Ultrastructural Effects of Thymidine Analogs on Melanosomes and Virus Activation in Cloned Hamster Melanoma Cells in Culture

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Melanosomes without the characteristic structure of normal melanocytes have been found in experimental melanomas and human nodular melanomas (1–3). They are associated with a unique type of premelanosome, the granular body (1, 2, 4, 5). Melanosomes and premelanosomes in melanomas are tyrosinase positive, but it has been shown that they also contain lysosomal enzymes (6–8). Novikoff et al. (6) proposed that melanosomes are modified lysosomes which tend to break down melanin in “melanosome-complexes” (5, 9) and become large autophagosomes, supporting an earlier view based on Drochman’s observations (10), but no physiochemical evidence of melanin degradation has been reported. In order to investigate the unusual pigmentation system, we examined the ultrastructure of four cell lines established from a transplantable hamster melanoma associated with a R-type virus (1, 11). These clones differ in degree of melanogenesis and virus formation.

One clone (MB line) closely resembled the most predominant cell found in the tumor in vivo (1). Ultrastructurally, melanosomes filled the cytoplasm and a few granular bodies were found (Figs. 1a, b). As colonies aged, large autophagosomes appeared, containing “melanosome complexes” and lamellated structures. Slight to moderate numbers of R-type viruses were observed in the cisternae of rough endoplasmic reticulum (RER) which intertwined with mitochondria, forming a characteristic relationship. Another clone (TD line) appeared similarly in the electron microscope except for an unpredictable tendency to produce large amounts of virus.

The other two clones were distinguished by a block in the melanization process, which appeared most dramatically in the KF line (Fig. 2a, b). These cells contained large golgi with many membrane-lined, smooth-surfaced vesicles (SSV) and granular bodies (premelanosomes), but virtually no melanosomes, and melanosome complexes were never seen, although large autophagosomes containing unidentifiable structures occurred in aged cultures. These cells were not completely amelanotic but Dopa- and Fontana-positive cells did not exceed more than 10%
of the total population. In addition, many lamellated bodies (lysosomes) were present in the cells and frequently in association with the premelanosomes. Viral particles also developed in profusion within the cisternae of the RER, but did not seem to be extruded from the cells. The fourth clone (WE line) was unpigmented at first but as the colonies aged pigment accumulated. Ultrastructurally massive
numbers of granular bodies and SSV filled the cytoplasm. But melanosome complexes and autophagosomes occurred only rarely and never became very large even in the oldest colonies. Viral particles were rarely seen; at the most one or two R-viruses were observed per grid. The cytoplasm was further altered by the absence of the mitochondria–RER relationship which is characteristic for this melanoma.

These findings indicate that pigmentation in melanoma cells proceeds by two basic mechanisms—progression and interruption of melanogenesis (Figs. 3 and 4). In heavily pigmented cells, as the MB line, melanogenesis continues from granular bodies through large melanosomes to melanosome complexes and ultimately autophagosomes; whereas in KF cells melanogenesis is interrupted by the appearance of lamellated structures within granular bodies and melanosomes during the early stages of melanin deposition, producing much lighter colonies.

Injection of cultured cells from each of the four clones subcutaneously in hamsters produced pigmented malignant melanomas. To date these new tumors have not been studied extensively, but clearly the system provides a method to investigate the possible role of R-type virus in melanoma formation as well as histodifferentiation within these cells.

In B-16 mouse melanoma, Silagi (12, 13) reported that 5-bromodeoxyuridine (BrdUR) treatment causes decreased pigment formation and loss of oncogenicity. Others (14–16) also found a high concentration of BrdUR and 5-iododeoxyuridine (IdUR) effective in turning on RNA oncogenic viruses. This technique was utilized to investigate the dual mechanisms of melanosome differentiation as well as virus formation in the melanoma cell clones. For the study we selected two lines suitable for this purpose, one with a complete melanin system and the other, a low virus producer.

**EXPERIMENTAL**

BrdUR was added in concentrations of 25, 50, and 100 μg/ml and iododeoxyuridine (IdUR) was added as 25 and 50 μg/ml to petri dish cultures of MB and WE cells in MEM containing 10% fetal-calf serum. After 48-hr incubation at 37°C in a humidified CO₂ chamber the cells were washed and along with untreated controls cultured for an additional 4–8 days.

Growing cultures were examined without staining by an Olympus Tissue Culture microscope. Some petri dishes were stained with Giemsa and others with Dopa to assess overall pigment formation. For electron microscopy cells were scraped off the plates with a rubber policeman, pelleted at 1000 rpm, and fixed in 3% glutaraldehyde buffered with phosphate. Specimens were postfixed in osmic acid for 2 hr, dehydrated in graded ethanol, treated in propylene oxide, and embedded in a mixture of Araldite and epon. Thin sections were cut with an LKB ultratome, stained with uranyl acetate and lead citrate, and examined on a Siemens Elmiskop 1A.

**RESULTS**

Both drugs caused a certain amount of cell death by 48 hr in each clone. The MB cells were generally killed by 100 μg/ml BrdUR while WE cells tolerated the drug better. Surviving cells appeared enlarged and continued to divide and grow to fill the dishes in 6–7 days.
Fig. 3. The process of melanin deposition. a. Vesicles and granular bodies (premelanosomes) in Golgi region. ×60,000. b. Slightly enlarged premelanosome just starting to pigment. ×56,500. c. Melanosomes with pigment deposition in foci but not on ribs. ×60,000. d. Larger more heavily pigmented melanosome. ×48,000.

Fig. 4. Lamellated structures and interruption of melanogenesis. a. Lamellated structures in granular bodies often seen in KF and WE line cells. ×54,000. b. Lamellated structure occurring in individual melanosomes. ×56,500. c. Formation of melanosome complex. ×51,750. d. Small autophagosome. ×56,500.

Both drugs produced the overall effect of lightening the colonies and this roughly followed a dose-response curve (Fig. 5). Colonies kept more than 9 days after drug removal tended to become repigmented and to escape the drug effect. Pigment inhibition also was reversed if the cells were subcultured in fresh media 4–6 days after drug exposure.
Fig. 5. Densitometric readings of MB and WE cell colonies grown in varying doses of BrdUR and measured with a scanning Photovolt densitometer, Model 52 C. All colonies, including MB treated with 100 μg/ml, had grown to confluence. The percentage density is related to the control taken as 100%. Colonies were scanned in two directions and the results averaged.

**MB Line**

The greatest cell modification was achieved with 50 μg/ml IdUR where melanization virtually came to a halt. Many granular and lamellated bodies filled the cell, usually in association with an expanded and irregularly dilated Golgi apparatus (Fig. 6). If melanosomes were seen, they were usually in conjunction with lamellated structures. Autophagosomes were not observed. Other cytoplasmic organelles appeared disorganized; the RER was reduced in amount and its relation to mitochondria distorted. Irregularly shaped microtubules of odd lengths became evident. But virus particles remained moderate in number and within the cisternae of RER. At 25 μg/ml IdUR also caused an increase in granular and lamellated bodies. Melanosomes were observed but the pigment foci tended to be less dense and gave the distinct impression of being laid down on small tubules (Fig. 7). Lamellated structures were often seen in the incomplete melanosomes. Autophagosomes and melanosome complexes clearly were reduced in number in treated cells compared to controls. BrdUR in tolerated doses produced a similar but lesser effect in reducing the melanization process in treated cells compared to IdUR-treated cells.

**WE Line**

Both BrdUR and IdUR produced essentially the same effects on melanogenesis in this cell line; at 25 μg/ml the Golgi appeared expanded with irregular dilatation and numerous vesicle formation (Fig. 8). While granular bodies were present in large numbers, there seemed to be an even greater profusion of lamellated bodies and frequently granular bodies contained lamellated structures. There were almost
no melanosomes after treatment with both drugs, but IdUR appeared to have a greater suppressive effect. Occasional melanosomes were seen in cells that seemed to have escaped the drug effects. Several other unusual organelles for this line were observed. These included annulate lamellae (Fig. 8, 9), microtubules of irregular size and shape (Fig. 10) and dilated rough endoplasmic reticulum. Increased num-
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Fig. 8. (WE line) Cells exposed to 25 μg/ml BrdUR show dilated Golgi (G), annulate lamellae (AL), and many lysosomal structures. ×28,500.

Fig. 9. (WE line) Another cell showing large annulate lamellae (AL) and a viral particle (♂) in the RER. ×24,700.

Numbers of virus particles were seen only in BrdUR-treated cells. The effect of virus turn-on occurred most notably after 100 μg/ml BrdUR. Typical R-type viruses appeared in 10 or more cells per grid and some cells contained large numbers, but always within the cisternae of RER (Fig. 11). A similar trend was seen with 50 μg/ml BrdUR but not with 25 μg/ml or with IdUR.
DISCUSSION

This study confirms the concept of dual control of pigment formation in melanoma cells. One determines formation of premelanosomes and deposition of melanoprotein to produce melanosomes and the other relates to appearance of lamellated structures and interruption of cell darkening. In these hamster cells, as in the mouse (12, 13), BrdUR and IdUR perturb histodifferentiation in favor
of cell lightening. In this system, IdUR was most effective. Ultrastructurally granular bodies and premelanosomes formed, but melanin was laid down sparsely or often not at all, suggesting that the lightening of melanoma cells after the treatment results partially from cessation of pigment deposition on the structure already formed. A closer examination of the premelanosomes and incompletely pigmented melanosomes leaves the impression that the basic morphologic unit for deposition of pigment may be a tubule rather than a granule, which originally gave it the name of granular body (3, 4). The tubular appearance may be artifactual due to drug therapy, possibly relating to blockade of pigment deposition or it may reflect the true morphology of this structure revealed by the relative lack of melanization.

Lamellated bodies (lysosomes) proliferated in large numbers in treated cells and premelanosomes frequently contained lamellated structures. This finding was demonstrated clearly in MB cells, because the cell line without the treatment does not produce lamellated structures in individual premelanosomes and melanosomes, but only in packaged melanosomes such as melanosome complexes and autophagosomes. Melanosome complexes and autophagosomes were severely limited in MB cells after IdUR or BrdUR. Similarly, in hepatoma cells synthesis of certain specific enzymes are inhibited by BrdUR while such lysosomal enzymes as acid phosphatase continue to be made at normal rates (18).

The explanation for coexistence of tyrosinase and lysosomal enzyme activity in these structures (6–8) has not been elucidated. Ohtaki and Seiji (18) found that while mouse liver degradative enzymes digested protein from isolated melanosomes they failed to release $^{14}$C from labeled melanin in these preparations. In normal melanocytes the two systems may coordinate to ready melanosome complexes for transfer to keratinocytes. But in melanomas where no transfer occurs the lysosomes seem to function to stop deposition of pigment. To date, contrary to Drochman’s suggestion (10) no melanin destructive effect has been described.

As in so many other systems of differentiating cells (12, 13, 19–21), the effects of these thymidine analogs were reversible. Prolonged culture or subculturing of the cells 4–6 days after drug treatment resulted in a greater than normal appearance of pigment and cell darkening.

BrdUR but not IdUR also turned on production of R-virus in a low-producer clone, the WE line. The virus remained within the cisternae of RER. In the mouse melanoma BrdUR turned on production of A and C particles of a murine leukemia virus, perhaps an incidental finding. The ultrastructure of the R-virus after the treatment was identical to that described for an H-virus of malignant hamster tissues (22–24). The presence or absence of virus did not seem to determine melanin synthesis, but there was a tendency for more virus to be present in cells producing fewer melanosomes.

**SUMMARY**

Two clones of hamster melanoma cells, one containing full melanization potential and the other a poor producer of R-type virus, were exposed in culture to BrdUR or IdUR for 48 hr and then washed and grown in fresh media for 4–8 days.

After some initial cell death, the surviving cells grew out well. All treated colonies became lighter than controls. Ultrastructurally, while premelanosomes appeared prominently in the cells, development of melanosomes, melanosome com-
plexes, and autophagosomes was curtailed, especially in IdUR-treated cells and particularly in the pigmented (MB) cell line. At the same time many lamellated bodies (lysosomes) were found in the cells and lamellated structures occurred in premelanosomes. These findings indicate a differential effect of thymidine analogs on the dual system controlling melanogenesis in these melanoma cells. Pigment progression, leading from premelanosomes to melanosome complexes is blocked while interruption of melanogenesis associated with appearance of lamellated structures within the pigmentary bodies continues.

R-virus turn was seen with BrdUR in high concentration, but not with IdUR. The virus appeared typical morphologically and remained in the RER. Virus formation did not seem to relate to pigment synthesis.

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