Pathology and Ultrastructural Characteristics of a Hypomelanotic Variant of Transplantable Hamster Melanoma With Elevated Tyrosinase Activity*

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A spontaneous, hypomelanotic variant (MI) of the highly melanotic transplantable hamster melanoma of Bomirski (Ma) is the subject of this report. Tyrosinase activity is 2-3 times higher, but melanin content significantly lower than in the parental Ma melanotic melanoma. Acid phosphatase activity is similar in both, but β-glucuronidase and arylsulfatase A are 2-3 times higher in the hypomelanotic variant.

Transplanted MI melanomas grow more slowly than the parental tumor, but metastasize with similar incidence and localization. Hypomelanotic variant melanoma cells, even those in grossly non necrotic parts of the transplants, show signs of low viability like swelling of the cytoplasm or cellular condensation, and disintegration. Autophagic vacuoles are numerous. They appear to be formed by enclosure of a portion of cytoplasm by cisternae of smooth endoplasmic reticulum or trans-Golgi network. These limiting cisternae contain tyrosinase as evidenced by deposition of electron dense reaction product on incubation with tyrosine or DOPA. Other sites of ultrastructural tyrosinase reaction are melanosomes and the smooth-surfaced cisternae and vesicles of the trans-Golgi network.

We postulate that low cell viability, associated with autophagosome formation, is the cause for the growth retardation of the MI variant, and that the lower melanin content of these tyrosinase-rich cells is due to sequestration of a substantial portion of newly synthesized enzyme into autophagic vacuoles before it has the chance of being incorporated into melanosomes. J Invest Dermatol 89:469-473, 1987

The Syrian hamster is the only laboratory animal for which the incidence of spontaneous melanomas has been determined [1,2]. The incidence of approximately 2% should place this animal high on the list of experimental models for the study of malignant transformation of melanocytes. Once established, transplantable melanomas in rodents possess a considerable degree of phenotypic stability over decades of passaging, but they occasionally undergo spontaneous changes that result in the appearance of a new tumor variant [3,4]. A transplantable black melanotic melanoma was derived in 1959 from a spontaneous cutaneous melanoma in a hamster, and has been maintained under the name of Ma melanotic melanoma [5]. Four years later, a spontaneous alteration in the Ma melanoma gave rise to an amelanotic tumor variant, and was passaged as Ab amelanotic melanoma [6]. In 1976, a brown variant was isolated from Ma and designated MI. This variant arose as partially depigmented foci, containing unusual, necrotic areas, within a transplant of the 104th passage of the black Ma melanoma. The deeply pigmented and partially depigmented tissues were passaged separately. From passage #116 on, the black transplants resumed a uniformly black color and no further changes have appeared since that time, 1977.

The melanoma line initiated with the partially depigmented tissue gave rise to tumors variable in color, from brown–black to light brown, all containing a vast central necrosis. After several passages the transplants assumed a brown color with some variation in depth of coloration. This appearance has persisted, indicating that a stable variant of transplantable hamster melanoma has been established.

The MI melanoma is, therefore, the third member of a family of transplantable hamster melanomas that are related by common origin. The three melanomas differ in several respects: (1) level of tyrosinase activity and melanin content; (2) type of energy-
yielding metabolism; (3) number of chromosomes; (4) transplantability; and (5) growth rate [4,7–12]. The phenotypic stability of the Ab and MI variants during many years of serial transplantation and propagation in animals, and the differences in chromosome number between them (Ab, hypertriploid; MI, hypotetraploid) and the parental Ma melanoma (near diploid) suggest that the two variants originated as the result of mutational events in some Ma transplants [4,11].

The MI variant has the intriguing characteristic of having a lower melanin content than the parental Ma melanoma but approximately 2 or 3 times as much tyrosinase activity [7,8]. The studies reported here delineate comparative pathologic, enzymatic and fine structural properties of the MI variant and its parental Ma melanoma.

MATERIALS AND METHODS

Animals and Tumors Randomly bred male Syrian golden hamsters, 5–8 months old, were used throughout for propagating and implanting tumor tissues. Bomirski MI and Ma melanomas were implanted subcutaneously into the flank region as tissue suspensions prepared with a hand-driven homogenizer [4,6]. The amount of tissue injected was 200 mg per hamster.

Determination of Tumor Growth and Sites of Metastasis

The latency period was estimated on the basis of formation of palpable tumors [8]. Tumor growth was expressed as a function of survival time of the host animals [4] and as an increase in tumor size, according to the method of Schrek [13]. According to this method, during the logarithmic phase of tumor growth, a linear plot representing a growth constant in mm/day was obtained by measuring the three main tumor dimensions a, b, and c and plotting them as V=abc versus time [8,9]. The growth constants in Table I are the arithmetic means of individual growth constants from 8–16 hamsters ± SE. Survival time was determined at three different occasions with 12 hamsters each time (total 36 hamsters) for each of the two tumor lines. Statistical estimates were made by means of variance analysis. On autopsy, the occurrence and location of metastases were determined macroscopically and, when negative, with the dissecting microscope.

Tyrosinase Activity

Tyrosinase (DOPA oxidase) activity in crude extracts of cells isolated from solid tumors by a non-enzymatic method (14) was measured as described before (15). Briefly, tumor tissue was dissected, freed from necrotic and connective tissues, and rinsed several times in Ham’s F10 medium (GIBCO, Grand Island, New York). The tissue was then cut up thoroughly with scissors, suspended in F10 medium, and filtered through gauze sponge. The resulting single cell suspensions were centrifuged for 5 min at 800 g, and the pellets were resuspended in F10 medium, after which the cells were counted in a hemocytometer. The suspensions were divided into portions, each containing 2 ×10^6 cells, centrifuged, and the cell pellets were frozen at ~70°C. After thawing, 10^6 cells were suspended in 1 ml 0.5% Triton X-100 in 0.1 M sodium phosphate buffer, pH 6.8, vortexed and incubated for 30 min at 0°C or room temperature. These extracts were centrifuged for 5 min at 800 g, and the supernatants were used for the assays. DOPA oxidase activity was measured spectrophotometrically at 475 nm, with 1 mM L-DOPA (Hoffmann-La Roche) as substrate in 0.1 M phosphate buffer, pH 6.8. Results are expressed as nmoles of dopachrome produced by 1×10^6 cells per min over the linear portion of the activity curve during the first 10 min of assay. Values represent means from 3 or 4 experiments ± SE.

Lysosomal Enzyme Activities: Acid Phosphatase, β-Glucuronidase and Arylsulfatase A

Tumor tissue, free of necrotic and gross connective tissue, was frozen and stored at –70°C (Fig 1). After 3–4 months the tissues were thawed, suspended in 3 volumes of 50 mM acetate buffer, pH 5.0, containing 0.5% Triton X-100, cut with scissors and homogenized. The homogenates were diluted 1:10 in 50 mM acetate buffer, pH 5.0, and 100 μL of this preparation were used for each enzyme assay. Acid phosphatase activity was measured by the method of Trouet [16], with β-glycerophosphate (Sigma, St. Louis, Missouri) as substrate, and expressed as nmoles of inorganic phosphate liberated per milligram of wet tissue during 1 h of incubation. β-Glucuronidase activity was assayed by the method of Stahl and Touster [17], with phenolphthalein mono-β-glucuronic acid (Sigma) as substrate, and expressed as nmoles of phenolphthalein released per milligram of wet tissue during 1 h of incubation. Arylsulfatase A activity was measured by the method of Breslow and Sloan [18], with p-nitrophenylsulfate (Sigma) as substrate, and expressed as nmoles of p-nitrophenol liberated per milligram of wet tissue during 1 h of incubation.

Histochemistry, Electron Microscopy, and Ultrastructural Cytochemistry

Histochemical DOPA- and tyrosine reactions were carried out according to the methods of Laidlaw [19] and Fitzpatrick and associates [20], respectively. Electron microscopy and ultrastructural tyrosine and DOPA cytochemistry were performed by methods described in previous papers [4,21].

| Table I. Parameters Characterizing Growth and Differentiation of Transplanted Bomirski Hamster Melanomas Ma and MI |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Tumor Variant  | Latency Period <sup>±</sup> (days) | Growth Constant <sup>±</sup> (mm/day) | Host Survival <sup>±</sup> (days) | Tyrosinase Activity <sup>±</sup> (DOPA Oxidase) (nmol/10<sup>6</sup> cells/min) |
| Ma              | 10 ± 0.5        | 1.16 ± 0.12     | 85 ± 3.1        | 4.64 ± 1.03     |
| MI              | 10 ± 0.45       | 0.77 ± 0.05     | 98 ± 4.3        | 10.83 ± 0.06    |

<sup>1</sup>Data reprinted from [8].
<sup>2</sup>Mean ± SE.
<sup>3</sup>See Materials and Methods.

Figure 1. Photograph of viable frozen tumor masses. The tissues were isolated from several transplants and freed of necrotic and connective tissues.
RESULTS AND DISCUSSION

Appearance and Growth Properties The gross appearance of viable, nonnecrotic Bomirski melanoma tissue and the contrast between the highly melanized parental Ma line and the hypomelanotic MI variant are shown in Fig 1. The dark aspects of the MI tumor in this black and white photograph are due to a reddish-orange color. Like that of the parental melanoma, transplantability of the MI variant in random-bred Syrian hamsters was 100%. Subcutaneous implants often grew to a large size (30 mm as calculated by Shrek's method). Thereafter, they generally ulcerated so that viable tissue formed only a thin peripheral shell. The MI melanoma grows slightly, but significantly slower than the Ma melanoma as evidenced by its lower growth constant ($p < 0.01$) and the longer survival time of the hamsters ($p < 0.05$) (Table I). The latency period was similar for both tumors.

Metastases were found in 92% of the hosts. Similar to Ma melanomas [4], they were localized most frequently in the lungs (86%) and lymph nodes (64%), less frequently (40%–10%) in the perivertebral connective tissue of the lumbo-sacral region, in the mediastinum, liver and kidneys and sporadically (below 10%) in the thoracic wall, mesentery, spleen, epididymis, and spine. The color of the metastases varied in the same animal from brown–black to light brown.

Histology and Ultrastructure Hypomelanotic melanoma cells formed nests within a vascularized connective tissue stroma. They were polyhedral and contained vesicular, pleomorphic nuclei. Mitotic figures were rare. Melanin occurred in the form of fine or coarse brown granules. The amount differed from cell to cell.

Histochemical DOPA and tyrosine reactions were strongly positive and also differed in intensity from cell to cell (data not shown).

At the level of ultrastructure, the fine granules of light microscopy were represented predominantly by spherical granular melanosomes of melanization stage III (Fig 2), which were similar to those described in the Ma melanotic melanoma [4,21] or in Ab melanoma cells, which, when grown in culture, develop melanosomes and form pigment [22,23]. These melanosomes lack a regular matrix and have disorderly deposits of melanin within an amorphous, tangled fibrillar or finely granular substance of medium electron opacity. Few MI melanosomes showed an organized fibrillar structure. At least some of the granular melanosomes are presumed to have originated as vesiculoglobular premelanosomes because a fair number of these structures was encountered free in the cytoplasm, not as part of autophagosomes (Fig 2, inset). A vesiculoglobular substrate is typical of phaeomelanosomes [24]. There were also hybrid, vesiculofibrillar melanosomes. Completely melanized, stage IV melanosomes were encountered occasionally following incubation with tyrosine or DOPA. The coarse brown granules of light microscopy were without doubt a reflection of the numerous compound melanosomes, as seen commonly in melanoma cells. In some melanoma cells, intracisternal viral particles (type R) typical to hamsters were observed (Fig 2, inset).

A distinguishing, fine structural feature of MI melanoma cells, besides an obvious difference in the number of melanosomes and amount of melanin, were ubiquitous autophagosomes in different stages of formation and with varying contents, which were bound

Figure 2. Electron micrograph of cytoplasm typical of variant MI. Arabic numerals 1–4 refer to autophagic vacuoles that are limited by smooth-surfaced membranous cisternae. Most of the melanosomes are granular, stage III. Arrow heads point to rare fibrillar melanosomes, stage II. Inset, upper right: Viral particles of type "R" in a cisterna of RER [39]. Inset, lower left: Vesiculoglobular bodies a and b, with and without melanin deposition. Bar = 0.5 μm.
by smooth–walled cisternae (Fig 2) that may have originated from smooth-surfaced endoplasmic reticulum (SER) or the trans-Golgi network of membranes and vesicles. The latter assumption is the more plausible one, because these cisternae contained histochemically responsive tyrosinase, as evidenced by the deposition of electron opaque reaction product on incubation with tyrosine (Fig 3A) or DOPA (Fig 3B). The tyrosinase-positive cisternal profiles were morphologically identical to the tyrosinase- and acid phosphatase–positive "GERL" of Novikoff, as demonstrated in murine melanomas [25].

Even after having been selected for by the taking of electron micrographs of strictly DOPA-positive cell profiles, tyrosinase-positive cisternae in Mi melanoma cells outnumbered those in Ma melanoma cells by 75 to 1, i.e., they were frequent in Mi and rare in Ma. In fact, we found only one in countless sections of Ma melanoma cells scanned. These findings were made originally in Gdansk in 1982 and repeated at Yale in 1986.

Autophagic vacuoles that contain cytoplasmic constituents, such as mitochondria or polysomes, in addition to melanosomes, and are limited by cisternae or by two fused cisternal membranes are distinct from compound melanosomes. The latter form by the fusion of individual melanosomes, are limited by a single membrane, and contain melanosomal matrix, with or without melanin, to the exclusion of other cytoplasmic constituents. Both types of giant granule may contain lysosomal enzymes and may indicate cell injury and portend cell death. Cellular autophagy has been reported as a reaction of sublethally altered cells [26] and of lethally altered melanocytes of chickens and mice that develop vitiligo [27]. Frequently, Mi melanoma cells showed frank signs of injury, commonly swelling of cisternae or, less commonly, condensation of cytoplasm with disintegration (apoptosis) of membranes or membranes and cytoplasm [30]. Autophagosomes and compound melanosomes have been observed previously in melanomas of mice, hamsters and human beings, but the process of their formation has not been described [25,31,32]. The presence of the limiting, smooth-walled cisternae suggests that in Mi melanoma cells autophagic vacuoles are formed by enclosure of portions of cytoplasm in a manner similar to that occurring in rat hepatocytes [33]. To our knowledge, ours is the first suggestion that such membranes may have had their origins from trans-Golgi cisternae.

Tyrosinase and Lysosomal Enzyme Activities as Related to Diminished Pigmentation and Viability

Tyrosinase (DOPA oxidase) activity was 2–3 times higher, but melanin content significantly lower in the Mi melanoma than in the Ma tumor (Table I, Fig 1). These actual data are in agreement with those reported in 1982–1983 [7,8]. Activities of three lysosomal marker enzymes are presented in Table II. Although acid phosphatase activities were similar in both tumors, β-glucuronidase and arylsulfatase A were 2–3 times higher in the Mi variant. Spontaneous depig-

Table II. Acid Phosphatase, β-Glucuronidase and Arylsulfatase A Activities in Bomirski Hamster Melanomas Ma and MI

<table>
<thead>
<tr>
<th>Melanoma Variant</th>
<th>Acid Phosphatase (nmol of reaction product per mg wet tissue per hour)</th>
<th>β-Glucuronidase</th>
<th>Arylsulfatase A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ma</td>
<td>44.6 ± 1.34</td>
<td>2.8 ± 0.36</td>
<td>15.5 ± 0.58</td>
</tr>
<tr>
<td>MI</td>
<td>38.3 ± 2.77</td>
<td>9.0 ± 0.45</td>
<td>38.4 ± 1.29</td>
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</tbody>
</table>

*Values represent the mean from 5 assays ± SE.
mation, giving rise to amelanotic or hypomelanotic melanoma lines, have occurred also in some other transplantable melanotic melanomas [34–36]. The growth rates of the more lightly colored melanomas, however, were greater than or at least the same as those of the parental melanotic melanomas. We are not aware of a melanoma variant lighter in color than the parental tumor but growing more slowly. The MI melanoma is the first reported tumor of this kind. It has been known for some time that tyrosinase, the crucial enzyme of melanization, is synthesized independently from melanosomes and transported to the latter via the Golgi system and associated smooth-surfaced cisternae and vesicles [25,37–39]. Previous and present biochemical analysis of Bomirski melanomas has shown that in the MI variant, tyrosinase activity is higher than in the parental, highly melanotic MA melanoma. The unusual location of tyrosinase in the limiting cisternae of autophagic vacuoles suggests that part of the tyrosinase of MI melanoma cells is being sequestered and held in these organelles and does not reach its usual destination, the melanosome. The lack of correlation between melanin content and tyrosinase activity, and the absence of fully melanized, stage IV melanomas, may be due to a diminished supply of tyrosinase to the pigment granules. Therefore, the processes leading to the formation of autophagic vacuoles, increases in lysosomal enzymes and assayable tyrosinase activity may be interrelated and jointly responsible for the retardation of growth, and finally necrosis, as well as the decrease in melanization of this tumor.

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REFERENCES