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Adaptation of Sinclair Swine Melanoma Cells to Long-Term Growth *in Vitro*[1]

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ABSTRACT

Sinclair swine melanoma usually regresses *in vivo*. In the present study, swine melanoma cells were adapted to long-term growth in culture. The morphology of cultured melanoma cells ranged from dendritic to cuboidal, similar to that described for human melanoma cells. Doubling times of the swine melanoma cells were also similar to those of human melanoma cells *in vitro*. 3,4-Dihydroxy-L-phenylalanine oxidase-positive cells were detected by light microscopy, and melanin and premelanosomes were detected by electron microscopy. Cell cultures could be propagated from progressing, partially regressed, and primary cutaneous lesions, as well as from visceral metastases. Thus, it appears that, under these cell culture conditions, Sinclair swine melanoma cells can be adapted to prolonged growth *in vitro*.

INTRODUCTION

The Sinclair miniature swine melanoma model represents a unique animal tumor system for basic studies on tumor development, growth, and regression. Melanocytic tumors develop spontaneously in the swine (4, 5); however, the most unique feature of this neoplastic system is the spontaneous regression of tumors that occurs in a high percentage of the affected animals (11). The Sinclair swine melanoma was first observed in 1967 in one animal of the Sinclair miniature swine breeding herd. Strafuss *et al.* (15) examined 100 of these swine of unstated ages and estimated the incidence of cutaneous melanocytic lesions at 21%. The melanocytic lesions were initially divided into 2 types: an exophytic "nevus-like melanoma" present in 11% of the animals; and a pigmented flat lentigo-like spot present in 15% of the animals (10, 15). A limited breeding experiment initiated to investigate the effect of selective breeding on the incidence of cutaneous exophytic melanomas indicated that the incidence of this tumor was 62% in newborn offspring of the affected parents (3). A recent large breeding study (4, 5) with this animal model confirmed that the incidence of cutaneous melanomas was increased by selective breeding. In this study, 85% of the progeny from selected matings developed cutaneous melanomas by 1 year of age; congenitally acquired melanomas represented 24% of the observed tumors, and 76% of the melanomas developed after birth.

Our studies were designed to evaluate the host and tumor cell characteristics that participate in the regression of Sinclair swine melanoma. One of the characteristics of a malignant neoplasm is infinite growth *in vitro*. Thus, these studies were designed to determine whether Sinclair swine melanoma cells could be adapted to long-term growth in culture. Eight cell cultures have been propagated from 10 to 14 months *in vitro* and have shown no signs of finite life spans. These results suggest that the cells isolated from swine melanoma can become permanent cell lines after propagation *in vitro*.

MATERIALS AND METHODS

Swine

The Sinclair melanoma swine used in this study were derived from miniature swine originally developed at the Hormel Institute of Minnesota. These swine were derived from melanoma female × melanoma male matings. All animals were housed indoors and given free access to water and a commercial swine diet that contained 18% protein. The swine were examined individually for the presence of melanocytic lesions within 72 hr after birth and at 2-week intervals thereafter. At the initial examination, each melanocytic lesion was measured carefully, and the location of each lesion was recorded on individual animal identification forms. At subsequent examinations, each previously identified lesion was measured, and each pig was examined thoroughly for new melanocytic lesions; all of the lesions were measured and their locations were recorded.

Establishment of Cell Cultures

All melanoma cell cultures were established from fresh specimens obtained at the time of necropsy or biopsy of primary and metastatic lesions. Tumor tissue was minced finely with curved scissors, the pieces were washed in medium to remove excess melanin pigment and debris, and the explants were placed in a 75-sq cm tissue culture flask containing a small amount of medium and incubated in a humidified 5% CO_2 atmosphere to allow optimal attachment of the explants to the bottom of the flasks. Additional medium (total volume, 20 ml) was then added, and the explants were fed with fresh medium twice weekly until subcultivation became necessary. The medium used for all cell culture procedures was CMEM[3] supplemented with L-glutamine, vitamins, 1% sodium pyruvate, 1% nonessential amino acids, 100 IU penicillin per ml, 100 μg

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[3] The abbreviations used are: CMEM, complete Eagle's minimal essential medium; DOPA, 3,4-dihydroxy-L-phenylalanine.

streptomycin per ml, 50 μg gentamicin per ml, and 20% fetal calf serum (1). Gentamicin was obtained from Microbiological Associates, Bethesda, Md.; all other components of the CMEM were obtained from KC Biological, Inc., Lenexa, Kans. Cultures were maintained at 37° in a humidified 5% CO_2 atmosphere.

Cell monolayers were rinsed with Hanks' balanced salt solution and overlaid with 2 ml of 0.05% trypsin and 0.02% EDTA (KC Biological, Inc.). The flasks were agitated to facilitate cell detachment and incubated briefly at 37°. The trypsin was inactivated with CMEM, and the suspension was plated at appropriate dilutions in new flasks.

Cells were frozen by the procedure below. Early as well as late subcultures were frozen to provide adequate representative populations for all *in vivo* and *in vitro* experiments.

Cryopreservation of Melanoma Cells

This method has been described previously (1). Tumor cells were suspended in CMEM containing 30% fetal calf serum and 12% dimethyl sulfoxide, and placed in plastic serum test tubes (A/S Nunc, Roskilde, Denmark). The suspension was left at room temperature for 30 min, placed in a −70° freezer for at least 2 hr, and then placed in liquid nitrogen until used. This method of freezing has been found to maintain a high degree of viability of both tumor cells and leukocytes. Frozen samples were thawed rapidly in a hot-water bath, diluted immediately with CMEM, washed by centrifugation, and tested for viability by trypan blue exclusion, by ability to grow in culture, and by transplantation to immunologically privileged sites.

Characterization of Cell Lines (2)

Morphology. Cell cultures were evaluated morphologically by: (*a*) phase-contrast microscopy while actively growing in tissue culture flasks; and (*b*) electron microscopy after fixation of actively growing cultures (9).

Doubling Time. For determination of doubling time, replicate culture dishes received inocula of 5×10^4 cells, and cells from 2 dishes were harvested separately each day and counted with a hemacytometer. Results were plotted on semilogarithmic paper, and doubling times were calculated from the curves.

Saturation Density. These data were obtained from the results of experiments done to determine doubling times. The numbers of cells present in confluent dishes which determine the plateau regions of the growth curves provided the maximum cell number for 35-mm dishes.

Plating Efficiency. Plating efficiencies on plastic were determined per inoculum by counting under a light microscope the colonies present in duplicate dishes 1 week after seeding.

Karyotyping. Exponentially growing cultures were incubated overnight with Colcemid (Grand Island Biological Co., Grand Island, New York), 0.06 μg/ml. Cells were harvested, and chromosome preparations were made according to standard techniques. The preparations were observed under a phase microscope, and at least 25 metaphases were analyzed for each cell type.

DOPA[3] Oxidase Reactivity. For the investigation of pigment-forming cells, DOPA oxidase reactivity was assessed by the method of Rodriguez and McGavran (12).

RESULTS

More than 70% of the swine melanoma specimens cultured have grown progressively. Eight specimens have been propagated from 10 to 14 months *in vitro* with no signs of slowing down in culture. These cultures have now been cryopreserved for future use. Salient features of the lesions and the swine from which they were obtained are shown in Table 1. Melanoma lesions that exhibited varieties of growth characteristics *in vivo* have been found similarly to manifest a range of growth patterns *in vitro*. For example, Lesion 4187-9 arose after birth from normal skin, was biopsied in its progressively growing stage, and produced a culture that has remained spindle shaped throughout 45 passages *in vitro*. Another spindle-shaped culture, Culture 3764-1, was derived from a lesion present after birth on a 16-month-old boar. The lesion had partially regressed at the time of necropsy. Culture 4222-1 was obtained from a tumor that developed after birth from a flat black spot. These cells exhibited an extremely unusual morphology *in vitro* and formed large round phagocytic-appearing structures.

Another morphological variation *in vitro* was an epithelioid cell type observed in several cultures. One of these cultures,

Table 1

Origin of Sinclair swine melanoma cells adapted to long-term growth in vitro

Cell cultures were established from fresh swine melanoma necropsy or biopsy specimens. Explants were prepared and plated in CMEM. At confluency, cells were detached with trypsin/EDTA and replated at appropriate dilutions into clean flasks.

Swine					
Lesion no.	Sex	Date of birth	Date specimen obtained	Development of lesion	No. of *in vitro* passages[a]
4187-9	Female	2/4/77	7/21/77 (sacrificed)	After birth from normal skin	46
4457-1	Male	6/3/77	9/22/77 (sacrificed)	Present at birth	28
3764-1	Male	5/26/76	9/30/77 (sacrificed)	Present at birth	19
4222-1	Female	2/7/77	10/4/77 (biopsied)	After birth from flat black spot	21
4293-1	Male	2/18/77	10/4/77 (biopsied)	Present at birth	22
4453-11	Male	6/3/77	10/14/77 (sacrificed)	After birth from normal skin	23
2839	Female	6/17/74	11/30/77 (biopsied)	At 3 yr of age	19
4609-6	Male	11/19/77	12/14/77 (sacrificed) visceral metastases	Present at birth	22

[a] Cultures were diluted 1/10 for subculturing. Therefore, each passage number represents approximately 3 generations.

Table 2

In vitro characterization of 2 Sinclair swine melanoma cell cultures

Cells were characterized from actively growing cultures which had been propagated *in vitro* for at least 8 passages (24 generations). Data are averages of duplicate dishes which agreed within the error listed.

Culture	Doubling time (hr)	Saturation density ($\times 10^4$ cells/sq cm)	Plating efficiency (%) 3000 cells/ dish	1000 cells/ dish	300 cells/ dish	Karyotype	Modal no.
4187	36 (5.0)[a]	3.9 (7.0)	30.5 (7.1)	28.9 (1.7)	25.7 (9.9)	Diploid	38
4453	34 (5.0)	2.8 (7.0)	40.1 (3.3)	39.1 (2.3)	30.07 (1.1)	Diploid	38

[a] Numbers in parentheses, percentage of error.

Culture 2839 (Fig. 1), was derived from a melanin-exuding lesion which arose on the jowl of a 3-year-old sow. Since the majority of Sinclair swine melanomas arise within 4 months after birth and 35% regress within 1 year, this lesion had an unusually late onset. This particular sow has been used extensively for melanoma matings and has consistently produced the most diseased offspring.

Selected *in vitro* growth characteristics of 2 swine melanoma cultures are illustrated in Table 2. The doubling times and plating efficiencies are very similar to those described for cultured human malignant melanomas (7). Karyotype analysis revealed both cell cultures to be diploid with 38 chromosomes, the normal chromosome number for domestic swine. The normal karyotype of Sinclair swine has not been determined but, since the herd was originally derived from domestic swine, it is quite likely that the normal karyotype is 38. No gross chromosomal abnormalities were detected in our analysis.

DOPA oxidase analysis of the cell cultures revealed DOPA-positive cells occurring with the frequency of about 1 in 200 to 1 in 500. Electron microscopy studies (Figs. 3 and 4) indicated the presence of melanin as well as premelanosomes in the swine melanoma cell cultures.

DISCUSSION

Our results indicate that Sinclair swine melanoma can be adapted to prolonged growth in culture. After *in vitro* propagation for a period of 10 to 14 months, melanoma specimens were cryopreserved; at this time, they demonstrated no signs of cessation of growth *in vitro.* Thus, it would seem that the factors operating *in vivo* to cause regression of Sinclair swine melanoma were not operating *in vitro.* Infinite growth in culture was not demonstrated by previous investigators who attempted to culture Sinclair swine melanoma cells (6). It is possible that different cell culture conditions and different methods of propagating the cell cultures might have accounted for the finite characteristics of the specimens previously cultured. The doubling times of our cell cultures were much shorter than were those of the swine melanoma cultures previously reported and were similar to the doubling times for human melanoma cell lines as reported by other investigators (7). Morphologies of the swine melanoma cell cultures were also very similar to those reported for human melanoma cell lines (7, 8) and ranged from elongated dendritic to triangular dendritic and cuboidal (Figs. 1 and 2).

Although not all the cells were macroscopically pigmented, DOPA oxidase histochemistry revealed DOPA-positive cells with a frequency of about 1 in 200 to 1 in 500. This frequency was very similar to that found in a culture of B-16 melanoma which is known to be highly pigmented. Although the B-16 melanoma is highly melanotic, it has been demonstrated that tyrosinase activity decreases with increased melanization of mature melanosomes (13, 14). Thus, it is not surprising that the number of DOPA-positive cells was low in the B-16 melanoma. Ultrastructural examination of the swine melanoma cell cultures revealed both melanin and premelanosomes. The premelanosomes and DOPA-positive cells were found in cells that had been cultured for 3 to 12 months. Artificial enhancement of pigment cell production has not yet been attempted.

Liao *et al.* (7, 8) reported difficulty in establishing epithelioid cell lines unless they adopted the technique of light trypsinization with EDTA. We have not used trypsinization of primary specimens but have cultured cells directly from explants of biopsy and necropsy specimens. It is thus possible that some of the cell lines were contaminated with fibroblasts. In an attempt to select more malignant subpopulations of melanoma cells, we have begun to transplant biopsy and necropsy specimens to the hamster cheek pouch, followed by adaptation of these cells to culture.

Adaptation of Sinclair swine melanoma cells to growth *in vitro* permits one to distinguish between host and tumor cell characteristics which might participate in the growth and regression of Sinclair swine melanoma. Cell culture techniques not only facilitate evaluation of growth characteristics in the absence of host milieu but also permit direct investigation of the effects of selected agents upon tumor cell growth. Adaptation of cells to growth in culture also permits the use of *in vitro* assays for cell-mediated and humoral immune responses which can further elucidate host factors participating in the *in vivo* growth and regression of this highly relevant tumor system.

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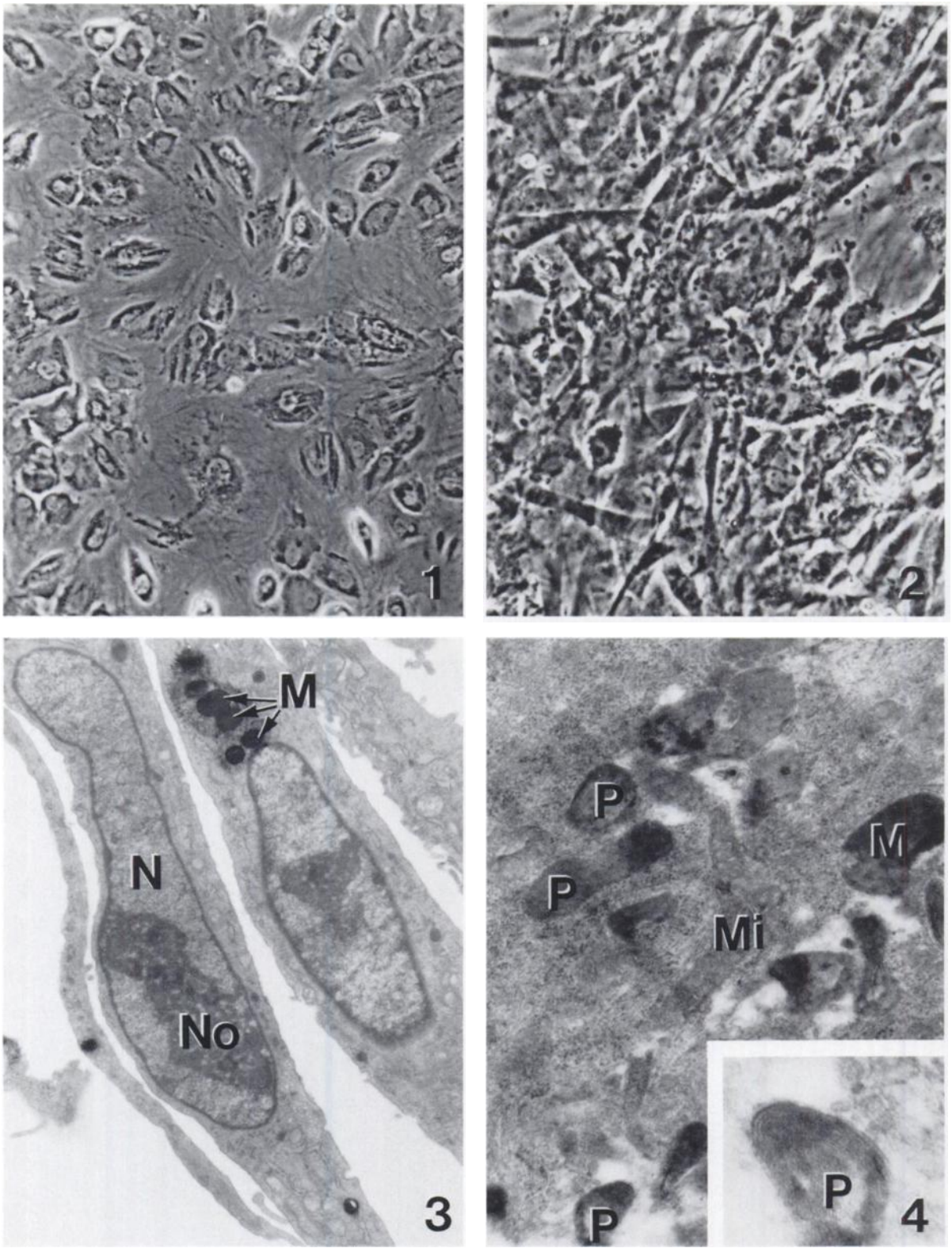

Fig. 1. Phase-contrast micrograph of Sinclair swine melanoma culture 2839, passage 4, illustrating the cuboidal morphology. × 160.

Fig. 2. Phase-contrast micrograph of Sinclair swine melanoma culture 4609, passage 2, demonstrating an elongated dendritic morphology. × 160.

Fig. 3. Electron micrograph of melanoma culture 2839, passage 5, illustrating mature melanosomes (*M*) present in the cell. *N*, nucleus; *No*, nucleolus. × 6,300.

Fig. 4. Electron micrograph of melanoma culture 4609, passage 23, demonstrating the presence of premelanosomes (*P*) and mature melanosomes (*M*) in the cell. *Mi*, mitochondrion. *Inset*, blowup of a premelanosome showing the characteristic striations. × 19,000.