The Isolation and Cytology of Two Pigment Cell Strains from B16 Mouse Melanomas*

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SUMMARY

Two cell lines (HFH-14, HFH-18) isolated from B16 mouse melanomas were established as monolayer cultures. These cells retained their capacity of melanin pigment formation after repeated passages in vitro. According to their morphology and developmental history, various cell types, namely, melanoblasts, melanocytes, and mature melanocytes, are identified. These cells when inoculated into C57BL mice produced typical melanotic tumors which closely resembled the original melanomas from which the cell strains were derived. These cells have all the characteristics of melanocytes and are considered to be true pigment cell strains.

Even though cell lines derived from mammalian melanomas have been established in vitro (2, 13, 14, 17, 19), among the available cell lines only a few retained the capacity of melanin pigment formation after prolonged cultivation in vitro. Foley et al. (1) have established several tissue culture strains from mammalian melanomas, both human and mouse; none of these is capable of maintaining the pigment content in these cells. Sloboda and Kopac (18), using one of Foley's S91 cell strains, failed to induce melanogenesis when these cells were exposed to agents which were known to stimulate melanin formation. Our experience was similar to Foley's and that of Sloboda and Kopac. Three years ago we were able to establish two cell lines from the Cloudman S91 mouse melanoma and one line from a human amelanotic melanoma. The mouse melanoma cells were originally moderately pigmented, but after several tissue culture passages the predominant cell type became epithelial-like, with complete loss of melanin pigment. Incorporation of melanogenic agents also failed to stimulate pigment production in either one of these cell strains.1 Recently, Moore et al. reported the isolation of a melanotic cell line from the golden hamster which continued to form pigment after a year in vitro (13, 14).

The present paper is to report the successful isolation of two cell lines which after prolonged in vitro passages retain their capacity of melanin pigment formation (6, 7, 9). These cells, we believe, represent a true pigment cell strain.

OBSERVATIONS

HISTORY AND CULTURE CHARACTERISTICS

The B16 mouse melanoma was originally supplied to us through the courtesy of Vernon Riley of the Sloan-Kettering Institute for Cancer Research. The tumors from which the cell lines were derived were at the fourteenth and eighteenth transplantations in C57BL mice in this laboratory.

Strain HFH-14.—The melanoma was excised from the thigh of the mouse 23 days after transplantation. The tumor was cut into small pieces and treated with a trypsin solution on a magnetic stirrer for 30 minutes. The resulting cell suspension was centrifuged for 10 minutes, and the cells were suspended in medium 199 containing neomycin, 20 μg/ml, and penicillin, 100 units/ml, and supplemented by 20 per cent fetal bovine serum. A suspension of 0.5 ml. packed cells in 10 ml. of medium was made, and 2 ml. of this was added to 15 ml. of medium in a Blake bottle (8 oz.) which was incubated at 37 C.

A good growth of cells was obtained in 24 hours; these were predominantly small, round, ovoid or stellate non-pigmented cells. These cells were fed daily and were subcultured twice at intervals of 6 days. During the second subculture the behavior of the cells changed radically. The pH rose to 7.8-8.0, and there were only scattered cells; these cells were small to large and predominantly

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Cultures were kept for a longer period of time, the number and stored in liquid nitrogen. At present, this cell line is and 210 days after isolation some of these cells were frozen starting from day 2. The number of pigmented cells varied. There were stellate cells, bipolar cells, epithelioid-like cells and polydendritic cells. Overlying the nonpigmented cells there were collections of small numbers of pigment-containing cells which were still relatively few in number, but definitely more than one could find in the first 4 days of incubation (Figs. 3, 4). Often the bottles were so crowded with sheets of cells that subculture had to be done in the next 2-3 days.

On the 44th subculture and 406 days after isolation some of these cells were frozen and stored in liquid nitrogen. So far the frozen cells have retained their morphological and culture characteristics when fresh cultures were initiated with the stored cells.

At present, after 17 months \textit{in vitro} and 53 subcultures, the culture pattern has changed somewhat. The appearance of the pigmented cells was delayed slightly, usually taking more than 2 weeks or longer to appear in appreciable numbers as the overlay. Other than this, the behavior of these cells was similar to that observed at their earlier age of \textit{in vitro} life.

\textbf{Strain HFH-18.}—Cell line HFH-18 was established similarly from a B16 mouse melanoma 18 days after transplantation; 19 days after culture there were mostly fibroblast-like cells; 26 days later a few pigmented plaques appeared. When these plaques enlarged, new plaques appeared at the same time in the next 8 days. On the 53rd day of culture the cells were subcultured; lightly pigmented cells appeared in 2 days; from then on, the cells were subcultured at intervals of 7 days. On their 22nd subculture and 210 days after isolation some of these cells were frozen and stored in liquid nitrogen. At present, this cell line is on the 39th subculture, and approximately 13 months after its first isolation its morphology and pigment-forming capacity remain essentially unchanged.

Since in general these two cell strains behaved in the same manner, the following description applies to both cell lines. The only difference between these two lines was that often strain HFH-18 had many more pigmented cells than strain HFH-14. This was true in all stages of growth starting from day 2.

The number of pigmented cells varied from bottle to bottle. Even among the cultures prepared from the same stock culture there were occasional bottles which had more or less pigment-containing cells than their sister cultures. It appears that the melanogenic potentialities of these cells are not uniform. Some cells have higher potentialities than others. By selection, one should be able to select strains which have high pigment-forming capacity and strains which have low melanogenic capacities. Cloning procedures are in progress with specific aims to select these clones for future propagation and experimentation.

If, instead of subculturing at the 7- to 10-day stage, the cultures were kept for a longer period of time, the number of the pigmented cells increased steadily. Also, the size of the pigmented cells increased with time (Figs. 5, 6).

When these cells were permitted to grow for a month or longer, they increased greatly in size and were approximately 5-20 times larger than the small, round, ovoid pigmented cells which appeared first in the cultures (Figs. 8-10). As the cells increased in size, their activity decreased. Other than the large amount of melanin pigment in the cytoplasm, these cells appeared physiologically and functionally inactive. They would remain fixed in morphology and in position for a long time until finally they became degenerated. They did not survive trypsinization.

\textbf{Cytology}

Under phase microscopy only two main groups of cells could be distinguished in the living cultures of B16 melanoma cell strains—namely, (a) pigment-containing cells and (b) nonpigmented cells.

Both groups of cells, pigmented and nonpigmented, varied greatly in size and shape (Figs. 7-10). As described in the previous section, the cell size and shape varied in different stages of their growth. There were small round or ovoid cells, triangular cells, fibroblast-like cells, as well as epithelioid-like cells. Occasionally there were also multinucleated giant cells.

With May-Grünwald-Giemsa staining (11) the following morphological types can be distinguished:

1. \textbf{Small round cells.}—These cells were characterized by their small size, round nuclei, and scanty cytoplasm, which may or may not contain melanin granules. The nuclei resembled those of the larger cells having two or more distinct nucleoli (Fig. 11). These cells, as mentioned in our earlier publications (5, 10), were believed to be young melanocytes and were seen in cultures of normal human skin, as well as in cultures of human melanomas. Occasionally these cells may be found inside the macrophages or epithelioid giant cells (Figs. 11, 12). Some of these cells may be seen to have a short, stublike process on one side or two on each side. The latter apparently represented the early unipolar or bipolar melanocytes.

2. \textbf{Bipolar and polydendritic cells.}—The main cell body, which was the part containing the nucleus of the bipolar cells, was about the same size as that of the small round cell. From the main cell body two slender processes of varying length came off from each of the two poles of the cell body (Fig. 13). The cell body of the polydendritic cells, including the dendrites, was much larger than the small round cells (Figs. 13, 14). Some of these larger cells were binucleated.

3. \textbf{Multinucleated epithelioid giant cells.}—These cells were very large and had abundant cytoplasm. They did not occur in large numbers, but were frequently found in these cultures. In some, the cytoplasm of the cell was vacuolated, contained melanin granules, and sometimes engulfed small round cells (Figs. 11, 12). These cells were found to be present in human (5, 10) as well as hamster melanoma cultures (16).

4. \textbf{Epithelial-like cells.}—These cells were often seen in small aggregates among the more spindle-shaped cells. They had large ovoid or oblong nuclei with two or more
distinct nucleoli or dense chromatin masses. Often the cytoplasm was vacuolated and sometimes contained melanin pigment in the perinuclear location (Figs. 13, 14).

When Masson's ammoniacal silver staining technic (12) was employed in staining cells of 7- to 8-day cultures, and when the stained preparations were compared with the control unstained preparations of the same cultures, always there were more cells containing black granules in the silver-stained preparations than in the unstained preparations. Since premelanin or the reduced melanin is stained black by this technic, the additional black-granule-containing cells in the stained preparations must represent the cells which contain premelanin.

The variation in size and shape of the pigmented cells was well illustrated in these stained preparations. They varied from small round cells, spindle- and stellate-shaped cells, to larger epithelial-like and polydendritic cells (Figs. 7-14).

The pigment in the polydendritic cells appeared to be most abundant in the perinuclear zone and in the tips of the dendritic processes.

The nonpigmented cells also varied in morphology. Predominantly they were small triangular-shaped cells, but spindle, fibroblast-like cells as well as epithelial-like cells were also frequently seen in small aggregates.

**Tumorigenicity**

When C57BL mice were given injections of cell suspensions of strain HFH-14/38 with or without Freund's incomplete adjuvant, typical melanotic tumors were produced. The histological sections of the original tumor and those of the tumors produced by the inoculated tissue culture cells were examined. These sections appeared to be similar. There were nonpigmented epithelial-like cells in the background, with aggregates of typical pigmented melanocytes overlaying the nonpigmented cells in sections of both. The pigmented cells were either round, ovoid, stellate, or spindle, but mostly polydendritic in shape, and they were larger than the nonpigmented epithelial-like cells. The pigmented cells possessed all the characteristic morphologic features of the melanocytes; however, it is probable that the nonpigmented cells represented the more undifferentiated melanoblasts. Also, the latter resembled the cells seen in the fresh subcultures of the cell strains.

**DISCUSSION**

When cells, regardless of origin, are grown in a tissue culture environment they resemble either fibroblasts or epithelial cells. By morphology alone it is not possible to identify the specific cell of origin. When cells from a tumor are grown in vitro, a mixed cell population consisting of the tumor cell proper as well as cells from the connective tissue stroma is obtained. When cell lines are isolated from such a mixed culture, one could not be certain which one (or several) of these cell types would eventually develop into the established line. A similar situation prevails when melanoma cells are grown. The embryonic or the undifferentiated melanoblasts resemble other types of undifferentiated cells and have no characteristic identifying features. However, when these cells differentiate into the pigment-containing melanocytes, it is possible to identify them.

Since, as far as we know, no extensive cytological examinations have been made on Foley's S91 strain and other cell strains mentioned above, it is not possible to make a detailed comparison with our own cell strains. The ultrastructure of one of the cell strains (HFH-14) was studied (6, 7). The identification of premelanosomes, melanosomes, and fully melanized melanin granules in these cells is additional confirmation that these cells are truly pigment-forming cells.

The pigment-forming capacity is the only characteristic which positively identifies a melanocyte. The cells from which the melanocytes are derived may be indirectly identified as the propigment cells or the melanoblasts.

Among all the cell lines so far reported to have originated from mammalian melanomas—i.e., S91 mouse melanoma cells (2, 17), Syrian hamster melanoma cells (13, 14), and human melanoma cells (2, 19)—only one cell strain (RPNI No. 1846), isolated by Moore et al. (13, 14), retained the pigment-producing capacity for a prolonged period in vitro and produced black tumor when reinjected to the hamsters. The nonpigmented cell strain 401 of Sanford et al. (17), when injected into DBA mice, did produce melanotic tumors in the animals. Here one could speculate that the host was an influencing factor in respect to melanogenesis in these cells. Thus this cell strain, by definition, should be classified as that of the amelanotic melanocyte.

We have had the privilege of obtaining information on cell strains which were isolated in various laboratories but have not been reported in the literature. Among these are the following: (a) an amelanotic melanoma cell strain of human origin which remained nonpigmented in vitro, (b) one cell strain isolated from Cloudman S91 melanoma

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Figs. 1-6.—Growth pattern following subculture. Monolayer on glass X 50.
Fig. 1.—2 days. Mostly scattered small round or stellate cells.
Fig. 2.—3 days. Cells in small aggregates.
Fig. 3.—5 days. Sheet formation. Mostly spindle or stellate cells with small numbers of epithelial-like cells.
Fig. 4.—7 days. More definite sheet formation. Occasional small spindle or stellate cells with melanin pigment.
Fig. 5.—12 days. More and larger pigment cells overlying the nonpigmented cells.
Fig. 6.—16 days. Large polydendritic and epithelial-like pigment cells.

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H. Schneider, personal communications.
FIGS. 7-10.—Strain HFH-18; 9-day monolayer culture. Unstained phase contrast microscopy. X 480. Note the great variation in size and shape of the pigment cells.

Fig. 7.—Bipolar melanocyte with a stublike third process appearing.

Fig. 8.—Almost all pigmented cells except one, which was nonpigmented.

Fig. 9.—One large plaque-like pigment cell with several short processes. Other cells were nonpigmented. Note the condensation of melanin granules in the perinuclear position and in the tips of the processes. Other cells were nonpigmented. Note the variation in size and shape of the nonpigmented cells. Some of the nuclei were irregular in shape, a few had more than one nucleus.

Fig. 10.—Two large pigment cells. Compare the tremendous size of these cells with one small round nonpigmented cell situated between two processes of the large pigment cells on the left upper part of the micrograph.
Figs. 11–14.—Strain HFH-14; 20-day monolayer culture. May-
Grünwald-Giemsa stain, × 300.

Fig. 11.—Mostly small, round, or ovoid cells with or without
melanin pigment. One large multinucleated giant cell with vac-
ulated cytoplasm and engulfed round cell.

Fig. 12.—Several spindle or bipolar pigment cells (arrows). A
large giant cell with two large nuclei and engulfed round cell in
center of the picture.

Fig. 13.—Pigmented and nonpigmented spindle and epithelial-
like cells. One epithelial-like pigment cell is shown with its
processes partly encircling two darkly pigmented small round
cells.

Fig. 14.—One tripolar pigment cell with secondary branching
in center of picture.
by Stubblefield\textsuperscript{4} which showed no evidence of melanin formation as monolayer cultures but when seeded on agar medium developed into black colonies. Presumably the black color represented melanin pigmentation, and (c) Sanford\textsuperscript{4} has isolated a strain of cells (NCTC strain 3960) from Cloudman melanoma which showed extensive melanin formation in culture. Also she has established a strain from the amelanotic melanoma (NCTC 3959) which showed little melanin formation. The former strain could thus be considered, like ours, the melanotic melanocyte strain, with the latter, the amelanotic strain. It is not absolutely certain that the latter strain is an amelanotic melanocyte strain unless pigmented tumor is produced by inoculation of these cells in the suitable host or premelanosomes demonstrated by means of electron microscopic examinations.

Hirsch and Zelickson (4) employing electron microscopy studied the tumors which were produced in DBA/2 mice from injections of Foley's S91 cells after prolonged passages in tissue culture. These tumors were noted to have a preponderance of melanosomes rather than the mature melanin granules and were characterized by complete absence of tyrosinase and a very low level of dopa oxidase activity. These features appear to fit the description of amelanotic melanocytes.

The production of typical melanotic tumors in the susceptible animals following injection of cultured cells of our B16 cell strains further proves that these cells are true representatives of the original tumor from which the cells were derived. The identical histology of these two tumors leaves little doubt that these tumors are the same.

When the growth pattern of the pigment cells in vitro is followed closely, one is impressed by their relatively constant morphological variations in different stages of development. In the early days of subculture the small round cells predominated; then, as incubation continued the larger and more complex forms increased. This picture follows closely the developmental stages observed in the fetal Negro skin (20) and in those of the human neonatal skin grown in tissue culture (10). Therefore, one can be reasonably sure that the size of the pigment cells is in inverse proportion to their age; the larger and the more complex their forms are, the older or the more mature they are. In short-term cultures of human melanomas, Cobb and Walker (1) described the melanocytes in one of several forms—i.e. (a) a small ovoid uni- or bipolar non-pigmented dopa-positive cell, (b) a large, multipolar cell with varicose processes and sometimes with cytoplasmic melanin, or (c) a dendritic cell with melanin. The authors felt, as we do, that the dendritic cells probably represent a late stage in the differentiation of pigment cells, whereas the small round or bipolar cells are the immature forms. The largest pigment-containing cells which are physiologically and functionally inactive are perhaps best interpreted as the senile forms of the melanocytes. Their morphology is reminiscent of the large melanophores in fish and amphibians. Gordon (3) observed similar transitional stages between the melanocyte and melanophore in the normal young platyfish. Similarly, in hormone-in-

\textsuperscript{4} E. Stubblefield, personal communications.
\textsuperscript{3} K. K. Sanford, personal communications.

References


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