Complex Mitochondrial DNA in Human Tumors

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INTRODUCTION

It is now well established that the mitochondria in eukaryotic cells contain DNA. The bulk of the DNA in mitochondria of animal cells, from sea urchins to humans, is in the form of a closed circular duplex (7) with a molecular weight of approximately $10^7$ daltons (1, 2, 12, 16). A variable proportion of mtDNA in animal cells is in the form of complex mtDNA. Such complex mtDNA occurs in 2 forms: catenanes, in which two or more monomer-length circles are topologically bonded as the links in a chain (8); and circular dimers, circular molecules that are twice the monomer size, and which consist of 2 monomer genomes in a tandem arrangement (3) (Fig. 1). Catenated molecules, first seen in HeLa cells (8), occur in the mtDNA of all animal cell tissues studied (5, 4, 14), including several tissue culture lines (10, 17). In tissues from laboratory animals, catenated dimers comprise from 5 to 9% of the mtDNA molecules; catenated higher oligomers, containing 3 or more monomer circles, make up from 0.5 to 2% of the mtDNA molecules. The mtDNA of cells in tissue culture has larger quantities of catenated molecules. In contrast, circular dimers, which comprise 12 to 50% of the mtDNA in leukocytes of patients with granulocytic leukemia (5), were not detected in normal human leukocytes, in leukocytes from patients with nonmalignant proliferations of granulocytes (6), or in a variety of tissues from healthy laboratory animals (4). They have been seen in only certain established cell lines, e.g., mouse L-cells and SV40-transformed BALB/c 3T3 cells (9, 10).

Recently, circular dimers have been reported in surgically removed human thyroid tissue and in beef thyroid (13). It was shown (6) that the frequency of the circular dimer in the peripheral leukocytes of patients with granulocytic leukemia was reduced when patients were treated with antileukemic drugs. This finding and the fact that circular dimers were absent in all other tissues studied at the time this investigation was begun suggested a correlation between this mtDNA form and human neoplasia. We investigated the mtDNA of human solid tumors in order to examine this correlation further.

The solid tumors from which the mtDNA was prepared were provided by several cooperating surgical pathologists. Freshly excised samples for which preliminary observation indicated a high probability of cancer were used. The samples were reasonably free of nonmalignant tissue and were grossly similar to that taken for pathological examination. The 21 tumors examined were taken at random; however, pathologists were asked to limit the samples to those which could be dispersed by the homogenization methods described below. A copy of the pathology report was made available for all samples tested. mtDNA was prepared from a human term placenta as a control sample.

The results of this preliminary survey extend the observation of the occurrence of the circular dimer form in human neoplastic cells. The presence of the dimer was unambiguous in 12 of 15 cases in which analysis was possible. However, the frequencies with which they were found were all well below those seen in granulocytic leukemia, several being just above our level of detection. The low frequencies may be characteristic of the tumor type, the pathology of the tumor, or may result from selective loss of mitochondria from malignant cells during cell fractionation or from the presence of contaminating nonmalignant tissue.

MATERIALS AND METHODS

Human Tissue. Samples of tumor tissue from surgical excisions were provided by several pathologists in the area...
Preparation of Mitochondria. Tumor tissue was minced into pieces approximately 3 to 5 cu mm and washed extensively in MS or TD buffer to remove blood and fatty tissue. Tumors containing some fibrous material were first homogenized with a large glass Dounce homogenizer fitted with a motor-driven nylon pestle. Other tumors were dispersed with a loose-fitting, motor-driven Thomas homogenizer. These first homogenates were filtered through cheesecloth and extensively homogenized in a glass homogenizer fitted with a motor-driven, tight-fitting Teflon pestle. Homogenizations were usually done in MS buffer; in some cases they were done in 0.01 M Tris (pH 7.5), 0.01 M NaCl, 0.001 M EDTA. In these cases, sucrose was added to 0.25 M immediately following homogenization. Phase-contrast microscopy at a magnification of ×400 was used to monitor the homogenization, which was continued until 50 to 75% of the cells were disrupted. Rehomogenization was sometimes carried out if the amount of crude mitochondria obtained was much less than expected for the size of the starting sample. Homogenates were centrifuged at 2500 rpm for 5 min in an International PR-6 centrifuge to remove cells, nuclei, and debris. After this centrifugation was repeated, the supernatant was centrifuged at 12,000 rpm for 20 min in a Sorvall SS 34 rotor to pellet mitochondria, which were then washed once with MS buffer. All steps in the above procedure were carried out at 4°.

The placenta was extensively washed with TD buffer, and large pieces of tissue were cut away from the membrane. These pieces were minced and washed with TD buffer and then were homogenized in the Dounce glass homogenizer. All further operations were as described for the tumor samples.

Purification of Mitochondria and Preparation of mtDNA. In all cases, sucrose gradients were used to purify mitochondria. Mitochondrial pellets were resuspended in MS buffer (5 ml/gradient tube) and layered onto a step gradient consisting of 1.0, 1.5, and 1.75 M sucrose layers, each containing 0.001 M EDTA, 0.01 M Tris (pH 7.5). The tubes were centrifuged in a SW25.1 or SW27 rotor at 22,000 to 25,000 rpm for 45 to 60 min at 4°. The mitochondria, appearing at the center interface, were collected and pelleted. In most cases the mitochondria were then treated with DNase I as previously described (4). This step was omitted when only a small mitochondrial pellet was recovered. Lysis of the mitochondria and preparation of the DNA with CsCl-ethidium bromide density gradient centrifugation have been described (15, 17). In cases in which DNase treatment had reduced the DNA in the upper band to less than 50% of the total, both fluorescent bands were collected and consolidated. In other cases, only the closed circular DNA in the lower band was taken for analysis.

Electron Microscopy. DNA from the CsCl-ethidium bromide gradients was used directly for electron microscopy when the concentrations warranted. Otherwise, the DNA was concentrated by pelleting overnight at 35,000 rpm, 20°, in polyallomer tubes. All but 0.2 ml was drawn off, and the tubes were agitated on a Vortex mixer and allowed to stand for 3 to 5 hr to resuspend the DNA. The preparation of electron microscope specimen grids and the scoring rules for classifying molecules have been previously described (4, 8). When the circular dimers were present at low frequency, they were photographed and measured to confirm the assignment.

RESULTS

Frequencies of Complex DNA. mtDNA was prepared from a total of 21 human tumor samples and from 1 human placenta. In the case of 6 samples, only a small amount of mtDNA, insufficient for satisfactory analysis, was recovered. In 3 cases, a limited number of molecules of the lower bands in ethidium bromide-CsCl gradients were scored, and the frequency of catenated dimers was less than 2%, instead of the usual 5 to 9%. Such results indicate that the mtDNA experienced considerable nicking prior to purification and that dimer molecules (both catenated and circular) were selectively removed. These species are double-sized targets for nicking and are removed at twice the rate of monomers. Extensive nicking thus results in lowering the frequency of dimers seen. Two samples afforded only about 600 scorables molecules. Although the level of catenated dimers was within the normal range and no unambiguous circular dimers were seen, the dimer level could have been in the 0.2 to 0.5% range seen in some of the other samples. More molecules would have been needed to enable us to make a definite statement about the presence (or absence) of the form.

The results for 15 samples and the placenta are given in Table 1. The samples are grouped as tumors of the germ line and as carcinomas, sarcomas, and tumors which do not easily fall into these categories. They are listed in order of decreasing frequency of the circular dimer.

The tumors present a wide range of values for all types of complex mtDNA. The circular dimer is unambiguously present in 12 of the 15 cases. In 6 of these, however, the frequency is very close to our level of detection. In only 2 cases was the level above 5%, and in no case was a frequency seen which approaches frequencies in cases of granulocytic leukemia. In 3 samples, circular dimers were not detected. The placental mtDNA extends our earlier report that the circular dimer is absent from normal tissue. One circular dimer was seen during the scoring of 3000 molecules, which indicates that whatever process results in dimer formation is under tight control in nonmalignant tissue. The detection of 1 molecule also gives some assurance that the investigator doing the scoring does see the circular dimer even if it is present at very low frequencies.

The frequencies of catenated dimers and higher oligomers in 12 of the tumor samples fall within the ranges seen...
Table 1

Complex mtDNA in human tumors

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Monomers</th>
<th>Circular dimers</th>
<th>Catenated dimers</th>
<th>Catenated oligomers</th>
<th>Molecules classified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teratocarcinoma, ovary (LB)</td>
<td>83.0 ± 2.5°</td>
<td>8.9 ± 1.8</td>
<td>6.2 ± 1.6</td>
<td>1.9 ± 0.9</td>
<td>884</td>
</tr>
<tr>
<td>Seminoma, testis (LB)</td>
<td>86.4 ± 1.8</td>
<td>0.3 ± 0.2</td>
<td>9.6 ± 1.5</td>
<td>3.7 ± 1.0</td>
<td>1445</td>
</tr>
<tr>
<td>Granulosa cell carcinoma, ovary</td>
<td>70.5 ± 2.6</td>
<td>5.1 ± 1.2</td>
<td>14.2 ± 1.9</td>
<td>10.2 ± 1.7</td>
<td>1164</td>
</tr>
<tr>
<td>Squamous cell carcinoma, metastatic, groin (LB)</td>
<td>90.7 ± 1.5</td>
<td>2.6 ± 0.8</td>
<td>5.9 ± 1.2</td>
<td>0.8 ± 0.5</td>
<td>1400</td>
</tr>
<tr>
<td>Squamous cell carcinoma, foot (LB)</td>
<td>91.4 ± 1.5</td>
<td>1.1 ± 0.6</td>
<td>6.1 ± 1.3</td>
<td>1.4 ± 0.6</td>
<td>1283</td>
</tr>
<tr>
<td>Adenocarcinoma, salivary gland (LB)</td>
<td>93.0 ± 1.1</td>
<td>0.8 ± 0.4</td>
<td>5.4 ± 1.0</td>
<td>0.8 ± 0.4</td>
<td>1953</td>
</tr>
<tr>
<td>Carcinoma, lung*</td>
<td>88.9 ± 1.3</td>
<td>0.2 ± 0.2</td>
<td>8.5 ± 1.2</td>
<td>2.4 ± 0.6</td>
<td>2040</td>
</tr>
<tr>
<td>Carcinoma, breast*</td>
<td>87.8 ± 1.1</td>
<td>0</td>
<td>9.2 ± 1.0</td>
<td>3.0 ± 0.6</td>
<td>3175</td>
</tr>
<tr>
<td>Neurofibroma or fibrosarcoma (LB)</td>
<td>90.6 ± 1.8</td>
<td>1.8 ± 0.8</td>
<td>6.3 ± 1.5</td>
<td>1.3 ± 0.7</td>
<td>1011</td>
</tr>
<tr>
<td>Rhabdomyosarcoma, alveolar*</td>
<td>88.6 ± 1.2</td>
<td>0.4 ± 0.2</td>
<td>8.6 ± 1.0</td>
<td>2.4 ± 0.3</td>
<td>2526</td>
</tr>
<tr>
<td>Liposarcoma, breast (LB)</td>
<td>96.8 ± 0.6</td>
<td>0.2 ± 0.2</td>
<td>2.8 ± 0.6</td>
<td>0.2 ± 0.2</td>
<td>2700</td>
</tr>
<tr>
<td>Glioblastoma, brain*</td>
<td>88.9 ± 1.4</td>
<td>0.4 ± 0.4</td>
<td>8.5 ± 1.7</td>
<td>2.2 ± 0.9</td>
<td>1000</td>
</tr>
<tr>
<td>Osteogenic sarcoma, tibia*</td>
<td>93.9 ± 1.2</td>
<td>0</td>
<td>4.9 ± 1.0</td>
<td>1.2 ± 0.5</td>
<td>1500</td>
</tr>
<tr>
<td>Mesothelioma, omentum* (LB)</td>
<td>96.5 ± 1.0</td>
<td>0.4 ± 0.3</td>
<td>3.0 ± 0.8</td>
<td>0.1 ± 0.1</td>
<td>1500</td>
</tr>
<tr>
<td>Myeloma, metastatic, ileum* (LB)</td>
<td>94.5 ± 0.8</td>
<td>0</td>
<td>4.9 ± 0.8</td>
<td>0.6 ± 0.3</td>
<td>2500</td>
</tr>
<tr>
<td>Placenta*</td>
<td>94.7 ± 0.8</td>
<td>0 (&lt;0.1)</td>
<td>4.7 ± 0.8</td>
<td>0.6 ± 0.3</td>
<td>3000</td>
</tr>
</tbody>
</table>

* LB, those scorings done on closed circular DNA only.
° The S.E. given are the statistical sampling errors at the 95% confidence limit.
* Diagnosis provided by the surgical pathology department of University of Southern California-County Medical Center, Los Angeles, Calif.
* Mean ± S.E.
* Diagnosis provided by the surgical pathology department of Huntington Memorial Hospital, Pasadena, Calif.
* Diagnosis provided by the surgical pathology department of City of Hope Medical Center, Duarte, Calif.
* Diagnosis provided by the surgical pathology department of White Memorial Hospital, Los Angeles, Calif.
* Diagnosis provided by the surgical pathology department of St. Luke Hospital, Pasadena, Calif.

in tissues from laboratory animals (4). Two samples had substantially lower frequencies of catenated dimers and very low frequencies of catenated higher oligomers. In both of these cases, only closed circular DNA was analyzed. Substantial nicking is a probable explanation of the low values. One sample, the granulosa cell carcinoma, contained very high frequencies of complex DNA. In addition to circular dimers, this sample contained the highest level of catenated dimers seen in tissue and the highest level of higher oligomers ever seen, roughly 10%. A more detailed analysis gives 5.2% trimers, 2.3% tetramers, 1% pentamers, and 1% oligomers containing more than 5 submolecules. We showed that these were not electron microscope artifacts by roughly fractionating the sample with a preparative CsCl velocity experiment. These large forms were found near the bottom of the gradient, and the material near the top contained very few such molecules. The molecules do not give the appearance of aggregation at a single point when seen in the electron microscope. In addition, the sample contained about 0.5% molecules which appeared to be circular dimers catenated to monomers. It is possible that these represent overlapping and noninterlocked monomers and dimers. This is unlikely, however, because they were not seen in samples from leukemic leukocytes which contained 12 to 50% circular dimers (6).

Buoyant Properties of the mtDNA. Prior to this work, values obtained in this laboratory for buoyant densities of human mtDNA were from leukemic (3) or HeLa (17) cells which have been growing in culture for many years. The neutral buoyant densities for mtDNA from the granulosa cell carcinoma and from placenta were found to be 1.700
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g/ml, in agreement with previously obtained values (5). The buoyant separations in alkaline CsCl of the separate single strands for mtDNA from the rhabdomyosarcoma and for the placental mtDNA were also determined and compared in the same manner. The difference in buoyant density of the 2 strands in each case was 0.041 g/ml, in agreement with previously obtained values (3, 17). The absolute values obtained for the separate strands of the placental mtDNA were within the experimental error of the values reported previously (3).

DISCUSSION

The results of this study extend the correlation between human neoplasia and the presence of the circular dimer form of mitochondrial DNA in many neoplastic cells. Detailed interpretation of the variation of frequencies of the form, as well as the meaning of the fact that 3 tumor samples were found to be free of circular dimers, is made difficult by several factors.

Normal Tissue. The frequencies found are very sensitive to the presence of normal tissue in the sample. All tumors contain some normal vascular and connective tissue, but this amount is variable. It is difficult to assess this contribution and, indeed, no direct attempt was made to do so. Detailed microscopic examination was of necessity done on other portions of the tumor, and we rely on the pathologist's gross examination for the assurance that our sample was not heavily contaminated with nontumorous cells. Some selection may have taken place in the homogenization of the sample. It was presumed that the softer tumor tissue would be more easily homogenized than the connective or vascular tissue, but in certain cases this may not have been valid. The sample from osteogenic sarcoma, for example, was very difficult to homogenize due to small particles of bone present in the tumor and to the bone that was removed with the tumor. It is possible that mitochondria from tumor tissue are more sensitive to the fractionation procedures and DNase treatments used, although we do not regard this as a strong possibility.

Tumor Physiology. Tumors vary in size, growth rate, oxygen supply, and age. Results by Nass (11) indicate that, at least in 1 line of cultured cells, the level of circular dimers may be influenced by the growth conditions of the cells. If this were the case generally, the fluctuation in dimer levels seen here could be strongly affected by such factors.

Degree of Differentiation or Malignancy. Pathologists often classify tumors on the basis of the differentiation of the cells in the tumor and generally accord less differentiated cells more "malignancy." No obvious correlation can be made in the cases surveyed here.

Type of Neoplasia. The correlation between neoplasia and the circular dimer may be determined by the cause of the cancer itself, or may be in part determined by the type of cell that gives rise to the cancer. In the absence of a general model for all the different types of cancers, and without several samples for each kind of cancer, we cannot analyze the variations seen here.

Paoletti et al. (13) recently found circular dimers in 4 human thyroids excised for a variety of reasons. The dimers ranged from 12 to 37% of the mtDNA mass (frequencies of 7 to 30%). One thyroid was apparently obtained at autopsy of a patient with acute lymphoblastic leukemia, the 2nd contained a follicular adenoma, the 3rd was removed because of thyroiditis, and the 4th was removed because of hyperthyroidism. These authors also found that beef thyroid contained a significant level of circular dimers. In view of the above results, they concluded that it was inappropriate to generalize [from the study of normal tissues by Clayton et al. (4)] that circular dimers were always absent in nonmalignant cells. It is not clear, however, that the nonmalignant thyroids used in the above-described study can be regarded as normal tissue. The results with beef thyroid could conceivably be due to the possible use of drugs by the commercial beef industry to hasten fattening prior to slaughter. It is also conceivable that some monomer-to-dimer conversion may occur after excision of the human thyroids or after the death of patients or animals. It has been reported (11) that such an event occurs in 1 line of mouse L-cells under certain abnormal physiological conditions.

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REFERENCES

Fig. 1. Electron micrographs of mtDNA molecules from human tumors; a, a supertwisted monomer lying entirely within a relaxed monomer; b, catenated dimer containing 1 relaxed and 1 supertwisted submolecule; c, catenated trimer; d, circular dimer with no cross-over points; e, circular dimer with 2 cross-over points, neither of which divides the molecule into 2 equal parts.
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