Light and Electron Microscopic Studies of a Transplantable Melanoma Associated with Virus-like Particles

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SUMMARY

A transplantable melanoma of Golden hamsters has been carried by the chunk method from the 15th through the 52nd generation and examined for growth characteristics and for light and electron microscopic appearance as well as for light microscopy histochemistry. Grossly, tumors were heavily pigmented and invasive, and grew rapidly to kill the animals in 4 to 6 weeks. The growth rate remained stable within the 3 years of study. Histologically, malignant cells formed Fontana-positive pigment and grew as a solid tumor about dilated vascular channels with a supporting reticulum. Very little inflammatory infiltrate occurred, but necrosis and hemorrhage were common. 3,4-Dihydroxy-L-phenylalanine oxidase and acid phosphatase activity were seen together in most viable cells. Ultrastructurally, in addition to premelanosomes and melanosomes, large polyribosomes and rough endoplasmic reticulum abounded in most cells. Large autophagic vacuoles containing melanin granules were seen frequently, suggesting that the cells did not discharge their melanin. Virus-like particles were observed in the rough endoplasmic reticulum in a majority of cells of every generation of tumor; they did not occur in inflammatory or endothelial cells, or in surrounding normal structures. Transplantation of the tumor in albino hamsters resulted in an identical pigmented tumor, except that it grew more slowly. Virus-like particles occurred in large amounts. The relationship of virus-like particles to melanoma formation remains unknown. They may transform cells to increase growth rate and account for the very rapid growth of heavily pigmented melanomas.

INTRODUCTION

Recently we reported the transmission of a pigmented melanoma in Golden hamsters by cell-free material and associated with the presence of VLP's within the cisternae of RER (12). Although VLP's have been described in other transplantable melanomas (7, 22, 28, 37), the sightings have been rare compared with the profusion of particles seen in the melanoma under study (12). This prompted a detailed gross and microscopic study of this tumor to determine how it differed from other transplantable melanomas. The tumor was revealed as a rapidly growing, heavily pigmented melanoma with a constant association of VLP's. The investigation also provided insight into melanophage formation and morphology of VLP's.

MATERIALS AND METHODS

Between 1966 and 1967 and starting with the 15th generation, a pigmented hamster melanoma was carried through the 52nd generation by the “chunk” method in which 30 to 100 cu mm diced tumor are buried subcutaneously in young adult hamsters. After sacrifice with ether anesthesia, the tumors were examined grossly, and fixed for LM and for EM or quick-frozen for special stains. Tissues for LM were fixed in buffered neutral formalin, dehydrated, embedded in paraffin, and cut at 4 ¿i. Every specimen was stained with hematoxylin and eosin and selected ones with Fontana for melanin, the Wilder stain for reticulum, and periodic acid-Schiff with diastase, colloidal iron, and toluidine blue for mucopolysaccharides. Frozen specimens were cut at 5 M on a Harris-International cryostat, treated with DOPA to detect tyrosinase activity, and stained for acid phosphatase by the azo dye method (3), or by a combined DOPA-acid phosphatase method suggested by Mishima (20). Tissues for EM were fixed in 3% phosphate-buffered glutaraldehyde (pH 7.4) overnight at 4°, washed 3 times in buffer, and...
postfixed for 2 hr in osmium tetroxide and also in phosphate buffer at pH 7.4. After dehydration, tissues were embedded in a mixture of Araldite and Epon and cut at 0.5 μ and 600 to 800 Å. Thick sections were stained with toluidine blue and examined by LM. Thin sections were stained with uranyl acetate and lead citrate and examined with a Siemens Elmskop 1A.

In addition, 2 tumors from hamsters bearing the originally transformed Greene melanoma (12) but reared in a different laboratory were examined. Also, the pigmented melanoma was transplanted to true albino hamsters and studied for changes in ultrastructural morphology after each of 4 generations.

RESULTS

Growth Characteristics. The pattern and rate of growth of the tumor remained stable during the period examined. Between 7 and 10 days after transplantation the dark transplant began to enlarge visibly. It grew very rapidly after that and by 3 weeks reached a size of 2 to 3 cm across or a volume of 12 to 18 ml. The hamsters lost weight, became emaciated, and died shortly thereafter, if they were not sacrificed.

Grossly, the tumor was unencapsulated and bound to the tissue that it had invaded. The surface glistened red-black and cut like jelly to reveal reddish brown areas in a black matrix. Ulceration occurred late, but some transplants became necrotic; the edges, however, remained invasive. Metastasis to local lymph nodes occurred, but widespread metastases were rare except on the few occasions when the melanoma was transplanted intraperitoneally; then it seeded and invaded all abdominal organs and tissues, including the diaphragm. Metastasis to the lung occurred under these circumstances.

In albino, the transplants grew more slowly and never reached the size seen in Golden hamsters, but grossly the tumors appeared similar and pigment formation was retained for at least 5 generations. These animals were sacrificed between 4 and 6 weeks.

LM. Malignant cells generally formed a solid tumor which invaded and destroyed surrounding tissue such as fat and muscle. Malignant cells radiated from around large vascular channels (Fig. 1) where mitoses were seen commonly. Wilder’s stain revealed a delicate reticular network in the proliferative area. Individual cells had a large, oval, or irregular pale-staining nucleus with 1 or several nucleoli and a distinct nuclear membrane. The cell contour was irregularly stellate, surrounding abundant cytoplasm which often appeared granular and vacuolated (Figs. 1 and 2). In addition, small amounts of pigment were detected in most cells (Fig. 2). Positive Fontana staining of paraffin-embedded specimens confirmed that the pigment was melanin.

From intact vascular channels the tumor cells became separated, vacuolated, and pyknotic. The network of reticulum was destroyed. Farther away the tumor showed complete disorganization and necrosis. The cells showed nuclear pyknosis with disruption of cell membranes and distribution of debris into the tissue. No inflammatory response occurred, but occasionally plasma cells appeared at the tumor edge. The ubiquitous mast cell generally was absent and the tumor matrix did not concentrate mucopolysaccharides.

Enzyme Histochemistry. The results of stains for tyrosinase and acid phosphatase indicated that most viable cells contained both activities. While it was apparent that cells containing the greatest number of large pigment granules showed mainly acid phosphatase activity and cells containing the smallest pigment granules showed mainly DOPA oxidase activity, a spectrum of cells between these extremes was readily detected showing both activities.

EM. In solid tumor areas viable melanoma cells were crowded (Fig. 3). Plasma membranes were closely apposed and interdigitated. Nuclei were large and oval or irregular with massive, granular nucleoli; chromatins was clumped and tended to marginate along a distinct nuclear membrane containing many nuclear pores. Mitoses were seen frequently and seemed normal. The cytoplasm appeared either pale or dark but characteristically contained many large polyribosomes in rosette forms (Fig. 4). The Golgi apparatus when seen was large and complex. Mitochondria were frequent, large, and irregularly shaped. RER was observed in most cells and sometimes was extensive and dilated; its connection to the nuclear membrane was readily apparent and often the RER ringed groups of mitochondria (Fig. 5), but connections at the plasma membrane were seldom seen. Melanosomes appeared oval or elongated with a characteristic fibrillar substructure which often was obliterated by electron-dense accumulations of melanin (Fig. 6). The number of melanosomes per cell varied considerably and they were distributed throughout the cytoplasm. No significant differences were noted in size or distribution of melanosomes in cells carried in Golden or albino hamsters. The bizarre variation in size observed in carcinogen-induced melanotic cells in white hamsters (31) did not occur. Premelanosomes were seen in profusion only near the Golgi and were often associated with round bodies having a granular matrix (Fig. 6), which are thought to be either premelanosomes cut through the end or a stage preceding premelanosome formation (20, 35). A distinctive feature of this melanoma was the widespread presence of autophagosomes, containing primarily melanosomes in various stages of melanization and myelin figures (Fig. 7). Sometimes these became very large and dominated the cytoplasm, but pure phagocytes without earlier stages of pre- or single melanosomes were rare and nearly all the cells seemed melanotic in nature. Lysosomes and dense bodies were noted mostly in dividing cells or in some dying cells.

VLP's were regularly seen in the malignant cells. They

Colony started from animals kindly supplied by Dr. Philippe Shubik, Director, Eppley Institute for Research in Cancer, University of Nebraska College of Medicine, Omaha, Neb.
were round or slightly oval and 90 to 100 mμ across (Fig. 8). The nucleoid was unevenly dense and spherical with fine projections extending outward toward the limiting membrane (Fig. 8). They appeared almost exclusively within the cisternae of RER and often formed rouleaux (Fig. 8). They were seen prominently in the perinuclear cisternae (Fig. 9), but not in ER attaching at the cell membrane. They were not observed in other cytoplasmic structures or free in the cytoplasm, nor were they recognized in the nucleus or nucleoli. No viral "nucleic acid factory" was detected in the cytoplasm. In dying cells, VLP's were occasionally seen in smooth ER that appeared to have lost ribosomes; and when the cells disintegrated they could be found free between cells, but none were detected with an additional membrane, as occurs when viral particles are extruded from the cell (4).

All melanomas examined contained VLP's, even the earliest tumor sent to our laboratory (15th generation) and those reared elsewhere. Cells in mitosis contained VLP's within RER and albino hamster tumors also showed numerous VLP's within RER. Their distribution bore no obvious relationship to melanosomes or the Golgi apparatus. VLP's were not seen in the few inflammatory cells observed or in endothelial or muscle cells. Their distribution seemed limited to pigment-forming cells and specifically localized to the RER, where they accumulated en masse.

On nearly every thin section some area of necrosis was observed. Viable cells became separated from each other and possessed numerous cytoplasmic microvilli. In dying cells, nuclear clumping and fragmentation were common. Mitochondria swelled, lost cristae, and ruptured. Myelin figures developed extensively in autophagosomes and lysosomes appeared, but phagocytosis of large dead cells and particles occurred uncommonly. Often the cell membrane was broken, and cytoplasmic material was distributed in intercellular spaces, along with erythrocytes leaked from blood vessels.

**DISCUSSION**

Previous LM (13, 14, 24, 30) and EM (8, 10, 11, 25, 34, 36) studies of transplantable melanomas have emphasized the variability in appearance from tumor to tumor. In general, pigment formation in melanomas is associated with a slowing down of tumor growth, while amelanotic melanomas grow very rapidly and metastasize widely. The classic, heavily pigmented Harding-Passey mouse melanoma grows slowly over 3 to 6 months and does not metastasize (1, 18). The melanomas induced by carcinogens in Golden hamsters also show a correlation between pigmentation and rate of growth and metastasis (6, 9, 30, 33). Fortner and Allen (13) initially described 2 pigmented and 4 amelanotic melanomas arising spontaneously in hamsters that were transplantable. These grew at variable rates, but the most pigmented grew most slowly, taking 2 to 3 months to reach a size of 3 x 1.5 cm (13, 14). Subsequently, Fortner et al. (15) described a 3rd transplantable amelanotic tumor and gave more details about the others. Again, the most deeply pigmented tumor grew the most slowly.

Demopoulos et al. (10) made a direct ultrastructural comparison between the slowly growing Cloudman S-91 and rapidly growing B-16 mouse melanomas. The B-16 melanoma was grossly darker and its cells contained many more melanosomes and "compound aggregates" (compound melanin granule-autophagosome) than the S-91. The B-16 killed the host in 3 weeks, but failed to metastasize (10). The tumor that we have studied appears brownish black; it shows a strongly positive Fontana reaction and the cells contain organelles involved in melanogenesis. It is an actively synthetic, heavily pigmented tumor, and is unusually rapidly growing and aggressive. The probable prototype of this melanoma, observed by Greene and Harvey (17), was a spontaneous pigmented hamster melanoma which grew slowly over 6 months and became amelanotic after transplantation. We presume that it later reverted to a pigmented state (12) and increased its growth rate.

Melanosomes in the present tumor were distributed throughout the cytoplasm and varied greatly in number from cell to cell, as did premelanosomes. We were impressed with the large number of compound melanin granules (melanin autophagosomes) seen in many cells. They outnumbered the premelanosomes. Drochmans (11) pointed out that normally the compound melanin granule forms after melanosomes are transferred from the melanocyte to other cells and, in essence, this is the most complex level of organization of melanin granules. It seems that in certain melanomas a block exists in normal melanocyte function so that melanosomes are not transferred. Gordon (16) similarly proposed that the melanoma cell of platyfish-swordtail hybrids is an arrested melanocyte that can not evolve into a melanophore and that this loss of differentiation is a biological mark of malignancy.

The failure of transfer leads to accumulation of melanin granules in large autophagosomes and in some cases gives melanocytes the superficial appearance of a melanophage. Our LM enzyme histochemical findings and ultrastructural observations support the idea that many cells called melanophages result from massive accumulations of autophagosomes in melanotic cells (11, 20, 27, 28). Both tyrosinase and acid phosphatase activity occur in the smooth ER adjacent to the Golgi region (28) and Seiji and Kikuchi (32) recently confirmed the presence of acid phosphatase in melanosomes of melanocytes in the B-16 melanoma.

The question about host influence on tumor color (18, 24) seems to have been solved by Hesselbach (19), who showed through extensive differential selection and transplantation that color change depends upon the tumor and not the host. In the albino hamster the tumor grew more slowly, suggesting the host has some control over growth rate. The tumor did not lose pigment, as
occurs in chemically induced melanomas in white hamsters (21, 29).

The nature of the VLP's which abound in the RER of virtually every melanoma cell is uncertain. Morphologically, they correspond most closely to the type C body of Bernhard and Granboulan (4), but this body has not previously been found in the cisternae of the RER. In any event, of the known oncogenic viruses they resemble a small, coated RNA virus (2, 4). The source of the VLP's also is uncertain. We have seen no densely staining cytoplasmic areas described by Bernhard and Tournier (5) as possible "virus factories" and, in fact, such areas may be antigen-antibody complexes and not viroplasm (23). It is curious that the VLP's congregate in the RER and apparently are not released from the cells. This may be another blocked cell function. They are, however, passed on during cell division. Bernhard and Tournier (5) observed a nearly identical VLP in a number of normal or malignantly transformed hamster cells and proposed it as a new apparent viral infection of the cells. Another VLP (the A type particles) observed by earlier workers (7, 37) in transplantable melanomas is not convincingly demonstrated and tends to occur in many types of normal cells (4). It is likely that they are, at best, noninfectious particles (4). In our material, the VLP has appeared only in malignant pigment-forming cells and never in surrounding tissue cells. Their presence may account for the paradox of a melanoma that grows very rapidly and yet is heavily pigmented. Similar VLP's have recently been seen in Fortner's melanoma (22), a tumor that has increased its growth rate over the years (14, 15), and they may be present in the rapidly growing B-16 melanomas as well (28). Whether or not these VLP's are causal in formation of transplantable melanomas, they may act to transform cells and increase their rate of growth. A further search for VLP's in melanomas seems indicated, especially in rapidly growing human melanomas.

ADDENDUM

Since submission, M. Takahashi and Y. Mishima (A Sequence of Virus-like Particle Formation in the Eristactoplasm of Greene's Malignant Melanoma Cells, Cancer, 24: 904-911, 1969) have confirmed our initial morphological observations (12) and speculated on the origin of the VLP.

ACKNOWLEDGMENTS

We wish to thank Miss Mary Benn for her assistance in this study.

REFERENCES


6. Chernozemski, I., and Raichev, R. Two Transplantable Lines from Melanomas Induced in Syrian Hamsters with 9,10-Dimethyl-1,2-benz (a)anthracence (DMBA), Neoplasma, 13: 577-582, 1966.


21. Illman, O., and Ghadially, F. N. Coat Color and Experimental


Fig. 1. Light microscopy. Plastic embedded. Melanoma and vascular channels. Mitosis, × 400.

Fig. 2. Light microscopy. Plastic embedded. Pigment in most cells. × 400.

Fig. 3. Electron micrograph, low power. Melanoma shows crowding of cells, uniformity of nuclear appearance, mitosis (.), and presence of melanosomes in most cells. × 2750.

Fig. 4. Electron micrograph, high power. Part of a melanoma cell shows nucleus (N), ribosome rosettes, some rough endoplasmic reticulum (ER), melanosomes (m), and microfibrils. × 30,250.

Fig. 5. Electron micrograph. Another cell shows intimate relationship of endoplasmic reticulum (ER) to mitochondria (M). VLP's (••) are seen in cisternae of ER. × 30,000.

Fig. 6. Electron micrograph, high power. A small granular body (g), premelanosomes (p), and melanosomes (m). × 46,750.

Fig. 7. Electron micrograph. VLP's (••), melanosomes (m), and autophagosomes (•). × 24,000.

Fig. 8. Electron micrograph. Accumulation of VLP's (••) in cisternae of RER. Note irregular density of the nucleoid with spokes projecting to the limiting membrane (•••) and the characteristic rouleaux formation (—). × 47,000.

Fig. 9. Electron micrograph. Accumulation of VLP's (••) within the perinuclear and RER cisternae. × 50,000.
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