Heterogeneity of mitochondrial energy metabolism in classical triphasic Wilms' tumor

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1. ABSTRACT

Metabolic changes are observed in a variety of tumors. The nature of the changes in aerobic energy metabolism differs between tumor types. Therefore, immunohistochemical staining, enzymatic measurements and immunoblot analysis were used to determine alterations of oxidative phosphorylation (OXPHOS) in classic triphasic Wilms’ Tumor (WT). Our studies revealed that the epithelial, stromal and blastemal elements of this tumor differ in their energy metabolism. Compared to unaffected kidney tissue, normal mitochondrial mass was observed in the epithelial and blastemal regions of WT, whereas the stroma showed a massive down-regulation of mitochondria, as indicated by low porin content, low citrate synthase activity, and reduced mtDNA copy number. All OXPHOS enzyme activities were reduced in all WT samples, with the exception of two epithelial-dominant cases, which showed up-regulation of complex III activity compared to control kidney tissues. Interestingly, our studies show that, even within a specific tumor entity, cell-type-specific alterations of aerobic energy metabolism can occur, although all cell types showed a clear tendency toward a reduced aerobic energy metabolism.

2. INTRODUCTION

Wilms’ tumor (WT) affects 1 in 10,000 children, thus accounting for 8% of childhood cancers. It is the fourth most common pediatric malignancy. WT is believed to result from malignant transformation of abnormally persistent renal stem cells that retain embryonic differentiation capacity. The tumors are classically triphasic, including epithelial, blastemal, and mesenchymal or stromal elements. Accordingly, the tumor mass consists of variable proportions of these tissue types, having structures resembling the fetal kidney such as blastema, mesenchymal stroma, and tubules. Stromal type WT often contain ectopic differentiation pattern into muscle, fat, chondrocytes and osteocytes.

As described by Otto Warburg, cancer cells preferentially utilize glycolytic pathways for energy generation while down-regulating their aerobic respiratory activity (1). Even in the presence of sufficient oxygen to fuel respiration, cancer cells predominantly use glycolysis to metabolize glucose. This seems to be a bioenergetic paradox, as glycolysis, when compared to oxidative phosphorylation (OXPHOS), is a less efficient pathway for ATP production (2, 3).
Altered metabolism of cancer cells confers a selective advantage for survival and proliferation in a unique tumor microenvironment. As the early tumor expands, it outgrows the diffusion limits of its local blood supply, leading to hypoxia and stabilization of the hypoxia-inducible transcription factor (HIF) (4-7). As reduced dependence on aerobic respiration becomes advantageous, cell metabolism is shifted toward glycolysis. Indeed, many prominent cancer-related proteins with important functions in cell signalling, transcription or tumor suppression, also perform tasks in cellular energy metabolism. For example, Ras, Akt, Myc and p53 have all been shown to regulate OXPHOS or glycolysis (8-19).

The mechanism by which cellular respiration becomes impaired in a tumor varies with the tumor type. Pheochromocytomas and paragangliomas are associated with succinate dehydrogenase (SDH) mutations. Oncocytomas, in contrast, are characterized by a specific loss of complex I, caused by pathogenic frameshift mutations in the mitochondrial DNA encoding complex I subunits (20, 21) (Zimmermann et al. 2010, this issue). Other tumors, such as renal cell carcinomas and neuroblastomas, exhibit a general down-regulation of the OXPHOS system (22, 23). The aim of the present study was to determine the specific alterations of aerobic energy metabolism in WTs, with attention on the three characteristic histological elements of this tumor type.

3. MATERIALS AND METHODS

3.1. Ethics

The study was approved by the ethics committee of the Medical University of Graz and was performed according to the Austrian Gene Technology Act and in accordance with the Helsinki Declaration of 1975 (revised 1983) and the guidelines of the Salzburg State Ethics Research Committee, being neither a clinical drug trial nor an epidemiological investigation. All patients signed an informed consent concerning the surgical removal and therapy of the tumors. Furthermore, the study did not extend to examination of individual case records. The anonymity of the patients’ samples has been ensured.

3.2. Samples

All tissues were frozen and stored in liquid nitrogen within 30 min after surgery. Tumor cell content and cellular composition of the samples were evaluated using hematoxylin-eosin-stained frozen sections. Tissue samples with a tumor cell content >90% were investigated. WTs from nine patients were obtained from either the University Hospital Salzburg, Austria or the Biobank of the Medical University of Graz, Austria. For immunohistochemical studies, two formalin-fixed paraffin-embedded WTs, from the Department of Pathology, Salzburg, Austria, were used. MtDNA copy number was determined in the nine WTs from the Department of Pathology, Salzburg. Furthermore, six stroma-type WT with a WT-1 mutation and six stroma-type WTs without a WT-1 mutation were kindly provided by the Institute of Human Genetics and Anthropology, Heinrich-Heine University, Duesseldorf for analysis of mtDNA copy number.

3.3. Spectrophotometric detection of OXPHOS enzyme and citrate synthase activities

WT tissues (20-100 mg) were homogenized with a tissue disintegrator (Ultraturrax, IKA, Staufen, Germany) in extraction buffer (20 mM Tris-HCl, pH 7.6, 250 mM sucrose, 40 mM KCl, 2 mM EGTA) and then homogenized with a motor-driven Teflon-glass homogenizer (Potter S, Braun, Melsungen, Germany). The homogenate was centrifuged at 600g for 10 min at 4°C. The postnuclear supernatant (600g homogenate) containing the mitochondrial fraction was used for measurement of enzyme activities and Western blot analysis. Citrate synthase activity was determined according to Serre (24) with modifications. Briefly, the reaction mixture contained 50 mM Tris-HCl, pH 8.1, 0.1% bovine serum albumin (BSA), 0.1% Triton X-100, 0.2 mM 5,5’-dithio-bis(2-nitrobenzoic acid), 0.15 mM acetyl-CoA and the 600g homogenate. After initially recording thiolase activity for 2 min, the citrate synthase reaction was started by addition of 0.5 mM oxaloacetate and was followed at 412 nm for 8 min. The mean nonspecific thiolase activity in neuroblastomas was 2% of the citrate synthase activity.

Enzyme activities of the OXPHOS complexes were determined as previously described (23, 25). Briefly, rotenone-sensitive complex I activity was measured spectrophotometrically as NADH/decylubiquinone oxidoreductase at 340 nm. The enzyme activities of citrate synthase and complex IV (ferrocytochrome c/oxygen oxidoreductase), and the oligomycin-sensitive ATPase activity of the F1F0 ATP synthase were determined by using buffer conditions as previously described by Rustin et al. (26). For the ATPase activity measurement, the whole reaction mixture was treated for 10 seconds with an ultrasonifer (Bio cell disruptor 250, Branson, Vienna, Austria). SDH activity was measured according to Rustin et al. (26) with the following modifications. The reaction mixture contained 50 mM potassium phosphate pH 7.8, 2 mM EDTA, 0.1% BSA, 3 µM rotenone, 80 µM 2,6-dichlorophenol, 50 µM decylubiquinone, 1 µM antimycin A, 0.2 mM ATP, 0.3 mM KCN and the 600g homogenate. The mixture was preincubated for 10 min at 37°C, started by addition of 10 mM succinate, and followed for 6 min at 600 nm.

The reaction mixture for the measurement of the complex III activity contained 50 mM potassium phosphate buffer, pH 7.8, 2 mM EDTA, pH 8.6, 0.3 mM KCN, 100 µM cytochrome c, 200 µM reduced decyl-ubiquinol. The reaction was started by addition of the 600g homogenate. After 3 – 4 min the reaction was inhibited by addition of 1 µM antimycin A. Antimycin A-insensitive activity was subtracted from total activity to calculate complex III activity. All spectrophotometric measurements (Uvicon 922, Kontron, Milan, Italy) were performed at 37°C.

3.4. Western blot analysis

The 600g homogenates were used for Western blot analysis. After protein quantification (Pierce BCA Protein Assay), a total of 5 µg of protein was separated on 10% acrylamide/bisacrylamide gels and transferred to nitrocellulose membranes (HybondTM-C Extra; Amersham...
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Table 1. Enzymatic activity of OXPHOS complexes and cellular composition of Wilms’ tumors (n=9)

<table>
<thead>
<tr>
<th></th>
<th>B-type</th>
<th>M-type</th>
<th>E-type</th>
<th>S-type</th>
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<tbody>
<tr>
<td>kidney (n=14)</td>
<td>2</td>
<td>6</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>110 ± 7</td>
<td>94</td>
<td>99</td>
<td>80</td>
</tr>
<tr>
<td>Complex I [mUnits/mg protein]</td>
<td>43 ± 2</td>
<td>13</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Complex II [mUnits/mg protein]</td>
<td>128 ± 11</td>
<td>12</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>Complex III [mUnits/mg protein]</td>
<td>159 ± 9</td>
<td>124</td>
<td>114</td>
<td>97</td>
</tr>
<tr>
<td>Complex IV [mUnits/mg protein]</td>
<td>160 ± 8</td>
<td>32</td>
<td>43</td>
<td>40</td>
</tr>
<tr>
<td>Complex V [mUnits/mg protein]</td>
<td>41 ± 4</td>
<td>17</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>mtDNA copy number</td>
<td>3910 ± 668</td>
<td>350</td>
<td>197</td>
<td>362</td>
</tr>
<tr>
<td>Blastem [%]</td>
<td>95</td>
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<td>85</td>
<td>70</td>
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<td>Epithelial [%]</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Stroma [%]</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>5</td>
</tr>
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</table>

Values are given as mean ± SEM. In the lower part of the table the cellular composition of the Wilms’ tumor samples is described. B-type: blastema-dominant. M: mixed. E-type: epithelia-dominant. S-type: stroma-dominant.

Biosciences) using CAPS buffer (10 mM 3-[cyclohexylamino]-1-propane sulfonic acid, pH 11; 10% methanol). The membranes were washed in Tris-buffered saline (TBS, pH 7.4) for 5 min, air-dried for 30 min, washed 10 min in TBS and blocked 1 h at room temperature in 5% fat-free milk powder dissolved in TBS. After washing with TBS + 0.1% Triton X-100, the membranes were incubated with the primary antibody solutions. Primary antibodies were diluted in 5% fat-free milk powder dissolved in TBS. The following primary antibody dilutions and incubation times were used: monoclonal mouse anti-SDHA 70 kD antibody (1:15,000, 2h, room temperature (RT); MitoSciences, Eugene, OR, USA), monoclonal mouse anti-core protein 2 antibody (1:2000, 2h, RT; MitoSciences), polyclonal rabbit anti-GAPDH antibody (1:5000, 1h, RT; Roche). After detection of SDHA 70 kD and Core 2, the secondary antibodies as follows: SDHA 70 kD and Core 2 antibodies were used: mouse monoclonal anti-complex I subunit NDUF5 (1:1000; Abcam, Cambridge, UK), mouse monoclonal anti-complex II subunit 70 kDa Fp (1:5000; MitoSciences), mouse monoclonal anti-complex III subunit core protein 2 (1:1500; MitoSciences), mouse monoclonal anti-complex IV subunit I (1:1000; MitoSciences), mouse monoclonal anti-complex V subunit alpha (1:2000; MitoSciences), and mouse monoclonal anti-porin 31HL (1:3000; MitoSciences). All antibodies were diluted in Dako antibody diluent with background-reducing components (Dako, Glostrup, Denmark). Immunohistochemical staining was performed as described previously (21).

3.6. Determination of mtDNA copy number

The mtDNA copy number was determined by quantitative PCR as previously described (27).

4. RESULTS

The activity of citrate synthase, which is a Krebs’ cycle enzyme was measured as a marker of the mitochondrial energy metabolism. The nine histologically distinct WT tissues displayed a massive down-regulation of citrate synthase activity in the stroma-dominant samples (cases 3 and 5; Table 1), resulting in a significant inverse correlation between the percentage of stromal cells in the WT sample and citrate synthase activity (p < 0.05). A weak depression of citrate synthase was observed in those samples in which the epithelial or blastemal parts predominated (cases 1, 2, 6 and 9). Case 8, an epithelium-dominant WT showed an increase in citrate synthase activity compared to normal kidney.

Next we analyzed the enzymatic activities of OXPHOS complexes I-V in tumor tissues and compared them to unaffected normal cortical kidney (Table 1). The activities of complex I, II, IV and V were lower in all WT cases compared to normal kidney (Table 1, Figure 1). The reduction of complex II in all WT samples was confirmed by Western blot analysis (Figure 1). Complex III activity was significantly reduced in blastema- and stroma-type WT samples (Table 1). The two epithelium-dominant WTs exhibited an up-regulation of complex III activity compared to control tissues (Table 1).

To elucidate the changes in the amounts of OXPHOS complexes and the mitochondrial mass at the cellular level in the three histological elements of classical WT, immunohistochemical staining was performed on paraffin-embedded tissue of cases 6 and 8. Staining of porin and the OXPHOS complexes showed both the mitochondrial mass (Figure 2 B-D) and the complexes of the respiratory chain to be strongly reduced in the stromal region (Figure 2 H, L, P, T, X) of these classical triphasic WTs compared to adjacent normal cortical kidney tissue (Figure 2 A, E, I, M, Q, U). Blastemal (Figure 2 G, K, O, S, W) and epithelial parts (Figure 2 F, J, N, R, V) showed stronger staining of the OXPHOS complexes in comparison to the stromal region, but the staining intensities were still less than those of control kidney tissue.

Irrespective of their cellular composition, all WTs (n=9) showed a significant reduction of mtDNA copy number compared to normal cortical kidney tissues (P<0.0001). In accordance with the low mitochondrial
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**Figure 1.** Western blot analysis of Wilms’ tumor and kidney tissue samples. Porin, complex II 70 kDa subunit Fp, GAPDH. K: normal kidney cortex tissue; 1-9 Wilms’ tumor samples.

**Figure 2.** Immunohistochemical analysis of the OXPHOS complexes and porin in a classic triphasic Wilms’ tumor. A, E, I, M, Q, U: cortical kidney tissue; B, F, J, N, R, V: epithelial region of a Wilms’ tumor; C, G, K, O, S, W: blastemal region of a Wilms’ tumor; D, H, L, P, T, X: stromal region of a Wilms’ tumor. A-D: porin; E-H: complex I subunit NDUFS4; I-L: complex II 70kDa subunit Fp; M-P: complex III subunit Core2; Q-T: complex IV subunit I; U-X: complex V subunit alpha. All images were taken from the same formalin-fixed paraffin-embedded tissue section of a classic triphasic Wilms’ tumor and the adjacent unaffected cortical kidney tissue. D: The red arrow highlights single blastemal cells found in the stroma. The black arrow marks the elongated spindle-shaped stromal cells.
content in stromal cells, the mtDNA copy number was lower in S-type tumors (217 +/- 36) than in B- or E-type (429 +/- 70).

In accordance with the low mtDNA content, a reduced porin content in most WTs versus control kidney tissue was detected by Western blot analysis (Figure 1), again indicating reduced mitochondrial mass.

5. DISCUSSION

A massive down-regulation of mitochondrial content was observed in the stromal region of classical triphasic WT compared to normal kidney tissue, whereas the epithelial and blastemal parts predominantly showed a moderate down-regulation of mitochondrial mass, as indicated by the levels of citrate synthase activity and porin expression. These observations are supported by the results of earlier ultrastructural studies of WT, which showed that mesenchymal cells contain few mitochondria while primitive epithelial cells possess numerous mitochondria (28).

The loss of mitochondrial mass and mtDNA in tumors seems to be a feature of stroma-rich tumor types, because ganglioneuroma, a stroma-dominant pediatric tumor, also has a strikingly low mitochondrial content (Feichtinger 2010, this issue). Low OXPHOS activity with retained mitochondria is found in blastemal cells of undifferentiated neuroblastoma (22), which is similar to our findings in the blastemal and epithelial parts of WT. Renal cell carcinoma, which is an epithelial neoplasm, also has low OXPHOS in spite of normal mitochondrial levels (23). The low mitochondrial content in stroma-rich tumors might explain their low susceptibility to apoptosis induced by chemotherapy. This is consistent with the fact that stroma-predominant WTs show a poor clinical response to chemotherapy (29-31). Furthermore, other stroma-rich tumors such as ganglioneuromas, breast cancer and pancreatic cancer have been reported to exhibit resistance to neoadjuvant chemotherapy (32-35).

Mutations in the transcription factor Wilms' tumor-1 (WT-1) have been found to be associated with WT in about 10-15% of patients. WT-1 plays a role in the mesenchymal-epithelial transition (MET) in the embryonic kidney (36), by inducing epithelial differentiation and E-cadherin expression (36-38). Baudry et al. found low E-cadherin expression in 72% of WTs (39), which in most cases could not be explained by a lack of WT-1 in WT. The expression of E-cadherin in primary WT shows a moderate to high degree of staining in dysplastic tubules, weaker staining in the blastema, and negligible staining in the stroma (40), which is strikingly similar to the pattern of expression of OXPHOS complexes in WT.

Interestingly, loss or reduction of mtDNA results in hypermethylation of the E-cadherin gene promoter (41). LNrh0-8 and MCFrh0 cells are cell lines derived from epithelial cancer cell lines that lose their epithelial features and gain a mesenchymal phenotype by EMT during mtDNA depletion. In both cell lines, repression of E-cadherin and induction of vimentin expression were observed after mtDNA depletion. In addition, the down-regulation of E-cadherin was shown to be due to hypermethylation of its promoter region in LNrh0-8 and MCFrh0 cells (42). We assume that the reduction of mtDNA copy number leading to a decline in OXPHOS activity in WT induces epigenetic changes in nuclear DNA, influencing EMT. MtDNA copy number in WT is not dependent on the presence of WT-1 mutations, since we did not find differences in mtDNA copy number in WT-1-positive and WT-1-negative tumor samples (data not shown). Because only 10-15% of sporadic (43, 44) WTs harbor a WT-1 mutation, but all show low mtDNA levels, low mtDNA and OXPHOS might be the main trigger for low E-cadherin expression.

Despite the role that OXPHOS might play in EMT, it is well known that the respiratory chain regulates apoptosis. Respiratory chain dysfunction in cancer cells may confer resistance to apoptotic cell death. It was recently shown that both partial as well as complete loss of the respiratory chain protects cells from mitochondrial-initiated apoptosis (45).

The mechanisms regulating mitochondrial content in EMT of WT remain to be elucidated. One candidate is p53, which is an important regulator of mitochondrial energy metabolism and glycolysis (46). Loss of wild-type p53 or aberrant expression of mutant p53 is indicative of progression of WT (47-49).

In summary, WT is an example of differential regulation of aerobic energy metabolism within tumor tissues. The transformation to an anaerobic phenotype accompanied by low activity of the OXPHOS system is present in all three histological elements of classical triphasic WT. However, a concomitant deficit of mitochondria is found only in the stromal compartment. The decline of mitochondrial mass that occurs during stromal differentiation might also explain, in part, the resistance of stroma-dominant WT to chemotherapy.

6. ACKNOWLEDGMENTS

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7. REFERENCES

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Key Words: OXPHOS, Respiratory Chain, Stromal, Blastema, Epithelial Mesenchymal Transition

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