Active and Inactive Metabolic Pathways in Tumor Spheroids: Determination by GC–MS

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Active metabolic pathways in three-dimensional cancer-cell cultures are potential chemotherapeutic targets that would be effective throughout tumors. Chaotic vasculature creates cellular regions in tumors with distinct metabolic behavior that are only present in aggregate cell masses. To quantify cancer cell metabolism, transformed mouse fibroblasts were grown as spheroids and fed isotopically labeled culture medium. Metabolite uptake and production rates were measured as functions of time. Gas chromatography–mass spectrometry was used to quantify the extent of labeling on amino acids present in cytoplasmic extracts. The labeling pattern identified several active and inactive metabolic pathways: Glutaminolysis was found to be active, and malic enzyme and gluconeogenesis were inactive. Transformed cells in spheroids were also found to actively synthesize serine, cysteine, alanine, aspartate, glutamate, and proline; and not synthesize glutamine. The activities of these pathways suggest that cancer cells consume glutamine for biosynthesis and not to provide cellular energy. Determining active metabolic pathways indicates how cells direct carbon flow and may lead to the discovery of novel molecular targets for anticancer therapy. © 2009 American Institute of Chemical Engineers Biotechnol. Prog., 26: 789–796, 2010

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Introduction
Understanding the metabolism of cancer cells in three-dimensional tissue could play a key role in the development of targeted cancer therapeutics.1,2 Identifying active metabolic pathways defines the behavior of cancer cells grown under physiological conditions and can identify enzymatic targets for therapy. Several metabolic pathways have previously been proposed as targets for anticancer agents.3–6 Inhibitors for the key enzymes in these pathways have been shown to reduce cancer cell growth and are promising therapeutics.6–11

Multicellular spheroids are circular clusters of cells grown suspended in culture medium.1,12,13 Spheroids mimic the distribution of cell populations in tumors and create more realistic cell–cell interactions than monolayer cultures. Similar to tumors in vivo, most cells in spheroids touch neighboring cells on all sides. In addition, the chaotic vasculature in tumors1,15 creates distinct populations of cells with unique physiologies based on their distance to the nutrient supply.12 The concentric layers of cell in spheroids replicate the distinct metabolic microenvironments that are arranged around blood vessels in tumors.12

The intracellular metabolism of cancer cells grown as spheroids can be rapidly investigated using stable 13C labeling and gas chromatography–mass spectrometry (GC–MS). In a typical isotope experiment, labeled substrates are introduced into cell culture systems and intracellular metabolite labeling is measured. Two common isotope measurement techniques are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy.1,2,16,17 The major advantage of MS is that it requires considerably less cellular material. Comparatively, NMR has the advantage of being able to identify specific labeling positions.1,2,16,19 The smaller sample sizes required for GC–MS enables experiments to be run more rapidly and reduces the use of expensive labeled substrates. Increased sensitivity also considerably reduces required culture sizes, reduces mammalian culture apparatus, and increases culture uniformity. Previously, isotopic labeling and GC–MS has been used to measure metabolism in numerous mammalian systems, including hepatic cells,18–20 cardiac cells,21 and glial cells.22

Here, we describe the use of 13C labeling and GC–MS analysis to investigate the metabolism of transformed fibroblasts cells grown as spheroids. Both small and large spheroids were grown to identify the metabolic differences between proliferating and quiescent cells. Basic metabolic behavior was determined by measuring the rates of metabolite uptake and production. The labeling patterns of cytosolic amino acids were determined and used to identify active and inactive metabolic pathways. Metabolic analysis of cancer cells in physiologically relevant, three-dimensional culture is important because it will help identify critical metabolic pathways and future targets for cancer therapeutics.
Methods

Cell and spheroid culture

Mouse embryonic ras-transformed fibroblasts derived from embryonic stem cells were a kind gift from Dr. R.
Johnson (University of California, San Diego). This artiﬁ-
cially transformed cell line does not directly mimic a speciﬁc
human tumor, but has been shown to form tumors in mice.23
Cells were grown in high-glucose Dulbecco-Modiﬁed Eagle
Medium (DMEM; 4.5 g/L glucose) containing 20 mM
HEPES buffer (pH 7.4) supplemented with 10% fetal bovine
serum. All reagents were obtained from Sigma-Aldrich (St.
Louis, MO) unless otherwise noted. Cultures were main-
tained at 37°C with 5% CO₂ in a humidiﬁed incubator.
Spheroids were formed by inoculating a single-cell suspen-
Sodium bicarbonate (3.7 g/L of each solvent was
used depending on the volume of the extract. The mixtures
were stirred and heated at 70°C for 30 min. This reaction
added a tert-butylidemethylsilyl (rBDMS) group to the car-
boxyl and amine ends of each amino acid25 (Figure 1A).
Analysis was performed by injecting 1.5 µL of derivatized
sample into a quadrupole GC–MS system (HP 5890 GC and
5894 MS). A single injection was made per sample. The GC
was programmed to initiate a thermal ramp starting at 140°C
and increase by 3°C per minute until reaching 250°C. The
mass spectrometer operated in electron impact ionization
mode and fragments were detected with a quadrupole
analyzer.

Correction for natural isotope abundance

The mass spectra for each amino acid were corrected for
the presence of natural isotopes. The isotope distribution
observed for each amino acid was caused by two mecha-
nisms: (1) incorporation of 13C atoms from labeled glucose
in the media and (2) the presence of natural isotopes. To
determine the extent of label incorporation, the distribution
of natural isotopes had to be subtracted from the observed
distribution. This subtraction is possible because natural iso-
tope distribution is constant and predictable.

After ionization in the spectrometer, many molecular frag-
ments are produced for each derivatized amino acid. Two
major ionization fragments are the R-butyl and the R-
COOBDMS fragments (Figure 1A). As examples, these two
fragments of glutamate have molecular weights of 330 and
432. The whole derivatized glutamate molecule contains
three rBDMS groups and weighs 489 g/mol. Because the
rBDMS group is relatively large (Figure 1A), the probability
of a fragment containing a naturally occurring, heavy isotope
of carbon, silicon, oxygen, or hydrogen is quite large. Spe-
ciﬁcally, the chance of the 432 fragment of glutamate con-
taining exactly one heavy atom is 23.3% (m1 in Figure 1B).
Figure 1B shows the entire predicted mass distribution for
unlabeled glutamate fragment 432. During analysis, only the
R-butyl fragment of each amino acid was used because it
was the most predominant. This fragment also provided the
most information, because it contained all of the original car-on atoms.

To determine the extent of labeling four vectors were
defined: The measured mass distribution for each amino
acid (D), the mass distribution of each amino acid frag-
ment due to natural isotope abundance (m), the extent of
labeling from isotopically labeled glucose (M), and a vec-
tor of residual errors (E). For example, the natural isotopic
content of the 432 R-butyl glutamate fragment (m1325) is
shown in Figure 1B. The following equation was used to
account for the distribution of natural isotopes on each la-
beled molecule:
Higher order terms were neglected, because peaks greater than \( M + 4 \) were not detected. The value of the \( M \) matrix was determined iteratively. An initial value was guessed and subsequently adjusted using a nonlinear solver until the sum of least squared errors \( \sum_{i=0}^{4} (E_i)^2 \) was minimized. Statistical significance was determined by averaging the labeling from four experiments and calculating the probability that the labeling fraction was zero.

\[
\begin{align*}
E_0 &= D_0 \\
E_1 &= D_1 \\
E_2 &= D_2 \\
E_3 &= D_3 \\
E_4 &= D_4 \\
(M_0 \cdot m_0 - M_1 \cdot m_1 - M_2 \cdot m_2 - M_3 \cdot m_3 - M_4 \cdot m_4)
\end{align*}
\]  

(1)

**Results and Discussion**

**Spheroid size and extracellular metabolism**

Small and large spheroids contained considerably different populations of cells and had measurably different extracellular metabolism. The average sizes of small and large spheroids were 425 and 755 \( \mu \text{m} \), respectively, and large spheroids contained almost 50% more cells per spheroid (Figure 2). In previous experiments with the same cell line, the thicknesses of the proliferating and quiescent regions were determined to be 75 and 40 \( \mu \text{m} \). Based on these measurements, the ratio of quiescent to proliferating cells in small and large spheroids was 24 and 37%, and large spheroids had 54% more quiescent cells (Figure 2A).

Rates of metabolite uptake and production were determined from extracellular concentration profiles (Figures 2B,C). By convention, \(^1,^2\) the absolute value of all fluxes are reported, i.e., glucose and glutamine were consumed and lactate and glutamate were produced (Figure 2D). Large spheroids had higher rates of glucose consumption and lactate production, and the rates of glutamate production and glutamine consumption were similar (Figure 2D). The differences in the extracellular fluxes of glucose and lactate indicate that larger spheroids have higher rates of glycolysis. However, the ratio of lactate produced per glucose molecule drops from 1.93 to 1.55 as spheroid size increases. There are several possibilities that could explain the decrease in this ratio: (1) increased aerobic metabolism facilitated by available oxygen and reuptake of lactate or 2) diversion of more glucose carbons into the nonoxidative reactions of the pentose phosphate pathway to synthesize ribose.

**Amino acid labeling in spheroids**

In the homogenized and derivatized cell extracts, 16 amino acids were detected at distinct elution times (Figure 3A) and mass spectra were identified with multiple fragmentation patterns (Figure 3B). As a representative example, the predominant glutamate peaks at 432 and 330 (Figure 3B) are the R-tbutyl and the R-COOtBDMS fragments. \(^1,^2^5\) A mass distribution was observed for each fragment (Figures 3C,D). In most fragments \( M, M + 1, M + 2, M + 3, M + 4, \) and \( M + 5 \) peaks were observed. The extent of labeling was determined by accounting for natural isotope abundance. For example, in the mass distribution for glutamate 432 (Figure 3E) the 433 peak contains two species: (1) singly labeled...
glutamate (M + 1, white bar) and (2) unlabeled glutamate (M + 0) with one additional mass unit due to naturally abundant isotopes (black bar). For all amino acids, the extent of labeling was identical for both the R-buty1 and R-COO\textsubscript{t}BDMS fragments.

The amino acid isotopomer distribution was almost identical for small and large spheroids (Figures 4 and 5). For both the essential (Figure 4) and nonessential (Figure 5) amino acids, no significant difference in isotope labeling was observed. This suggests that despite differences in spheroid size, quiescent cell content, and extracellular metabolism (Figure 2), intracellular metabolism was similar for both spheroid sizes. No labeling was observed on any essential amino acids (Figure 4), because as expected, mammalian cells cannot synthesize these. The apparent labeling of lysine was not significantly greater than zero (P = 1.3) and was an experimental aberration. These results with essential amino acids provide a check of the method used to subtract naturally abundant isotopes: The observed mass distributions matched that expected due to natural abundance.

**Labeling of nonessential amino acids**

Labeling patterns observed on nonessential amino acids (Figure 5) suggest which intracellular pathways were active in cells grown as spheroids (Figure 6, Table 1). The metabolic map includes the pathways of primary metabolism, including glycolysis, the tricarboxylic acid (TCA) cycle, transport of molecules in and out of mitochondria, and the synthesis of nonessential amino acids (Figure 6). These results describe the intracellular metabolism of transformed cells under cell culture conditions; in vivo metabolism will vary depending on many variables including time, metabolic microenvironment, and tumor site. In culture, at least one other cell line, MCF7 breast cancer cells, has been shown to have similar patterns of pathway activity.\textsuperscript{1} For analysis, amino acids were grouped into those synthesized from glycolysis metabolites (Figure 5A), and those synthesized from TCA-cycle metabolites (Figure 5B).

The presence of M + 1 labeling on serine (P < 0.01) and cysteine (P < 0.05; Figure 5A) indicates that serine synthesis [pathway (a) in Figure 6] and cysteine synthesis [pathway (b)] are both active. Serine is synthesized from 3-phosphoglycerate by a pathway that includes the enzymes 3-phosphoglycerate dehydrogenase, and phosphoserine phosphatase. The carbon backbone of cysteine is formed from serine by the enzymes cystathionine-\(\beta\)-synthase, and cystathionine-\(\gamma\)-lyase. In spheroids, these cysteine synthesis enzymes were highly active because the cysteine pool contained relatively the same fraction of labeled molecules as the serine pool (Figure 5A). The metabolic precursor 3-phosphoglycerate was most likely labeled on the third carbon following feeding with 1-\textsuperscript{13}C glucose. Synthesis pathways (a) and (b) would have transferred label to the side-chain carbons (carbon 3) of both serine and cysteine. The synthesis of glycine by serine hydroxymethyl-transferase [pathway (c)] removes the side chain carbon (carbon 3) from serine. Lack of glycine labeling could have been caused by removal of labeled carbon or inactivity of pathway (c).
First carbon (M + 1) labeling of alanine (P < 0.01) indicates that alanine aminotransferase [pathway (d)] is active. The M + 1 labeling on alanine (0.403; 99% CI 0.370–0.436; Figure 5A) was significantly (P < 0.01) less than 50%, indicating that pyruvate labeling was diluted. Because alanine is derived directly from pyruvate, the labeling pattern of alanine equals the labeling of pyruvate. Without dilution, the extent of pyruvate M + 1 labeling would have been expected to be 50%, because two pyruvate molecules are synthesized from each glucose molecule. Two mechanisms could contribute to dilution of pyruvate labeling: (1) The pentose phosphate pathway (e) and (2) protein turnover. If the pentose phosphate pathway was active, pyruvate labeling would have been diminished, because glucose-6-phosphate dehydrogenase removes the labeled first carbon from glucose. Protein turnover would have reduced pyruvate labeling by producing unlabeled amino acids. The breakdown of cysteine, serine, glycine, alanine, threonine, and tryptophan would all dilute pyruvate labeling. The M + 1 labeling on alanine indicates that at least one of these two pathways is active; however, GC–MS data alone cannot isolate their individual activities. Based on previous NMR studies, it is probable that both pathways are active.\textsuperscript{1,2}

\textbf{TCA metabolites}

Four of the detected nonessential amino acids are synthesized from TCA cycle metabolites: Aspartate, glutamate, glutamine, and proline (Figure 5B). Aspartate is synthesized from oxaloacetate (OAA), and glutamate, glutamine, and proline are synthesized from z-ketoglutarate (Figure 6). Single (M + 1) labeling was observed for aspartate (P < 0.01), glutamate (P < 0.01), and proline (P < 0.01) indicating that aspartate aminotransferase [pathway (f)], aminotransferase [pathway (g)], and pyroline-2-carboxylate reductase [pathway (h)] were all active. The lack of any labeling on glutamine (Figure 5B) indicates that glutamine synthetase was inactive and not producing glutamine [pathway (i)]. Because glutamine was consumed (Figure 2), glutaminase [pathway (j)] was active in the reverse direction producing glutamate from glutamine.
Two of the detected amino acids, glutamate and proline, had significant \( P < 0.01 \) double labeling \( \left( M + 2 \right) \). The most predominant source of double labeling in primary metabolism is the action of citrate synthase \([\text{pathway (k)}]\), which joins one four-carbon molecule of OAA with one two-carbon molecule of acetyl-CoA. If both molecules are labeled, a doubly labeled molecule of citrate is produced. The double labeling of glutamate and proline indicates that (1) the TCA cycle is active in both small and large spheroids, (2) labeled OAA was produced, and (3) much of the cell’s glutamate and proline are produced from a well-mixed \( \alpha \)-ketoglutarate pool.

The extent of double labeling on glutamate and proline suggests the rate of diluting pathways into TCA-cycle intermediates is proportional to the rate of the TCA cycle. These diluting fluxes include the degradation of unlabeled proteins, the catabolism of many amino acids, and the breakdown of unlabeled fatty acids into acetyl-CoA.\(^1,2,26,27\) If these diluting fluxes were slower than the TCA cycle, then the fraction of doubly labeled metabolites would be greater and there would be more higher order, multiply labeled \( \left( M + 3 \right. \) and \( M + 4 \) \) amino acids. Conversely, if the diluting fluxes were considerably faster, labeled OAA would not be produced and no doubly labeled species would have been observed.

Figure 4. Isotopomer distribution for essential amino acids. Ordinate labels indicate mass number.

Figure 5. Isotopomer distribution for nonessential amino acids produced from (A) glycolysis and (B) TCA-cycle metabolites. Ordinate labels indicate mass number. Singly labeled species \( \left( 1, M + 1 \right) \) were observed for alanine, serine, cysteine, aspartate, glutamate, and proline; and doubly labeled species \( \left( 2, M + 2 \right) \) were observed for glutamate and proline \( \left( ^* P < 0.05; ^* P < 0.01; n = 4 \right) \).
Gluconeogenesis, malic enzyme, and glutaminolysis

The absence of significant double labeling (M + 2) on serine, cysteine, and alanine (Figure 5A) indicates that the pathways that connect TCA-cycle metabolites with glycolysis metabolites were minimally active. The primary connecting pathways are phosphoenolpyruvate carboxykinase (PEPCK) and malic enzyme [pathways (l) and (m), respectively]. PEPCK, the initial enzyme of gluconeogenesis, was completely inactive because serine, cysteine, and hence the combined cytosolic-3-phosphoglycerate (3GP) and phosphoenolpyruvate (PEP) pool did not contain any doubly labeled species. It is not surprising that gluconeogenesis is inactive in cultures adequately supplied with glucose.

The absence of significant double labeling on alanine and pyruvate suggests that extramitochondrial malic enzyme [pathway (m)] was minimally active. Reduced activity of malic enzyme suggests that consumed glutamine carbons were used predominantly for biosynthesis. Glutamine consumption is an anaplerotic reaction that increases the pool of TCA-cycle metabolites. At metabolic steady state, anaplerotic reactions must be balanced by carbon flux out of the mitochondria. Complete oxidation of these carbons to CO₂ requires the activity of PEPCK or malic enzyme to produce pyruvate. When these pathways are inactive, glutamine cannot be converted into pyruvate and glutamine consumption must increase flux to cytosolic pools of citrate and aspartate, which are in turn used to synthesize nucleosides, proteins, lipids, and cholesterol. Although this evidence for glutamine being utilized primarily for biosynthesis is indirect, this phenomenon has been observed previously in multiple cell lines. This observation does not indicate that ras-transformed fibroblasts cannot use glutamine for energy production, only that they are not doing so under these conditions. Similar rates of glutamine consumption in small and large spheroids (Figure 2D), and the absence of any labeling differences (Figure 5) suggests that rates of biosynthesis are similar in both spheroid sizes.

Conclusions

Using GC–MS, we have determined whether 13 critical metabolic pathways are active or inactive (Table 1). GC–MS is a powerful technique because it requires considerably less cell material (100-fold less spheroids) to obtain measurable results compared with alternate techniques, i.e., NMR. The most significant results were that (1) cells actively synthesize serine, cysteine, alanine, aspartate, glutamate, and proline; (2) cells do not synthesize glutamine; (3) glutaminolysis was active; and (4) malic enzyme and gluconeogenesis were inactive. The activities of these pathways suggest that cancer cells consume glutamine for biosynthesis and not to provide cellular energy. Determining which pathways are active under physiological meaningful conditions is useful because

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Primary Enzyme(s)</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Serine synthesis</td>
<td>3-Phosphoglycerate dehydrogenase, phosphoserine phosphatase</td>
<td>Active</td>
</tr>
<tr>
<td>(b) Cysteine synthesis</td>
<td>Cystathionine-β-synthase, cystathionine-γ-lyase</td>
<td>Active</td>
</tr>
<tr>
<td>(c) Glycine synthesis</td>
<td>Serine hydroxymethyl-transferase</td>
<td>N.D.</td>
</tr>
<tr>
<td>(d) Alanine synthesis</td>
<td>Alanine aminotransferase</td>
<td>Active</td>
</tr>
<tr>
<td>(e) Pentose phosphate pathway or</td>
<td>Glucose 6-phosphate dehydrogenase, numerous proteases</td>
<td>Active</td>
</tr>
<tr>
<td>protein turnover</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(f) Aspartate synthesis</td>
<td>Aspartate aminotransferase</td>
<td>Active</td>
</tr>
<tr>
<td>(g) Glutamate synthesis</td>
<td>Glutamate dehydrogenase</td>
<td>Active</td>
</tr>
<tr>
<td>(h) Proline synthesis</td>
<td>Pyroline-2-carboxylate reductase</td>
<td>Active</td>
</tr>
<tr>
<td>(i) Glutamine synthesis</td>
<td>Glutamine synthetase</td>
<td>Inactive</td>
</tr>
<tr>
<td>(j) Glutamine degradation</td>
<td>Glutaminase</td>
<td>Active</td>
</tr>
<tr>
<td>(k) TCA cycle</td>
<td>Citrate synthase</td>
<td>Active</td>
</tr>
<tr>
<td>(l) Gluconeogenesis</td>
<td>Phosphoenolpyruvate carboxykinase</td>
<td>Inactive</td>
</tr>
<tr>
<td>(m) TCA-cycle intermediate catabolism</td>
<td>Malic enzyme</td>
<td>Inactive</td>
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</table>

N.D., not determined.
it indicates how cells direct carbon flow, and because it may lead to the discovery of molecular targets that could act as anticancer therapeutics.

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