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Functional and molecular analysis of mitochondria in thyroid oncocytoma

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Summary. We report a functional and molecular analysis of nine oncocytic tumors of the human thyroid. In all the abundance of mitochondria observed ultrastructurally was accompanied by an increase in enzymatic activities of respiratory complexes I (NADH dehydrogenase), II (succinate dehydrogenase) IV (cytochrome c oxidase), and V (ATPase). Western blot analysis failed to detect uncoupling protein in the tumors. The elevated respiratory enzyme activities were paralleled by an increase in the mitochondrial DNA content. Restriction analysis of mitochondrial DNA gave no indication of heteroplasmy or other gross alterations. We conclude that the mitochondrial proliferation in oncocytic tumors is probably not the result of a compensatory mechanism for the deficiency in enzyme complexes of the mitochondrial respiratory chain.

Key words: Mitochondria – Mitochondrial DNA – Respiratory enzymes – Uncoupling protein – Thyroid oncocytic tumor

Introduction

Oncocytic change has been reported in a variety of human tissues and in some epithelial neoplasms and tumors of the kidney, salivary gland and thyroid (Cotton 1990; Hamperl 1936; 1962). The common feature of oncocytic tumors is an extreme abundance of mitochondria within the transformed cells that is often associated with increased activity of a number of enzymes, including cytochrome c oxidase, succinate dehydrogenase, DPNH- and TPNH diaphorase (Fischer 1961; Lindsay and Arico 1963; Tandler 1966; Tremblay and Pearse 1959). Additionally, ultrastructural studies of oncocytic tumors have shown that these organelles are often morphologically abnormal (Eble and Hull 1984; Goebel et al. 1980; Tandler 1966). Morphological changes in mitochondria have sometimes been accompanied by mitochondrial malfunction (Capaldi 1988) and it has been suggested that mitochondrial proliferation may represent a mechanism to compensate for this mitochondrial deficiency (Fischer 1961; Altmann 1990), like the physiological response to elevated energy demand (Williams 1986). This idea is further supported by the observation that myopathies resulting from a mitochondrial disorder are often characterized by increased numbers of these organelles (for review see: Capaldi 1988).

Mitochondrial accumulation requires the expression of both nuclear and mitochondrial encoded proteins (for review see: Attardi and Schatz 1988) although the factors triggering this event are unknown.

Thyroid hormone, which has been implicated in mitochondrial biogenesis (Nelson 1987), is immunologically detectable in less than one-third of oncocytic tissues, even in the thyroid (Schaeffer and Ormanns 1983). While this argues against a close association between synthesis of thyroid hormone and oncocytic change, it does not exclude the possibility that oncocytomas are especially responsive to the hormone due to the expression of the respective receptors. Probably differential expression of such receptors accounts for the tissue-specific influence on mitochondrial abundance by thyroid hormone. While hypothyroid animals show a decrease in muscle mitochondria (Dudley et al. 1987), no change in the number of those organelles is observed in the liver in either hypo-, or hyperthroid rats (Jacovcic et al. 1978). To our knowledge there are no reports of a correlation between hyperthyroidism and oncocytic change in thyroid tumors.

The present study is designed to gain further insight in the molecular characteristics of oncocytic tumors. In a comparative study, the ultrastructure and respiratory enzyme activities of several oncocytic tumors of the thyroid have been related to a qualitative and quantitative analysis of mitochondrial DNA (mtDNA), which encodes some essential subunits of the oligomeric complexes of the respiratory chain (for a review see: Attardi and Schatz 1988). Only recently, a number of mitochon-

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drial disorders have been found to result from structural rearrangements or point mutations in mtDNA (Holt et al. 1988, 1989; Poulton et al. 1989). In two renal oncocytic tumors, a restriction fragment length polymorphism in mtDNA was detected (Kovacs et al. 1989) the functional significance of which, however, remains to be substantiated by further studies.

Material and methods

Surgically resected oncocytic thyroid tumors from nine patients were sampled. In two instances tumor and control tissue were obtained from the same patients and thyroid tissue from autopsies was also used for controls.

Fresh tumor tissue was immediately frozen at −70° C and the diagnosis was confirmed later following the examination of paraffin sections stained by HE.

Electron microscopy. For electron microscopy tissues were fixed in 3.5% glutaraldehyde and 1% osmium tetroxide in sodium phosphate buffer, pH 7.3. After dehydration, tissues were stained with uranyl acetate in 96% ethanol, and embedded in Araldite (Serva, Heidelberg). Ultrathin sections were cut with an LKB Ultrotome (Type 8802A) ultramicrotome, stained with lead citrate, and examined in a Zeiss EM10 electron microscope.

Enzyme histochemistry. For histochemical studies, 10 µm serial sections were cut on a cryostat (−18° C), placed on clean slides, and dried briefly at room temperature. The sections were then incubated in appropriate substrates for the demonstration of the following enzymes: cytochrome c oxidase, succinate dehydrogenase, total NADH dehydrogenases, and adenosine triphosphate synthase (ATPase). Reactions in the presence or absence of inhibitors and control incubations were performed according to Loijda et al. (1976).

Western blot analysis. Mitochondria were isolated from tumor tissues and controls according to the "two step procedure" of Bogenhagen and Clayton (1974). Mitochondria of brown fat tissue obtained at post-mortem examination of a newborn baby served as a control for the presence of uncoupling protein (UCP) in human mitochondria.

Mitochondrial proteins were separated on a 12% SDS-polyacrylamide gel (Laemmli 1970). Western blotting and immune decoration was carried out as described earlier (Ebner et al. 1988). Rabbit-anti-hamster-UCP-serum was used (1:200 dilution) to detect human UCP.

DNA extraction. Total cellular DNA was isolated from tumor tissues or controls according to standard procedures (Ausubel and Frederick 1987). Tissue samples were rinsed in phosphate-buffered saline (PBS), cut into small pieces and homogenized. Homogenates were incubated at 37° C in ten volumes of 10 mM Tris/HCl, pH 7.4, 10 mM EDTA, 150 mM NaCl, 1 mg/ml Proteinase K, and 0.4% SDS until completely lysed. After phenol extraction, DNA was precipitated with two volumes ethanol.

DNA probes. HeLa cell mtDNA was isolated from mitochondria which had been purified by the "two step procedure" of Bogenhagen and Clayton (1974). Recombinant plasmid pmt34 containing HeLa cell mtDNA fragment pos. 10254–12878, according to the nomenclature of Anderson et al. (1981), was generated by PCR amplification (Saiki et al. 1988) using 22-mer oligonucleotide primers and a geneAmp®-kit obtained from Perkin-Elmer (Norwalk, CT). The amplified mtDNA segment was cloned as a XbaI/EcoRV-restriction fragment into the bluescript plasmid (Stratagene, La Jolla, Calif) pAct1, a derivative of pBR322 with a 1250 bp PstI-fragment of hamster actin cDNA, was used to detect the human single copy actin gene (Dodemont et al. 1982).

DNA probes were labeled with [^{32}P]-dATP (3000 Ci/mmole) using the random primed DNA labeling kit obtained from Boehringer (Mannheim).

Quantitation of mtDNA. The proportion of mtDNA for tumor and control samples was determined in relation to nuclear DNA. Samples (10 µg) of total cellular DNA were digested with PvuII, separated on an 1% agarose gel and blotted on an Hybond N filter (Amersham, Braunschweig, FRG). The filter was hybridized to labeled pAct1 nuclear gene probe and autoradiographed. Afterwards, the filter was rehybridized to labeled pmt34 mtDNA probe. DNA was quantitated by desitometric scanning of the hybridization signals with an LKB 2222-010 UltroScan XL densitometer. The signal ratio of mtDNA to actin DNA was determined and compared with that of controls.

Southern blot analysis. Samples (10 µg) of total cellular DNA were digested with several restriction endonucleases according to the specifications of the manufacturers. DNA restriction fragments were electrophoretically separated on 0.8% agarose gels, blotted onto Hybond N filters and hybridized to labeled HeLa mtDNA or pmt34, respectively.

Results

Ultrastructure. Six of the seven tumors examined revealed a cell morphology very similar to that shown in Fig. 1. Numerous mitochondria, most showing structural anomalies, were evenly distributed in the cytoplasm, while other organelles were hardly visible. The most striking features were mitochondrial cristae arranged in parallel stacks within the organelle, or swollen mitochondria with desintegrating cristae. All types of structural aberrations were found within a single tumor, but within an individual cell only one discrete morphological shape of mitochondria was observed (Fig. 1). In one of the tumors (no. 7, see Table 1), single cells containing only few mitochondria were located amongst the majority of typical mitochondria-rich cells. In one case (tumor 8) no increase in the number of mitochondria was found.

Enzyme histochemistry

Respiratory enzyme activities in tumors and control tissues were graded arbitrarily on a scale of + (=dye deposition within controls) to +++ (strong staining within tumor cells; see Fig. 2 and Table 1). High levels, compared with controls, of cytochrome c oxidase (complex IV), succinate dehydrogenase (complex II) and NADH dehydrogenase (complex I) as well as moderately elevated levels of mitochondrial ATPase in the oncocytic cells were observed. While most of the tumors consisted exclusively of cells with elevated enzyme levels, some tumors showed highly reactive foci surrounded by cells with normal enzyme activities (nos. 3, 4, 8). In one tumor (no. 7) small areas of histochemically inert cells were interspersed within the majority of cells which showed high activities for all the enzymes tested. No differences in histochemical enzyme activities were found between oncocytic adenomas and carcinomas (Table 1).

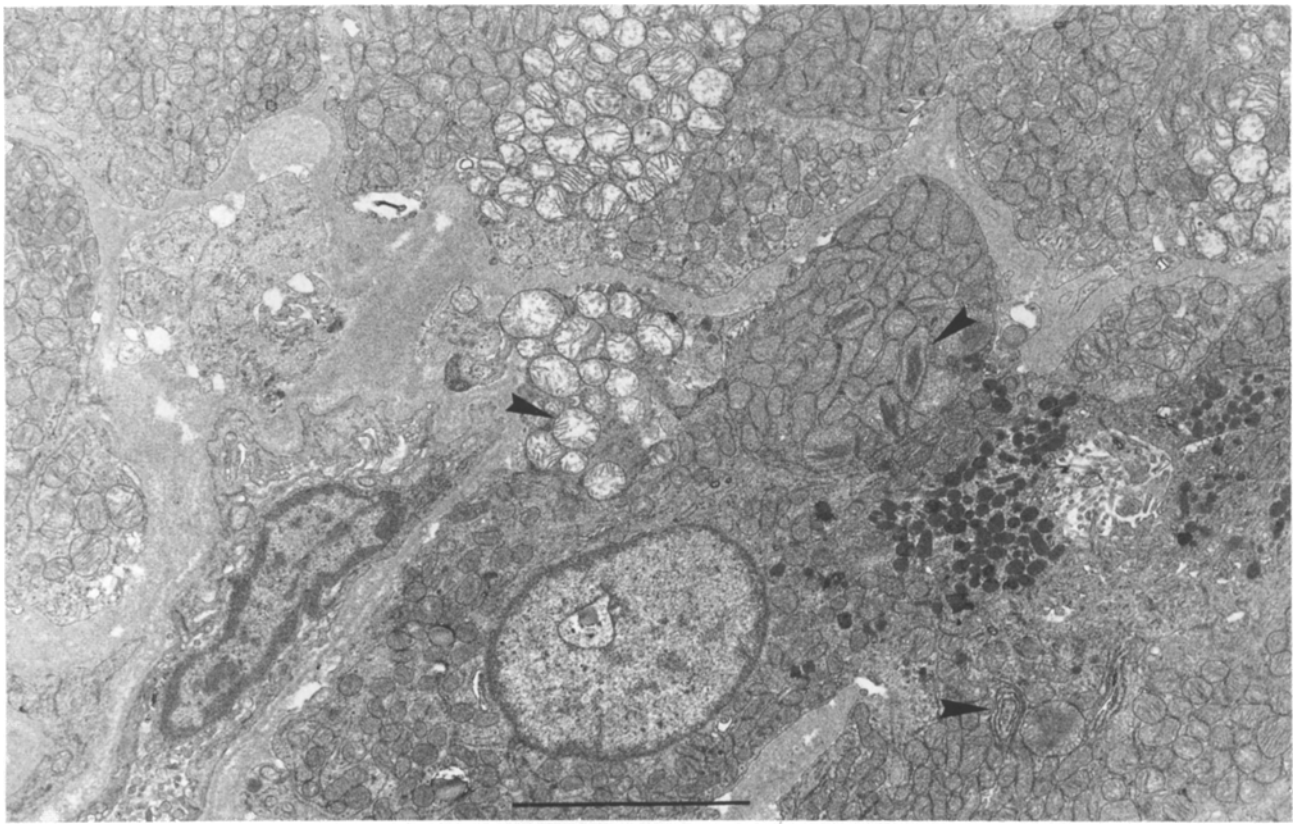

Fig. 1. Electron micrograph of the oncocytic tumor 9. The cytoplasm of the oncocytes is tightly packed with mitochondria; other organelles are hardly visible. Different cells contain mitochondria with a condensed or swollen matrix and cristae. *Arrows* indicate mitochondria with different morphology. Magnification ×6700. Bar marker represents 5 μm

Table 1. Comparative analysis of thyroid oncocytic tumors

Patient no.	Diagnosis (oncocytic tissue)	Activity of mitochondrial enzymes				Abundance of mitochondria	Relative increase in mtDNA (× fold control)
		COX	SDH	ND	ATPase		
1	Ca	+++	+++	+++	++	+++	4.5×
2	Ca	+++	+++	+++	++	+++	9×
3	Ad	++	++	+	+	nd	7×
4	Ad	++	++	+	+	+++	nd
5	Ad	nd	nd	nd	nd	+++	9×
6	Ad	+++	+++	+++	++	nd	5.5×
7	Ad	+++	+++	+++	++	+++	nd
8	Ca	++	++	++	+	+	0.7×
9	Ca	+++	+++	+++	++	+++	3×

Patient no.	Diagnosis	Activity of mitochondrial enzymes				Abundance of mitochondria	Relative increase in mtDNA
		COX	SDH	ND	ATPase		
6	thy	+	+	+	+	nd	nd
9	thy	+	+	+	+	nd	1
10*	thy	+	+	+	+	nd	1

Pathological diagnosis: adenoma (Ad); carcinoma (Ca); thyroid tissue control (thy). Enzyme activities of cytochrome c oxidase (COX), succinate dehydrogenase (SDH), total NADH dehydrogenase (ND) and adenosine 5′-triphosphatase (ATPase) were determined histochemically. Activities are indicated by + (=activity of controls), ++ (=focal high activity) and +++ (high activity) Mitochondrial abundance was determined by ultrastructural examination. nd=not determined; *=oncocytoma-free autopsy case

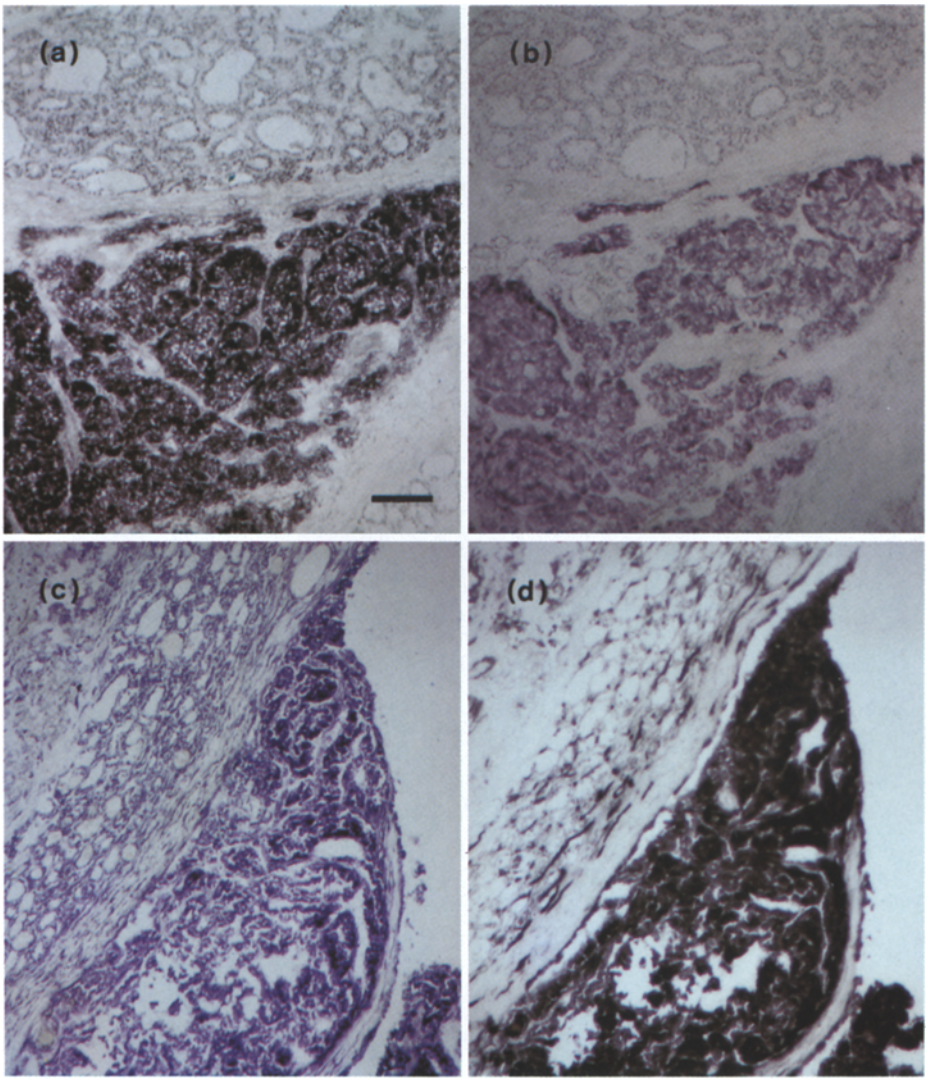

Fig. 2a–d. Histochemical demonstration of mitochondrial enzyme activities. Frozen section of thyroid oncocytic tumor 9 (see Table 1) consisting of tumor (dark stained areas) and normal thyroid tissue. Histochemical staining results from enzyme activity of cytochrome c oxidase **a**, succinate dehydrogenase **b**, total NADH dehydrogenase **c** and ATPase **d**. Bar marker represents 100 µm

Analysis of uncoupling protein. There was no indication of a particularly high energy demand in oncocytic tumors. Their high activities of respiratory enzymes are in apparent contradiction to their slow growth characteristics, unless one assumes an uncoupling of ATP synthesis from the electron flow in the respiratory chain. Such an uncoupling is naturally observed in the brown fat of hibernating animals or newborn infants due to the presence of uncoupling protein (UCP). To address the question whether UCP is expressed in oncocytic tumors, we isolated mitochondria from five tumors and analysed them by immune blotting with an antiserum directed against hamster-UCP. Human UCP is recognized by this antiserum as shown by the signal obtained with the brown fat control tissue (Fig. 3, lane c). In contrast, UCP was not detectable in any of the oncocytic tumors tested (Fig. 3, lanes a and b; only two of the five tumors are shown). Therefore an uncoupling of the respiratory chain due to the presence of uncoupling protein seems very unlikely.

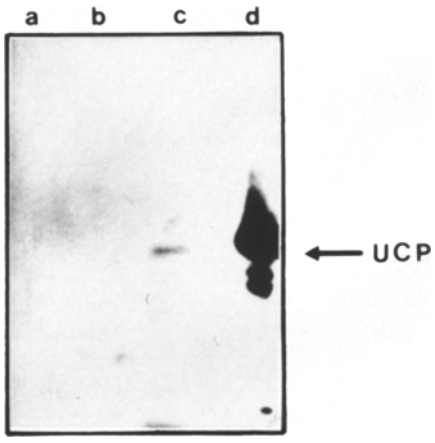

Fig. 3. Western blot analysis of uncoupling protein in oncocytic tumors. 100 µg mitochondrial protein isolated from oncocytic tumor 6 (*lane a*), no. 7 (*lane b*), human brown fat (*lane c*) and 5 µg of purified hamster UCP (*lane d*) were run on a 12% SDS-polyacrylamide gel, Western-blotted and immunodecorated with anti-hamster UCP-serum. The position of UCP is indicated by an *arrow*

Analysis of mtDNA

Quantitation of mtDNA in oncocytic tissues. We next asked whether the abundance of mitochondria in oncocytic tumors is accompanied by a parallel increase in the mtDNA content or whether the total number of mtDNA molecules per cell remains constant, in which case the average copy number of mtDNA molecules per organelle would be reduced. To address this question we quantitated mtDNA in relation to nuclear DNA. The result of this examination, together with that of the respiratory enzyme activities and the observed abundance in mitochondria is compiled in Table 1. An elevated mtDNA content, ranging from three- to nine-fold higher than in controls, was seen in all tumor samples with high enzymatic activities and/or large numbers of mitochondria. Only one tumor (no. 8) failed to show an increase in mtDNA. This may be due to the fact that this tumor was a mosaic with respect to mitochondrial function. In addition, electron micrographs did not show abundant mitochondria (see above). The results clearly show that the increase in the number of mitochondria was accompanied by an increase in mtDNA. Again, we were unable to see any differences between oncocytic adenomas and carcinomas and concluded that there is no relationship between the amount of mtDNA and the biological behaviour of oncocytomas.

Restriction analysis of mtDNA. Mitochondrial disorders are sometimes associated with mitochondrial heteroplasmy, i.e. the presence of a mixed population of intact and altered mtDNA molecules. To look for alterations in the mitochondrial genome of oncocytic tumors, restriction enzyme analysis using various restriction endonucleases (listed in the legend of Fig. 4) was carried out.

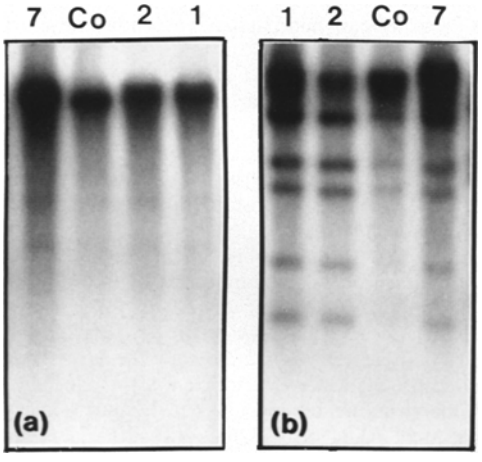

Fig. 4a, b. Restriction analysis of mtDNA. DNA was isolated from oncocytic tumors 1, 2, 7 and from thyroid control (Co) and cleaved with BamHI **a** and ApaI **b**, respectively. DNA fragments were Southern-blotted and probed with labeled purified HeLa mtDNA. The upper band in **b** represents uncut mtDNA. Similarly, mtDNA was digested with the restriction endonucleases AvaI, AvaII, BclI, EcoRI, EcoRV, HindIII, HpaI, HincII, KpnI, NcoI, NsiI, PstI, PvuII and XbaI. In none was a restriction fragment length polymorphism detected

The recognition sequences of these enzymes are randomly distributed throughout the mtDNA. The restriction patterns of all tumor DNAs proved to be identical with that of controls, and was in line with the expected pattern derived from the standard mtDNA sequence (Anderson et al. 1981). A representative example of restriction analysis is given in Fig. 4, which shows the DNA fragments obtained after digestion of mtDNA with BamHI (1 recognition site) and by ApaI (5 recognition sites) from three tumors (nos. 1–3) and one control sample (Co).

In conclusion, our analysis of mtDNA in oncocytic tumors gives no indication of the presence of a subpopulation of mtDNA molecules with gross structural alterations.

Discussion

The data presented in this paper show that in thyroid oncocytic tumors the abundance of mitochondria is paralleled by high activities of respiratory chain complexes I, II, IV and V (ATPase). All of these complexes – with the exception of complex II – are composed of subunits which are either encoded by mtDNA or by nuclear genes (Attardi and Schatz 1988). Clearly the genetic information for the respective structural genes, as well as for all the components required for expression of these genes, must be intact. In line with this conclusion restriction analysis of mtDNA of oncocytic tumors gave no indication of gross alterations. Similarly, we failed to detect any alteration after selective amplification of various DNA segments from mtDNA by the polymerase chain reaction (PCR) (Ebner, unpublished results). Point mutations or very small deletions, however, would escape detection in either type of analysis.

Altered mtDNA molecules often result from DNA-deletions (Holt et al. 1988, 1989; Schon et al. 1989) or duplications (Poulton et al. 1989) and have been found together with wild type mtDNA in several mitochondrial disorders ("heteroplasmy"). The concomittant abundance in mitochondria in affected tissues is best interpreted as a compensation mechanism to maintain the energy supply in the form of ATP synthesis provided by mitochondrial respiration. Our histochemical data show that respiratory enzymes are functional as is mitochondrial ATPase. This result does not, however, exclude the possibility that ATP synthesis might be uncoupled from the electron flow in the respiratory chain. The failure to immunologically detect uncoupling protein (UCP) argues against an USP-mediated mechanism at least in those five oncocytomas which were probed with an antiserum directed against UCP. Uncoupling by other processes, e.g. by accumulation of certain fatty acids (Luvisetto et al. 1987), remains possible, however.

The present study is the first to show that the mitochondrial abundance in oncocytic tumors is accompanied by an increase in the content of mitochondrial DNA. Only recently it was reported that high cellular levels of mtDNA preceed increased expression of mito-

chondrial functions in striated muscle (Williams 1986). From that point of view the observed elevation of mtDNA could represent the primary cause of oncocytic transformation. The accumulation of organelles and the high activities of respiratory enzymes would represent a secondary phenomenon. Major determinants in the regulation of mtDNA replication are the D-loop region of mtDNA, where replication is initiated (Clayton 1982), and the nuclearly encoded enzymes involved in the replication process. DNA alterations in both mtDNA or in nuclear genes could therefore affect mtDNA replication. For example, a cis-acting mutation in the D-loop region could enhance the affinity for components of the mitochondrial replication machinery, while a mutation in the nuclear gene encoding mtDNA polymerase could lead to a higher processivity of the enzyme. Similar effects may result from mutated components which indirectly affect mDNA replication, e.g. transcription factors or hormone receptors involved in the expression of mitochondrial proteins.

Our experiments do not allow an answer to the question whether all cells or only a subset of cells within the tumor are responsible for the elevated enzymatic activities and for the increase in mtDNA content, respectively. The observation of neighboring cells with mitochondria of different morphology (see Fig. 1) may hint at the latter possibility. To elucidate this problem it will be necessary to perform enzymatic and molecular analyses at the single cell level.

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References

Altmann HW (1990) Onkozytäre Hepatozyten. Der Pathologe 11:137–142

Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457–465

Attardi G, Schatz G (1988) Biogenesis of mitochondria. Ann Rev Cell Biol 4:289–333

Ausubel M, Frederick M (eds) (1987) Current protocols in molecular biology. J. Wiley and Sons, New York

Bogenhagen D, Clayton DA (1974) The number of mitochondrial deoxyribonucleic acid genomes in mouse L cells and human HeLa cells. J Biol Chem 249:7991–7995

Capaldi RA (1988) Mitochondrial myopathies and respiratory chain proteins. Trends Bio Sci 13:144–147

Clayton DA (1982) Replication of animal mitochondrial DNA. Cell 28:693–705

Cotton DWK (1990) Oncocytomas. Histopathology 16:507–509

Dodemont HJ, Soriano P, Quax WJ, Ramaekers F, Lenstra JA, Groenen AM, Bernardi G, Bloemendal H (1982) The genes coding for the cytoskeletal proteins actin and vimentin in warm-blooded vertebrates. EMBO J 1:167–171

Dudley GA, Tullson PC, Terjung RL (1987) Influence of mitochondrial content on the sensitivity of respiratory control. J Biol Chem 262:9109–9114

Eble JN, Hull MT (1984) Morphologic features of renal oncocytoma. Hum Pathol 15:1054–1061

Ebner D, Raabe T, Siddell SG (1988) Identification of the coronavirus MHV/JHM mRNA 4 product. J Gen Virol 69:1041–1050

Fischer R (1961) Über den histochemischen Nachweis oxydativer Enzyme in Onkocyten verschiedener Organe. Virchows Arch [A] 334:445–452

Goebel HH, Schulz F, Rama B (1980) Ultrastructurally abnormal mitochondria in the pituitary oncocytoma. Acta Neurochir 51:195–201

Hamperl H (1936) Über das Vorkommen von Onkocyten in verschiedenen Organen und ihren Geschwülsten. Virchows Arch [A] 298:327–375

Hamperl H (1962) Onkocyten und Onkocytome. Virchows Arch [A] 335:452–483

Holt IJ, Harding AE, Morgan-Hughes JA (1988) Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature 331:717–719

Holt IJ, Harding AE, Cooper JM, Schapira AHV, Toscano A, Clark JB, Morgan-Hughes JA (1989) Mitochondrial myopathies: clinical and biochemical features of 30 patients with major deletions of muscle mitochondrial DNA. Ann Neurol 26:699–708

Jakovcic S, Swift HH, Gross NJ, Rabinowitz M (1978) Biochemical and stereological analysis of rat liver mitochondria in different thyroid states. J Cell Biol 77:887–901

Kovacs G, Welter C, Wilkens L, Blin N, Deriese W (1989) Renal oncocytoma: a phenotypic and genotypic entity of renal parenchymal tumors. Am J Pathol 134:967–971

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lindsay S, Arico IM (1963) Enzyme histochemistry of the human thyroid gland. Arch Pathol 75:627–647

Loijda Z, Gossrau R, Schiebler TH (1976) Enzymhistochemische Methoden. Springer, Berlin Heidelberg New York

Luvisetto S, Pietrobon D, Azzone GF (1987) Uncoupling of oxidative phosphorylation. 1. Protonophoric effects account partially for uncoupling. Biochemistry 26:7332–7338

Nelson BD (1987) Biogenesis of mammalian mitochondria. Curr Top Bioenerg 15:221–272

Poulton J, Deadman ME, Gardiner RM (1989) Duplications of mitochondrial DNA in mitochondrial myopathy. Lancet I:236–240

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Schaeffer R, Ormanns W (1983) Immunohistochemical demonstration of thyreoglobulin in oncocytic carcinoma of the thyroid. Acta Endocrinol [Suppl] 252:42–43

Schon EA, Rizzuto R, Moraes CT, Nakase H, Zeviani M, DiMauro S (1989) A direct repeat is a hotspot for large-scale deletion of human mitochondrial DNA. Science 244:346–349

Tandler B (1956) Fine structure of oncocytes in human salivary glands. Virchows Arch [A] 341:317–326

Tremblay G, Pearse AGE (1959) A cytochemical study of oxydative enzymes in the parathyroid oxyphilic cell and their functional significance. Br J Exp Pathol 40:66–70

Williams RS (1986) Mitochondrial gene expression in mammalian striated muscle. J Biol Chem 261:12390–12394