Mitochondrial complex I is deficient in renal oncocytomas

Hélène Simonnet¹,⁴, Jocelyne Demont¹, Kathy Pfeiffer², Leila Gueneache¹, Raymonde Bouvier³, Ulrich Brandt², Hermann Schägger² and Catherine Godinot¹

¹CGMC (Center of Molecular and Cell Genetics), Unit 5534 of the CNRS and the University Lyon 1 Claude Bernard, Villeurbanne, France, ²Biochemie I, ZBC, Universitätssklinikum, Frankfurt am Main, Germany and ³Service of Anatomy and of Cytopathology, Hôpital Edouard Herriot, Lyon, France

Renal oncocytomas are benign tumors characterized by dense accumulation of mitochondria the cause of which remains unknown so far. Consistently, mitochondrial DNA content and the amounts and catalytic activities of several oxidative phosphorylation (OXPHOS) complexes were known to be increased in these tumors, but it was not ascertained that the OXPHOS system was functional. Here we investigated mitochondrial complex I and found that its NADH dehydrogenase activity and protein content were specifically decreased in oncocytomas, in stark contrast with the parallel decrease of all respiratory chain complexes in other, malignant, renal tumors. We conclude that deficiency of complex I in oncocytomas might be the early event causing the increased mitochondrial biogenesis, attempting to compensate for the loss of OXPHOS function. Since other tumors were found to be linked to mitochondrial deficiencies like genetic alterations of fumarate hydratase or succinate dehydrogenase, oncocytoma could be the third type of benign tumor associated with impairment of mitochondrial ATP production in an oxidative, quiescent tissue. Besides, complex I enzyme activity was moderately decreased in the vicinity of oncocytomas, when compared with normal tissue adjacent to other renal tumors. This suggested that oncocytomas are the result of at least two serial modifications altering the mitochondrial respiratory chain.

Introduction

The different types of renal tumors are caused by various specific genomic alterations determining their phenotype and their aggressiveness. Oncocytomas are benign tumors characterized by dense cytoplasmic accumulation of mitochondria (1) the cause of which remains to be elucidated. They also occur in thyroid gland (Hurthle cell tumor), salivary gland and in adrenal gland (2). Renal oncocytomas originate from the collecting duct (3). They are associated with heterogeneous chromosomal changes: a first subgroup includes tumors with frequent loss of chromosomes Y and 1, another one includes translocations comprising the breakpoint region 11q13, and a third subgroup comprises tumors with various genetic abnormalities such as monosomies, trisomies and loss of heterozygosity in several chromosomes. Mutations of the tumor suppressors VHL or p53 are infrequent or absent (1). In spite of this genetic variability, phenotypes are remarkably similar, with a low mitotic index, leading in the quiescent kidney parenchyma to a clear-brown tumor and, under the microscope, a granular, eosinophilic cytoplasm stacked up with mitochondria (1,4).

The most frequent tumors in kidney are clear cell renal cell carcinomas (CCRCCs), originating from the proximal tubule. Their common feature is abnormality of the tumor suppressor protein pVHL (von Hippel-Lindau) (1,5), a ubiquitin-ligase down-regulating HIF-1 (the hypoxia-inducible transcription factor) (6). Their metastatic capacity and resistance to antimitotic drugs make them very aggressive (7). Chromophilic (or tubulopapillary) tumors are renal cell carcinomas less aggressive than CCRCCs, and also originate from the proximal tubule. They are due to a constitutive activation of the proto-oncogene product c-MET, the hepatocyte growth factor receptor (8).

When kidney tumors were tested for their oxidative phosphorylation (OXPHOS) capacity, a striking inverse correlation was found between several OXPHOS enzymes (activity and protein content) and the aggressiveness of the tumor type (9). Namely, complex IV (cytochrome c oxidase, or COX), complex II (succinate dehydrogenase), complexes II and III and complex V (ATPase/ATP-Synthase), were negatively correlated with the seriousness of the tumor type and with its Fuhrman’s grade. All these activities were decreased in malignant carcinomas and increased in the benign oncocytomas. The high mtDNA and citrate synthase contents in oncocytomas reflected their dense accumulation of mitochondria.

The cause of mitochondria accumulation in oncocytomas was so far unknown, but a similar feature has already been observed in mitochondrial pathologies. In fact, aggregations of abnormal mitochondria under the sarcolemma of type I fibers has been described as early as 1962 by Luft et al. (10). The proliferation of these abnormal mitochondria has been named ‘ragged red fibers’ (RRF) (11). RRF is a characteristic histological feature of skeletal muscle presenting mtDNA deletions, duplications or mitochondrial tRNA point mutations and it is generally accepted that the proliferation of mitochondria in this pathology is initiated by a deficient mitochondrial ATP synthesis, and followed by an up-regulation mechanism increasing the transcription of nuclear genes coding for mitochondrial proteins (12). Therefore, the question was whether accumulated mitochondria in renal oncocytomas are fully functional or if they are the result of an attempt to restore some deficiency. In thyroid oncocytomas, Savagner et al. (13) have shown that oxygen consumption is defective in spite of the mitochondria accumulation. In our previous studies (9), complex I activity had not been analyzed, but two dimensional-PAGE performed on one oncocytoma suggested that

Abbreviations: CCRCCs, clear cell renal cell carcinomas; OXPHOS, oxidative phosphorylation; pVHL, von Hippel-Lindau tumor suppressor protein.
complex I might be decreased while all other OXPHOS complexes were increased.

In the present work, we focused on the analysis of respiratory chain complex I, measuring its NADH dehydrogenase activity, the complex I protein content, and the immunoreactivity of its 75 kDa subunit in oncocytomas, in chromophilic tumors, in CCRCCs, and in their respective adjacent normal tissue. The results confirm that there is a specific deficiency of complex I in oncocytomas.

Materials and methods

Biopsies

Kidneys were surgically removed for renal tumor. After examination by the pathologist, normal cortex and tumor tissue samples (100–400 mg) were dissected and immediately frozen in liquid nitrogen and stored until utilization. Tumor grades were determined according to the Fuhrman’s classification (4).

NADH dehydrogenase enzyme activity

Frozen tissue samples (50–150 mg) were minced on a cooled glass plate and then homogenized with a Teflon or a glass Potter in 200–500 µl of medium containing 66 mM sucrose, 222 mM mannitol, 10 mM HEPEs buffered with KOH at pH 7.4, 1.2 mg/ml bovine serum albumin (fatty acid free), 1.1 mM EDTA, 0.04 mM pepstatin, and 0.4 mM epsilon-amino caproic acid. The remaining debris were sedimented by a pulse centrifugation and the supernatant homogenate was passed through a 16-gauge needle. NADH dehydrogenase enzyme activity determinations were performed using the spectrophotometric methods of Rustin et al. (14). Briefly, 1–20 µl homogenate were osmotically lysed in 0.8 M water for 3 min at 37°C, then 0.2 M NADH in 10 mM Tris–HCl, pH 8.0, 0.05 mM decylubiquinone and 0.3 mM KCN were added. The total NADH: decylubiquinone oxidoreductase activity was recorded at 340 nm for >2 min, then the appropriate inhibitor was added to the cuvette. Mitochondrial complex I activity was inhibited by 0.3 µM rotenone. In preliminary studies, this concentration was found to be the lowest dose able to induce maximal inhibition of NADH dehydrogenase in normal kidney cortex, this tissue containing one of the largest amounts of mitochondria. Non-mitochondrial NADH oxidoreductases were also determined using another specific inhibitor of the respiratory chain, KCN. NADH:O2 oxidoreductase activity was recorded in the absence of decylubiquinone and of KCN. The contributing mitochondrial activity related to endogenous cytochrome c was then inhibited by 0.3 mM KCN.

Citrate synthase. Citrate synthase activity was determined according to Srere (15).

Protein content. Protein content was determined using the Bradford Coomassie reagent from BioradTM.

Two-dimensional resolution of the OXPHOS complexes

Crude mitochondria from 10 mg of kidney (wt weight) were solubilized by dodecylmaltoside and the native OXPHOS complexes separated by Blue Native-PAGE using a 5–13% acrylamide gradient gel, as described previously (16). The subunits of the complexes were then resolved in a second dimension by a 16% acrylamide, Tricine–SDS gel, and Coomassie stained (16).

Immunological detection of the 75 kDa subunit

Five percent each of the crude mitochondrial extracts used for BN-PAGE and two-dimensional resolution as described above were used for SDS–PAGE corresponding to 0.5 mg kidney tissue (or ~15 µg extracted mitochondrial protein). The mitochondrial proteins were resolved by a 10% acrylamide SDS gel according to Laemmli (17), and transferred to PVDF membranes according to Schägger (18). The PVDF membrane was incubated first with a polyclonal rabbit antibody against the 75 kDa subunit of bovine complex I, and secondly with a peroxidase-labeled anti-rabbit antibody. The peroxidase activity was then monitored using the ECL reagent according to the manufacturer’s instructions (Amersham Pharmacia BiotechTM, Uppsala, Sweden).

Results

Specific decrease of mitochondrial complex I in oncocytomas

The enzyme activity of respiratory complex I was determined as the NADH: decylubiquinone reductase activity that could be suppressed by 0.3 µM rotenone. Figure 1 shows that complex I activity was decreased in each of the five tested oncocytomas.

Similarly, we found decreased complex I protein amounts in all tested oncocytomas, when mitochondria were analyzed by two-dimensional PAGE, again in stark contrast to the increase in other complexes such as cytochrome oxidase (complex IV) or ATP synthase (complex V) (Figure 2). To better visualize the reduction of complex I in oncocytomas, we used an antibody against the 75 kDa subunit of complex I for immunodetection. A marked decrease of the 75 kDa subunit again indicated complex I deficiency in each of the seven tested oncocytomas (Figure 3).

Altogether a pronounced complex I deficiency was evident in all oncocytomas tested (n = 8) although the enzymatic, electrophoretic or immunologic analyses could not be performed in some of the patients because of the small size of some samples.

Complex I activity in different types of renal tumors and in adjacent tissues

Mitochondrial complex I enzyme activity was measured in several types of renal tumors and compared with non-tumoral tissue from the same kidneys, termed normal tissue. The results are shown in Figure 4 and in Table I.

In normal tissue of tumor-bearing kidneys, the rotenone-sensitive NADH: decylubiquinone oxidoreductase activities were (nmol min⁻¹ mg protein⁻¹): 50.9 ± 15.2 (n = 5) in tissues adjacent to low grade CCRCCs, 79.3 ± 67.2 (n = 5) in tissues adjacent to high grade CCRCCs, 50.7 ± 11.2 (n = 3) in the vicinity of chromophilic renal cell carcinomas, and only 21.2 ± 6.1 (n = 5) in tissues adjacent to renal oncocytomas (Figure 4). Therefore, there is a significant reduction of NADH dehydrogenase in the vicinity of oncocytomas (P < 0.05, according to the Kruskall and Wallis’ non-parametric test). Similarly, the CN-sensitive NADH:O2 oxidoreductase activity was reduced in tissues adjacent to oncocytomas (P < 0.05, Kruskall and Wallis’ test) (Table I).
Fig. 2. Two-dimensional resolution of OXPHOS complexes from oncocytomas by Blue Native PAGE/SDS-PAGE. Mitochondria were extracted from 10 mg of frozen tissue from four oncocytomas (right) and from their four normal counterparts (left) and were submitted to two-dimensional PAGE, firstly under non-denaturing conditions (horizontally), and secondly under SDS-denaturing conditions separating the protein subunits (vertically). The location of complex I is indicated by the boxed area. The largest and most intensely stained complex I subunits (75, 51 and 42 kDa) were detected in all normal tissues adjacent to oncocytoma tissue (left) but were below the detection limit in all oncocytomas (right). (A–D) correspond to oncocytoma patients 3 and 4, and to new patients 5 and 6.
In tumor samples, the rotenone-sensitive, NADH:decylubiquinone oxidoreductase activity was decreased in each of the 18 samples tested from the four types of renal tumors. The mean values were (in nmol min$^{-1}$ mg protein$^{-1}$): 23.4 ± 8.3 (5) in low-grade CCRCCs, 12.0 ± 11.7 (5) in high-grade CCRCCs, 15.2 ± 6.8 (3) in chromophilic tumors and 13.8 ± 5.2 (5) in oncocytomas. The average in all tumor tissues was 32 ± 20% of the value in normal corresponding tissue. In addition, a western blot followed by immunodetection of the 75 kDa subunit of complex I evidenced a marked decrease of the tumor protein amount for each of the 18 samples from four types of renal tumors (Figures 3 and 5), in oncocytomas and in malignant renal cell carcinomas.

These results showed that, in contrast with the parallel decrease of all OXPHOS complexes in renal carcinomas (9 and this work), there is an isolated complex I deficiency in oncocytomas.

**Discussion**

This work shows for the first time that one of the OXPHOS complexes, complex I, is selectively and constantly decreased in renal oncocytomas. This comes in stark contrast to the...
grades 1 and 2: lanes 1–5; grades 3 and 4: lanes 6–9; chromophilic tumors: lanes 10 and 11.

Fig. 5. Generally decreased amounts of the 75 kDa protein subunit of respiratory complex I in malignant renal carcinomas. The 75 kDa subunit of complex I was immunodetected in tumor tissues (T) and in their normal counterpart (N) from the same kidney, as described in Figure 3. Clear cell renal cell carcinoma, oncocytomas. In agreement, decreased oxygen consumption oxidative phosphorylation cannot be fully functional, although NADH oxidation, expressed in nmol min⁻¹ mg protein⁻¹ (mean ± SD), was first measured in the absence of exogenous decylubiquinone and of KCN as described in Materials and methods, and secondly after addition of KCN in the cuvette. The CN-sensitive activity represents the difference between the rates measured before and after KCN addition. CCRCC: clear cell renal cell carcinoma, low- (low) or high-grade (high).

Table I. Cyanide-sensitive oxidation of NADH in renal tumors and their adjacent normal tissue

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<thead>
<tr>
<th></th>
<th>With KCN</th>
<th>CN-sensitive</th>
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<tr>
<td></td>
<td>NADH:O₂ oxidase</td>
<td>Tumor</td>
</tr>
<tr>
<td></td>
<td>Without KCN</td>
<td></td>
</tr>
<tr>
<td>CCRCC, low</td>
<td>38.4 ± 17.3 (5)</td>
<td>28.0 ± 10.0 (5)</td>
</tr>
<tr>
<td>CCRCC, high</td>
<td>35.8 ± 24.2 (5)</td>
<td>25.8 ± 20.2 (5)</td>
</tr>
<tr>
<td>Chromophilic</td>
<td>18.9 ± 11.7 (3)</td>
<td>14.0 ± 10.0 (3)</td>
</tr>
<tr>
<td>Oncocytomas</td>
<td>9.4 ± 3.5 (5)</td>
<td>3.8 ± 4.4 (5)</td>
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parallel decrease of respiratory chain complexes observed in other, malignant, renal tumors. Since complex I is deficient, oxidative phosphorylation cannot be fully functional, although all other OXPHOS complexes were found to be increased in oncocytomas. In agreement, decreased oxygen consumption and defective mitochondrial ATP production was reported by Savagner et al. (13) in another type of oncocytoma, the thyroid oxyphilic tumors. Hence the induction of mitochondrial biogenesis, of citrate synthase and of mitochondrial DNA/nuclear DNA in oncocytomas (3,4,9) is not likely to be a primary event, but rather a response mechanism attempting to compensate for inefficient oxidative phosphorylation. It is reminiscent of the increased biogenesis of abnormal mitochondria occurring in response to alterations of mtDNA in mitochondrial pathologies (12) and suggests that the decrease of complex I protein and activity in renal oncocytomas could be causal to the accumulation of mitochondria, the hallmark of this relatively benign tumor.

The correlation between complex I deficiency and tumoral growth arises two major questions. Is tumor formation due to the defective complex I or to some other unidentified alteration? And how can such a heterogeneous group of genetic alterations as that observed in oncocytomas (1,19) be related to the observed unique complex I deficiency?

The respiratory chain NADH dehydrogenase is the largest OXPHOS complex. It is L-shaped and made up of at least 45 different subunits as reviewed by Kerscher et al. (20), Walker (21), Schoffner and Wallace (22), Smeitink et al. (23), Chomyn (24) and Duborjal et al. (25). Although the mechanism of electron transport and proton translocation by complex I are not elucidated so far, all genes coding for the human subunits have been sequenced, including the seven genes encoded by the mitochondrial DNA and the 36 nuclear genes (23). Genetic alteration in any of these subunits could result in the same complex I deficiency if the modified protein could not be used for assembly of the complex, or if the stability of the complex was disturbed. Factors, which are very likely required for assembly of complex I but not found in the assembled complex, are further candidates for genetic alterations that would also result in complex I deficiency.

Previous studies (26) provided no evidence that mtDNA alterations were responsible for the pathogenesis of oncocytomas. For example, a specific deletion (‘common deletion’), which can be observed in mitochondrial disorders, was no more frequent in tumors than in normal tissues. In addition the mtDNA size estimated by segmental amplification (26) or by Southern blot (9) was found to be normal indicating that large mtDNA deletions are not commonly present in these tumors. However systematic sequencing of mitochondria-encoded complex I genes has never been performed in oncocytomas.

Nuclear gene sequences encoding and regulating the expression of complex I subunits and of their known assembly factors have also not been analyzed in oncocytomas so far. However, a great number of nuclear chromosomal rearrangements have been described (1). As none of these different genetic alterations was found in all or even a majority of the tested oncocytomas (1,26), it seems possible that they end at a common unique deficiency of complex I assembly, by affecting one of the 36 nuclear genes coding for complex I subunits that are scattered over many different chromosomes (23). Sequencing the 43 genes encoding complex I in several patients bearing oncocytomas represents a hard task but is now under progress in our laboratory. Alternatively, a unique, genetic defect specific to oncocytomas could have remained undetected until now. In the latter case, the observed nuclear genome alterations would be additional defects, since additional variable genetic events are frequent in other adult renal tumor types (1). The genetic analysis of familial forms of oncocytomas (19,27) should bring out important information to address this question. Moreover, it might be advisable to search for an additional alteration in the tumors itself but in adjacent areas, since tissue adjacent to oncocytomas differed from normal renal parenchyma with respect to complex I activity. This
alteration is detectable in enzyme activity but not in protein content (see Figures 2 and 4), and is not associated with mitochondria accumulation. It could be induced by oncocy-toma, but more probably represents an early event. Indeed, it is again restricted to complex I activity since the other OXPHOS activities were not decreased in tissues adjacent to oncocyto-mas (9). Identification of the gene(s) involved in tumor patho-genesis could help to understand whether complex I deficiency is a required step in tumoral growth of oncocytomas.

Complex I decrease could be caused by a side effect of another alteration also inducing a loss of growth control by an independent mechanism. However, it seems feasible that complex I deficiency itself could cause directly or indirectly the increase of cell growth since other genetic defects affecting mitochondria have been recently found to be associated with benign tumors. A first example is fumarate hydratase familial impairment, which could cause an imbalance of NADH supply to the respiratory chain, and which is assumed to be a true tumor suppressor gene in familial uterine fibroid tumors (28). A second example is the familial impairment of several subunits of respiratory chain complex II, which leads to a unique phenotype of paraganglioma (29,30). The common feature of these tissues (kidney, uterine myometrium and parasympa-thetic ganglia) is their low rate of renewal. Interestingly, both myometrum and kidney are susceptible to develop tumors caused by alteration of a cell cycle inhibitor and tumor suppressor gene, TSC-2 (31,32), showing that they share at least in part the same pathway of cell cycle regulation. If genetic defects could be disclosed in the genes encoding the complex I subunits and if reproducing these defects in an ex vivo model could increase mitochondrial biogenesis and tumorigenic capacity, it would be a third example for a defect associated with mitochondrial ATP production that induces a benign tumor growth in a quiescent tissue type.

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