic and metastatic 21MT-2 cell line. The advantages of this model system are that all of the breast cell lines were grown under the same conditions and have been characterized previously in terms of tumorigenesis, metastatic potential, erbB2 expression, estrogen receptor status, and rhodamine 123 uptake (2, 3). With this model system, we have begun to identify the changes in PC and oxidative metabolism that occur with breast epithelial cell transformation and progression. Such studies will allow us to understand the biochemical mechanisms involved in breast cell tumorigenesis and progression. A basic understanding of these mechanisms may ultimately lead to novel drug therapies and new prognostic factors for breast cancer.

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References

Pyruvate Utilization, Phosphocholine and Adenosine Triphosphate (ATP) Are Markers of Human Breast Tumor Progression: A $^{31}$P- and $^{13}$C-Nuclear Magnetic Resonance (NMR) Spectroscopy Study

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