Quantitative Study of Melanosome and Mitochondrial Populations in Pigmented and Amelanotic S-91 Mouse Melanomas

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SUMMARY

The relative numbers of mitochondria and melanosomes were enumerated in pigmented and amelanotic S-91 mouse melanomas. There were 6 times more mitochondria per sq μm of cytoplasm in amelanotic S-91 tumors than in melanotic S-91 tumors. This relative paucity of mitochondria in pigmented S-91 melanomas is significant in view of the identical in vivo growth rates of the two tumors and also because the two tumor types are so closely related in terms of common origin and ease of producing one from the other by serial selective transplantation. The respiratory rate of melanotic S-91 melanomas is only about 30% less than that of nonpigmented S-91 tumors. The 6-fold difference in mitochondrial numbers and the ultrastructural similarities of the mitochondria in the two tumor types suggest that the respiratory capacity of the melanotic S-91 melanocytes is attributable in part to melanosomes. These organelles contain high levels of tyrosinase activity, an enzyme that is coupled directly to molecular oxygen. This quantitative study of mitochondria and melanosome populations in these two closely related melanomas is harmonious with the concept that tyrosinase and melanosomes play a vital respiratory role in the relatively mitochondria-poor pigmented S-91 melanomas.

INTRODUCTION

Melanosomes are unique organelles in terms of their enzyme content and variable ultrastructural features, which are dependent on the activity of tyrosinase, the principle enzyme in the melanin-biosynthetic pathway. Melanosomes are indolent and structurally uniform in normal adult melanocytes in the eyes and skin, generally requiring UV stimulation to activate their tyrosinase and then only for a brief period of time (5). Malignant melanocytes in pigmented melanomas have a broader spectrum of melanosomes than normal melanocytes do, because in melanomas these organelles have active tyrosinase and consequently a high turnover rate (2). Young melanosomes are characterized by longitudinal rods with distinctive periodicities. As melanization ensues, cross-bars link the longitudinal rods, and the resultant inner structure of melanosomes becomes that of a latticework. With further melanization, the polymeric pigment obscures this inner structure, and the individual melanized melanosomes are gathered into autophagic vacuoles and expelled from the cell (2). In normal adult melanocytes, the young and intermediate melanosomes are rarely seen; the melanosomes are generally heavily melanized.

Biochemically, tyrosinase in these organelles oxidizes tyrosine to DOPA² and then also oxidizes the DOPA to DOPA quinone. Malignant pigmented melanocytes have high active levels of this enzyme, in sharp contrast to normal adult melanocytes, which have little to no demonstrable activity (4). Amelanotic S-91 melanomas (S-91A) show no measurable tyrosinase and have only a few young melanosomes that do not melanize (6). Furthermore, the amelanotic S-91 tumor is not sensitive to the growth- and respiration-inhibiting effects of tyrosinase-blocking agents, whereas pigmented S-91 melanomas (S-91P) are sensitive (1, 8). Pigmented S-91 melanomas rely on tyrosinase activity for almost one-half of their oxidative phosphorylation (8). This is in harmony with the proposal that tyrosinase and melanosomes play a vital respiratory role in some pigmented melanomas (1, 8).

Previous fractionation studies with S-91P tumors reveal that melanosome fractions that are free of mitochondrial contamination can be obtained (3). In studies that have been completed and are being prepared for publication, approximately 50 to 60% of the total succinoxidase activity is recovered in the mitochondria-free melanosome fractions.

Since melanosomes in S-91P melanomas seem to carry on some of the functions of mitochondria, the question arises as to whether melanosomes, in a sense, "replace" mitochondria. Amelanotic S-91 tumors are readily derived from S-91P melanomas by selective serial transplantation of the very pale transplant fragments. These 2 tumors, the S-91A and S-91P, are identical in their biological malignant potential and serve as valid comparative tissues. Their respiratory rates are known (1) and, since there is a direct correlation between mitochondrial numbers and cellular respiratory capacity (7), it was felt that the mitochondrial population in S-91P and S-91A might be compared, in order to help answer the question of whether S-91P tumors are relatively poor in mitochondria.

MATERIALS AND METHODS

Heavily pigmented and amelanotic S-91 mouse melanomas, carried s.c. in separate DBA/2 mice, were used. The original tumors were obtained from Dr. Mark Woods of the National Cancer Institute.

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2 The abbreviation used is: DOPA, 3,4-dihydroxyphenylalanine.
Cancer Institute, NIH, Bethesda, Md. The melanotic and
amelanotic S-91 tumors were subsequently maintained by
selective serial transplantation of black and white fragments,
respectively, in separate groups of male DBA/2 mice.

Five melanotic and 2 amelanotic tumors were used in this
study when they reached a volume of 1 cm³, approximately
ten days after transplantation.

Fresh tumor was obtained immediately after the sacrifice of
the host mouse by cervical fracture. Only firm, viable portions
of the tumors were sampled. They were then minced and fixed
in Veronal-acetate-buffered 1% OsO₄ (pH 7.4 to 7.5) for 1 hr
at 5°. After dehydration in graded alcohols and propylene
oxide, the tissue fragments were embedded in Epon 812 and
sectioned on the LKB Ultratome III with glass knives. Thick
sections, 1 to 3 μm, were stained with toluidine blue and
examined by light microscopy for the final area selected for
ultrathin sections. These ultrathin sections were mounted on
Formvar-coated copper grids and then stained with 5% uranyl
acetate in absolute methanol for 20 min. Fifteen blocks were
prepared from each tumor. The 1-mm fragments for each
block were selected from different areas of the excised tumor
mass. Five grids were prepared from each block. The best of
the 5 grids was then used for enumeration of mitochondria
and melanosomes for each block. Since a different block face
was used for the counting, there was no overlap. Only 1
section of the serial sections from a block were counted.

The grids were studied in a Hitachi HU11B electron
microscope which was equipped with 2 liquid nitrogen
anticontamination and cooling devices. Random cytoplasmic
areas were chosen and all exposures made at a direct
magnification of ×32,000. Care was exercised to avoid
ectroplasmic area, and the average density of mitochondria and
melanosomes per sq μm was calculated. The areas
photographed excluded nuclei. A part of a nucleus was
allowed in the electron micrograph if it did not encroach on
more than about 10% of the area. The final photographs were
evaluated and computations done separately by 2 individuals.
Linear measurements of mitochondrial sizes were made on
10% of the S-91P and 5% of the S-91A melanomas. The largest
single diameter of these mitochondria was enumerated, and
their average and standard deviations were calculated.

RESULTS

A total area of 637 sq μm was evaluated in each tumor
(type. There were 179 electron micrographs, each representing
3.57 sq μm of cytoplasm. Much more area than this was
examined in the S-91P and S-91A melanomas, but electron
micrographs with more than 10% of the area encumbered by
nucleus, capillaries, or nonmelanocyte cells were excluded.
There was no obvious clustering of mitochondria or
melanosomes around nuclei, and therefore there was probably
no error introduced by excluding some of the paranuclear
cytoplasm.

The results are summarized in Table 1. The melanosomes
inside autophagic vacuoles were frequent in the cytoplasm of
S-91P melanocytes. Macrophages were infrequent in the
tumors examined, probably because of the early harvesting of
the transplants. The macrophages were readily identifiable by
the large numbers of melanin granule-laden phagosomes that
they ingested following expulsion from melanocytes. Further
differences between melanocytes and macrophages were the
large numbers of lysosomes and abundant granular
endoplasmic reticulum in the latter. Nuclear and nucleolar
differences also aided in the distinction. Other connective
tissue elements, such as fibroblasts, were easily identified and
were present in small numbers (Figs. 1 and 2).

The average mitochondrial size, expressed as the greatest
single diameter, was slightly greater in the S-91A melanocytes,
as summarized in Table 1. Since the mitochondria varied in
their plane of sectioning from area to area and from block to
block, the greatest single diameter actually represents a
composite diameter. In the S-91A, 5% of the mitochondria
were measured, and in the S-91P, 10% were measured. There
were no readily observable differences between the S-91A and
S-91P mitochondria in terms of numbers, shape, or size of
cristae.

DISCUSSION

The foregoing data show that there is a striking paucity of
mitochondria in pigmented S-91 melanocytes, relative to
amelanotic S-91 melanocytes. Amelanotic S-91 tumor cells
contained only sparse young melanosomes in contrast to S-91P
melanocytes.

The comparison of S-91A and S-91P mitochondrial numbers
is valid because these tumors can readily be obtained from

| Table 1 |
| Mitochondrial and melanosome populations in S-91A and S-91P melanoma |

<table>
<thead>
<tr>
<th></th>
<th>Total area photographeda (sq μm)</th>
<th>Mitochondria</th>
<th>Melanosomes</th>
<th>Av. size of mitochondria b (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no.</td>
<td>No./sq μm</td>
<td>Total no.</td>
<td>No./sq μm</td>
</tr>
<tr>
<td>S-91A</td>
<td>637</td>
<td>1800</td>
<td>2.8</td>
<td>173</td>
</tr>
<tr>
<td>S-91P</td>
<td>637</td>
<td>286</td>
<td>0.45</td>
<td>2373</td>
</tr>
</tbody>
</table>

a There were 179 electron micrographs taken, each representing a cytoplasmic area of 3.57 sq μm.
b The greatest single diameter of 5% of the S-91A mitochondria and 10% of the S-91P mitochondria was measured.
c Average ± S.D.
Mitochondrial Numbers in Pigmented Melanomas

Each other by simple, selective serial transplantation. The comparison acquires importance when viewed in the light of their nearly identical growth rates in vivo in DBA mice. The respiratory rates ($QO_2$) of these tumors have been investigated previously (1). The $QO_2$ of S-91A melanomas is 6.8, and that of S-91P tumors is 4.7 (1). The 30% greater respiratory rate of S-91A tumor is in part a reflection of the larger number of mitochondria per unit area of cytoplasm. The question, however, is what accounts for the $QO_2$ of 4.7 in melanotic S-91 tumor cells? Could it all be accounted for by their few mitochondria, or do other organelles possibly contribute? There is general agreement that the level of oxidative respiration correlates well with the number of mitochondria in a cell under physiological conditions (7). The S-91A melanocytes have about 600% more mitochondria per unit area of cytoplasm than do S-91P tumor cells, yet the respiratory rate of S-91A is only 30% greater. This implies one of several possibilities: the S-91A mitochondria are relatively inefficient, or many are not functioning; S-91P mitochondria are relatively very efficient; another organelle, possibly the melanosome, is responsible for some of the S-91P oxidative respiration. The 1st 2 possibilities seem unlikely because ultrastructurally the mitochondria of S-91P and S-91A cells were not significantly different in terms of numbers, size, or shape of cristae. Nor were there differences in matrix density or other changes to suggest large difference in oxidative respiration. The ultrastructural appearances of mitochondria have been well correlated with their function (7). If there were substantial functional differences between S-91P and S-91A mitochondria, then they would have had strikingly different ultrastructural appearances (7).

Melanosomes in S-91P tumors contain high levels of tyrosinase activity. This enzyme is coupled directly to molecular oxygen, and this accounts for some of the oxidative respiration of S-91P tumors. Previous studies with specific tyrosinase inhibitors revealed that this enzyme accounts for nearly 50% of the $QO_2$ of S-91P melanocytes (1) and for about 50% of the ATP formed in vitro (8). The data in the present study are therefore harmonious with the concept that tyrosinase and melanosomes play a vital respiratory role in S-91P tumors, especially since there appears to be a relative paucity of mitochondria in these tumors.

REFERENCES

Fig. 1. Portion of cytoplasm of amelanotic S-91 melanocyte. The density of mitochondria and the paucity of melanosomes is representative. X 40,050.

Fig. 2. Portion of cytoplasm of pigmented S-91 melanocyte. Compared to amelanotic cells, there is a low density of mitochondria. Although the mitochondria seem smaller in this figure compared to those of the preceding figure, this is not representative. The figures were chosen for comparison of density of organelles. X 37,100.
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