Selective Aberration and Pigment Loss in Melanosomes of Malignant Melanoma Cells in Vitro by Glycosylation Inhibitors: Premelanosomes as Glycoprotein*

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We have found that glucosamine (1 mg/ml) or tunicamycin (0.2–0.4 μg/ml), specific inhibitors of lipid carrier-dependent glycosylation of protein, when added to cultured B-16 melanoma cells produce a marked loss of pigmentation, accompanied by distinctive biochemical melanin, although their population is not substantially altered melanosomes containing little or no melanin, although their population is not substantially reduced. Within the melanogenic compartments, selective aberration of melanosomes is seen, that is, deformity, bulging, and segregation of their interior membrane, as well as the intramelanosomal formation of irregularly concentric lamellar structure. No apparent structural deformity of Golgi apparatus, Golgi-associated endoplasmic reticulum of lysosome (GERL), and coated vesicles has been observed. Further, no substantial alteration is seen in mitochondria or other cellular structures. Quantitative analysis of altered and nonaltered melanosomes has revealed that the ratio of altered premelanosomes to the total number increases to 44% in glucosamine-treated cells and to 99.5% in tunicamycin-treated cells, compared to 13% in the control.

Electron microscopic dopa reaction has also revealed that these altered melanosomes seem to exhibit a weakly positive or a negative dopa reaction in both glucosamine- and tunicamycin-treated melanoma cells although a number of dopa-positive altered melanosomes are still seen. However, GERL and coated vesicles show no apparent decrease in dopa reaction. It may be concluded that glycoprotein synthesis is integral to the formation of normal melanosomes and to their specific melanizing function, which could be impaired by inhibition of the synthesis of asparagin-linked mannose-containing sugar chains. This results in retrogressive changes in the premelanosomal tyrosinase during its maturation process.

In 1980, we reported much higher tyrosinase activity in the Golgi-associated endoplasmic reticulum of lysosome (GERL) and coated vesicle fraction than that in the premelanosome fraction of the pigment cells [1]. It has been found that tyrosinase synthesized by ribosomes is condensed and activated in the GERL-coated vesicle system, acquiring the capacity to form dopa-melanin in vitro. Thus, the transfer system of the activated tyrosinases into premelanosomes by the possible function of coated vesicles is presumed to be necessary for the initiation of melanogenesis in vivo.

Tyrosinase has been shown to exist in 3 forms, T₁, T₂, and T₃ in the intracelluar melanogenic compartments [2–5], depending on its stage of maturation, which primarily involves its glycosylation. Further we have recently found the carbohydrate moiety of T₂ possesses melanogenically essential properties different from those of T₁, the synthesis of which is not interrupted by current glycosylation inhibitors [6]. The core structure of N-glycosidically linked oligosaccharides is a key structure of T₂ tyrosinase for its melanization-inducing function in premelanosomes [6].

It has been shown that the carbohydrate moieties of glycoproteins in various cellular membranes have a role in the
cellular or intracellular recognition process [7-9]. N-glycosidically linked oligosaccharide chains in hydrolytic enzymes are reported to function intracellularly as a signal for their transport into specific organelles such as lysosomes [8].

Although the precise association between these multiple forms of tyrosinase and melanosome structure is not known, it was stated that tyrosinase in mice is either directly or indirectly involved in forming the melanosome matrix [10]. The above evidence led us to investigate the specific function of each melanogenic subcellular compartment such as GERL, coated vesicles, and premelanosomes, by their dynamics following the selective inhibition of glycosylation using glucosamine (Glc) and tunicamycin (TM). Glc [11] has been reported to inhibit the multiplication of various enveloped animal viruses by interfering with the synthesis of viral glycoproteins. TM [12,13], a novel glucosamine-containing antibiotic, selectively inhibits the synthesis of N-acetyl-glucosaminyl-pyrophosphoryl-poly-isoprenol which is involved in the synthesis of the core sequence of N-glycosidically linked oligosaccharides.

As we predicted, the addition of Glc and TM to the cultured B-16 melanotic melanoma cells has been found to induce unpigmented melanoma cells. In the present paper, marked ultrastructural aberration and pigment loss which are selectively induced in melanosomes by the glycosylation inhibitors will be described together with accompanying changes in the tyrosinase activity of the corresponding melanogenic subcellular compartments.

**Fig. 1.** Disappearance of the melanin pigment in a pellet of glycosylation inhibitor-treated B-16 cells in comparison with those of untreated B-16 cells. The cells were cultured with the glycosylation inhibitors for 11-26 days and trypsinized for pelleting. A, Glc (1 mg/ml)-treated B-16 cells (GL+) on day 11 of culture in comparison with control B-16 cells (B-16). B, TM (0.1, 0.2, and 0.4 mg/ml)-treated B-16 cells on days 10, 22, and 26 of culture. Dose-dependent inhibitory effect on pigmentation is seen. B-16 = control culture on day 7 of culture.

**Fig. 2.** Light microscopic appearance of cultured B-16 melanoma cells. In comparison with the control (a), the glycosylation inhibitors induced unpigmented cells, resulting in the loss of pigment. The dopa reaction is, however, distinctly positive for Glc- and TM-induced unpigmented cells, although 0.4 μg/ml TM-treated cells exhibit a much weaker reaction. a, Control B-16 cells. b, Glc (1 mg/ml)-induced unpigmented cells. c, TM (0.2 μg/ml)-induced unpigmented cells. d, TM (0.4 μg/ml)-induced unpigmented cells. e-h, Respective dopa-reacted cells. X 245.
FIG 3. Representative premelanosomes exhibiting various degrees of structural alteration which is used as the evaluation criteria for quantitative analysis of melanosome aberration induced by glycosylation inhibitors. — = Absent, 1+ = up to 3%, 2+ = 31-70%, and 3+ = 71-100% of premelanosome field. GTA-OsO₄, Bar = 1 µm.

FIG 4. Control cultured B-16 mouse melanotic melanoma cells exhibiting numerous highly melanized spheroid melanosomes (m). GTA-OsO₄, Bar = 1 µm. Inset, Numerous partially melanized melanosomes (m) with well-developed filamentous interiors with periodicity (arrow) in various stages of differentiation. GTA-OsO₄, Bar = 1 µm.

FIG 5. a, Glc-treated B-16 melanoma cells exhibit a complete absence of melanization in the premelanosomes where a filamentous interior with periodicity is generally lost. These premelanosomes (pm) have finely granular homogenous interiors with various degrees of multiconcentric lamellar figures (arrows). GTA-OsO₄, Bar = 1 µm. b, Glc-induced unpigmented melanoma cells exhibit selective aberration of premelanosomes (arrows). GTA-OsO₄, Bar = 1 µm.
ABERRATION AND PIGMENT LOSS BY GLYCOSYLATION INHIBITORS

MATERIALS AND METHODS

Cells
B-16 mouse melanotic melanoma cells were cultured in Eagle's minimal essential medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml

Fig 6. a, Control B-16 mouse melanotic melanoma cells contain dopa-positive premelanosomes (pm) with deposition of newly formed dopa-melanin in the filamentous interiors with regular periodicity. A number of GERL (gl) and coated vesicles (cv) exhibit a strongly dopa-positive reaction. GTA-dopa (37°C, 5 h)-OsO₄, Bar = 1 μm. b, Glc-induced unpigmented melanoma cells contain weakly dopa-positive premelanosomes (pm) in areas, although the premelanosomes have no naturally occurring melanin. Coated vesicles (cv) still exhibit strongly positive dopa reaction. GTA-dopa (37°C, 5 h)-OsO₄, Bar = 1 μm.

Fig 7. a, Glc-induced unpigmented melanoma cells contain strongly dopa-positive GERL (gl). G = Golgi apparatus. GTA-dopa (37°C, 5 h)-OsO₄, Bar = 1 μm. b, Glc-induced unpigmented cells retain dopa-positive reactions in coated vesicles (cv). A fine spike structure (arrows) at their periphery is seen. GTA-dopa (37°C, 5 h)-OsO₄, Bar = 0.1 μm.
Table I. Quantitative analysis of population and structural alteration of melanosomes in unmelanized B-16 melanoma cells induced by glycosylation inhibitors in vitro

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Both types of melanosomes</th>
<th>Melanosomes with melanin</th>
<th>Melanosomes without melanin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2016 (100%)</td>
<td>645 (32.0%)</td>
<td>1371 (68.0%)</td>
</tr>
<tr>
<td>Glc 1 mg/ml</td>
<td>1504 (100%)</td>
<td>15 (1.0%)</td>
<td>1489 (99.0%)</td>
</tr>
<tr>
<td>TM 0.2 μg/ml</td>
<td>3200 (100%)</td>
<td>139 (4.4%)</td>
<td>3061 (95.6%)</td>
</tr>
<tr>
<td>TM 0.4 μg/ml</td>
<td>2558 (100%)</td>
<td>123 (4.8%)</td>
<td>2435 (95.2%)</td>
</tr>
</tbody>
</table>

Abbreviations: Glc = glucosamine, TM = tunicamycin.

* Number of melanosomes per 150 cytoplasmic 64-μm² fields from different cells.

Table II. Quantitative analysis of dopa-positive premelanosomes and coated vesicles in unmelanized B-16 melanoma cells induced by glycosylation inhibitors

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Total number of melanosomes</th>
<th>Subclassification according to extent of dopa reaction</th>
<th>Total number of dopa-positive coated vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3555 (100%)</td>
<td>-- 1+ 2+ 3+</td>
<td>502</td>
</tr>
<tr>
<td>Glc 1 mg/ml</td>
<td>3001 (100%)</td>
<td>-- 1+ 2+ 3+</td>
<td>605</td>
</tr>
<tr>
<td>TM 0.2 μg/ml</td>
<td>4194 (100%)</td>
<td>-- 1+ 2+ 3+</td>
<td>523</td>
</tr>
<tr>
<td>TM 0.4 μg/ml</td>
<td>3527 (100%)</td>
<td>-- 1+ 2+ 3+</td>
<td>209</td>
</tr>
</tbody>
</table>

Abbreviations: Glc = glucosamine, TM = tunicamycin.

* Number of melanosomes or coated vesicles per 150 cytoplasmic 64-μm² fields from different cells.

Electron Microscopy and Cytochemistry

Cultured cells were washed twice with Hanks' balanced salt solution and fixed in situ with 2.5% glutaraldehyde in Hanks' buffer for 60 min at 4°C. After being washed twice with the buffer for 10 min, the cells were postfixed with 1% uranyl acetate for 60 min and then 1% osmium tetroxide for 30 min. The cells were dehydrated with graded ethanol and embedded in Epon 812. For the electron microscopic dopa reaction, cultured cells were fixed with 2.5% glutaraldehyde in Hanks' buffer for 30 min, washed twice with the same buffer for 10 min, and incubated in streptomycin, at 37°C with 5% CO₂/95% air atmosphere. Glucosamine hydrochloride was added to give a concentration of 1 mg/ml culture medium immediately after cell seeding. TM experiments were carried out 24 h after cell seeding using the culture medium containing 0.1, 0.2, and 0.4 μg/ml TM. Thereafter all medium was replaced with the inhibitor-containing fresh medium twice a week during the cell growth for present experiments.

Complete loss of pigmentation was induced with a slight growth inhibition in B-16 melanoma cells after 10 days of culture in the presence of 1 mg/ml Glc (Fig 1A). Similar pigment loss has also been obtained after continuous addition of 0.1, 0.2, and 0.4 μg/ml TM. The degree of pigment loss was parallel to the incubation concentrations of TM (Fig 1B). Corresponding to the macroscopic pigment loss, phase contrast light microscopic examination of unpigmented cells induced by Glc and TM also revealed almost complete pigment loss without substantial morphologic changes of the cells (Fig 2a–d). However, dopa reactions [1] of these unpigmented melanoma cells induced by the glycosylation inhibitors all showed distinctly positive reactions (Fig 2e–h). These glycosylation inhibitor-induced unpigmented melanoma cells have been used in this study. Biochemical characterization of these cells has been described elsewhere [6].

Fig 8. Like control B-16 cells, there are also a number of dopa-negative early premelanosomes (pm) in addition to retained dopa reactivity in many premelanosomes after Glc treatments. GTA-dopa (37°C, 5 h)-OsO₄. Bar = 1 μm.
0.1% L-3,4-dihydroxy-phenylalanine (dopa) (Sigma Chemical Co.) solution in 0.1 M phosphate buffer, pH 6.8, for 5 h at 37°C, and postfixed in the same manner as described above [1]. Thin sections were cut with a Porter-Blum MT-1 ultratome, stained with lead citrate, and examined under a JOEL 100CX microscope.

Quantitative Analysis of the Population and Structural Alterations of Melanosomes

Electron microscopic quantitative analysis of melanosomes has been carried out by counting and classifying melanosomes using the following criteria. One hundred and fifty 64 μm² cytoplasmic fields of different melanoma cells were counted; 64 μm² comprises one field on the electron micrograph with 18,300 magnification. Each sample is quantitatively analyzed for melanized and unmelanized melanosomes. Thereafter, the melanized melanosomes are analyzed for the degree of structural aberration by their subclassification according to the extent of the area occupied by abnormal lamellar or vesicular figures in their interior as follows (Fig 3): — = absent, + = up to 30% of premelanosome field, 2+ = 31-70%, and 3+ = 71-100%.

Tyrosinase activities of premelanosomes and coated vesicles were evaluated by counting and classifying them according to the degree of dopa reactivity in 150 of the cytoplasmic fields. The degree of dopa reactivity of premelanosomes was evaluated by the electron density due to induced dopa-melanin within their structures as follows: — = absent, + = slight, 2+ = moderate, 3+ = marked.

RESULTS

Electron Microscopy of Glc-Induced Unpigmented Melanoma Cells

Electron microscopy of the control melanoma cells shows that a large number of melanosomes in various stages of development are distributed throughout the cytoplasm (Fig 4). Cells which contain only early premelanosomes in abundance are scarcely seen. A well-developed Golgi apparatus and a large number of coated vesicles are present.

Following 10 days of cell culture with the addition of 1 mg/ml Glc immediately after seeding, melanization in the melanosomes of Glc-induced unpigmented melanoma cells (Fig 5) is seen to disappear. These melanosomes have generally lost their characteristic internal membrane structures with periodicity, and show altered, rather homogenous, finely granular interiors, often accompanied by various degrees of abnormal, multiconcentric lamellar figures.

Quantitative analysis of the population and structural alteration of melanosomes (Table I) has revealed that while the production of melanosomes does not greatly diminish (1504 compared to 2016 in the control), melanizing melanosomes almost completely disappear (99.0%) after glycosylation inhibition. This interrupted melanization is accompanied by the complete loss of normally seen periodicity of the interior membrane within the melanosomes; 43.7% of those unmelanized melanosomes further exhibit the formulation of unique concentric lamellar figures.

Electron Microscopic Dopa Reaction of Glc-Induced Unpigmented Melanoma Cells

In the control cells (Fig 6a), induced intracytoplasmic melanization following dopa reaction is localized in the tubular and cisternal portions of the smooth endoplasmic reticulum called GERL as well as in some premelanosomes, but not in the Golgi apparatus as previously found in the in vivo system [1]. In melanized premelanosomes the deposition of newly formed dopa-melanin after the reaction is difficult to identify. On the other hand, there are a number (12.3%) of dopa-negative premelanosomes [1] in spite of the presence of positive GERL and coated vesicles in the same cell (Table II).

In the Glc-induced unpigmented cells, despite a marked and widely distributed aberration of premelanosomes with concomitant loss of in vivo melanization, the reaction product, dopa-melanin is still seen in a considerable number of the premelanosomes (51.2%) although the degree of the reaction is generally reduced (Fig 6b). However, GERL and coated vesicles retain their dopa reactivity both in intensity and reactive population (Fig 7 a,b).

Simulating the control cell, there are early premelanosomes without dopa-melanin deposition, and moreover their numbers

Fig 9. a, TM (0.2 μg/ml) induces almost complete loss of melanization throughout the premelanosomes (pm) and produces marked aberration of premelanosome structures such as irregular lamellar (double arrow) and vesicular (single arrows) figures. GTA-OsO₄, Bar = 1 μm. b, TM (0.2 μg/ml)-induced unpigmented melanoma cells exhibit premelanosomes (pm) with irregular lamellar and vesicular figures (double arrows) in which a few remaining melanin particles (single arrow) are occasionally found. GTA-OsO₄. Bar = 1 μm.
vesicles, an increased number of tyrosinase-negative premelanosomes (48.8%) is seen parallel to an increase of altered premelanosomes (43.7%) (Table I).

Electron Microscopy of TM-Induced Unpigmented Melanoma Cells

In contrast to the untreated control cells, the cells after treatment with 0.2 and 0.4 μg/ml TM almost completely lose their partially and fully melanized melanosomes (Figs 9, 10), retaining only 4.4-4.8% compared to 32.0% in the control (Table I). Except for a few premelanosomes retaining relatively normal structure, almost all premelanosomes (89.5-100%) exhibit marked ultrastructural alteration, which may be described as unmelanized concentric lamellar type with various degrees of asymmetry, multiconcentric type, multivesicular type, and bulging type. No apparent structural abnormalities in other subcellular organelles including GERL and coated vesicles are seen. The population density of premelanosomes is not substantially decreased. In comparison to Glc treatment (1 mg/ml), TM treatment (0.2 or 0.4 μg/ml) induces more marked premelanosome aberration with a rather empty interior.

Electron Microscopic Dopa Reaction of TM-Induced Unpigmented Melanoma Cells

TM-induced unpigmented melanoma cells exhibit distinct dopa-positive reactions in GERL and coated vesicles in the normal range of their population, while a considerable number of premelanosomes (30-70%) are still dopa-positive, though weak, despite almost complete pigment loss and structural aberration in vivo (Figs 11, 12). However, the total number of unmelanized and dopa-negative premelanosomes also distinctly increases (30-70%) compared to the control (12.3%) (Table II). A more distinct decrease in the formation of dopa-melanin after the reaction is seen with 0.4 μg/ml TM treatment than with 0.2 μg/ml TM treatment. Concentric lamellar and multiconcentric types of premelanosomes seem to retain some dopa reactivity, while multivesicular and bulging types of premelanosomes more often lose dopa reactivity.
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Fig 12. a, Electron microscopic dopa reaction of TM (0.4 μg/ml)-induced unpigmented cells exhibits weakly dopa-positive premelanosomes (pm) despite their structural aberration (arrows) and pigment loss. GTA-dopa (37°C, 5 h)-OsO₄. Bar = 1 μm. b, Electron microscopic dopa reaction of TM (0.4 μg/ml)-induced unpigmented cells exhibits numerous aberrated premelanosomes without (pm) and with (arrows) dopa-induced melanin particles. GTA-dopa (37°C, 5 h)-OsO₄. Bar = 1 μm. c, Electron microscopic dopa reaction of TM (0.4 μg/ml)-induced unpigmented cells exhibits strongly dopa-positive GERL (gl) in the vicinity of the Golgi apparatus (G) despite the fact that aberrated bulging premelanosomes (pm) exhibit no or hardly any positive dopa reactivity. GTA-dopa (37°C, 5 h)-OsO₄. Bar = 1 μm.

DISCUSSION

The melanogenic function of tyrosinase may be divided into 2 different stages of maturation. Melanization proceeds only in premelanosomes in vivo, while the GERL-coated vesicle system possesses the capacity to form dopa-melanin in vitro. The intracellular events found in the glycosylation inhibitor-induced interrupted melanogenesis are (1) loss of melanin in melanosomes, (2) the number of premelanosomes formed does not greatly decrease, (3) marked selective aberration of the melanosome structure, (4) GERL and coated vesicles retain their tyrosinase activity capable of forming dopa-melanin in vitro. In order to correlate further these electron microscopic observations with the biochemical findings [6], we have undertaken quantitative analysis of the status of melanogenesis in the various subcellular compartments before and after glycosylation inhibitor treatments. It has been found that although Glc and TM produce up to 99% and 95.2% unpigmented premelanosomes, respectively, approximately ½ and ⅓, respectively, of these unpigmented premelanosomes are found to be still dopa-positive suggesting disturbed maturation of tyrosinase to acquire an in vivo melanizing function in melanosomes.
In addition, dopa-negative unmelanized premelanosomes increase from 12.3% to 48.8% and 70.1% after treatment with Glc and TM, respectively.

In analyzing the genesis of almost complete inhibition of melanization in premelanosomes by the inhibitors, it may be noted that carbohydrate-rich T₃ is reported to be only a form of tyrosinase in membrane-bound conditions, and is capable of melanization in the specialized membranous structure [3]. Our parallel biochemical studies [6] reveal a loss or marked decrease of T₃ in the melanosome-containing fraction as well as in the small-granule fractions despite no decrease in the total tyrosinase activity in this experimental system. Quevedo [10] reported that in most cases where T₂ and T₃ are reduced or absent within hair bulbs of mice, the matrix of the melanosomes exhibits significant defects in the ultrastructure. Furthermore, the hypopigmentation of tyrosinase-positive oculocutaneous albinism [14] and Chédiak-Higashi syndrome [15] have been reported to be related to a defective function of the melanosome membrane. Thus it seems reasonable to assume that T₁ and T₃ are both able to form dopa-melanin in vitro as seen in the GERLs, coated vesicles, and premelanosomes, while in vivo melanization requires T₃ and its specific activation by the function of the melanosome membrane.

Concerning the increased ratio of dopa-negative premelanosomes to the total number of premelanosomes, it can be hypothesized that the inhibitors cause the partial interruption in tyrosinase transfer to premelanosomes from GERLs and coated vesicles which lose T₃ but still contain preactivated dopa-positive T₁ tyrosinase even after the treatments. This interrupted transfer may be due to the observed structural alteration in the melanosomal membrane which results in the abolishment of the tyrosinase-accepting function, possibly the tyrosinase receptor.

Recent investigations in the function of sugar chains of membrane surface glycoprotein provide increasing evidence of their role as signals for secretion, fusion, transportation, and receptor functions in various cellular events [7-9]. It should be mentioned that in our experimental system the incorporation rate of [¹⁴C]-mannose is highest in melanosomal fractions with 3-fold of the total cells and this high incorporation can be inhibited by the glycosylation inhibitors [6].

The specific function of the outer limiting as well as inner membranous structures of melanosomes has been the subject for speculation in the past. However, little evidence is provided. Thus, the specific effects of the marked structural alteration of melanosomes, induced by glycosylation inhibitors, would provide notable information. It has also been shown that carbohydrates contribute to the structure of the premelanosomal matrix [16]. Our present evidence seems to suggest that structurally impaired premelanosomes still can receive transportable tyrosinase to some extent, as seen by the dopa reaction, but the TM- or Glc-induced unmelanized cells have lost their ability presumably in GERL to form mature tyrosinase capable of in vivo melanization.

The above evidence, together with biochemical findings regarding T₃ and total tyrosinase activity [6], would indicate that glycoprotein synthesis is an integral part of the normal structure of melanosomes and their specific function, which could be impaired by the inhibition of the synthesis of the asparagin-linked mannose-containing sugar chain. This impairment is accompanied by retrogressive changes in the premelanosomal tyrosinase to that of GERL-coated vesicles, during its maturation process, losing in vivo melanizing capacity.

REFERENCES