Characterization of a Differentiated Cat Melanoma Cell Line

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ABSTRACT

We have established several long-term cultures of a naturally occurring metastatic melanoma of a domestic cat. The cells are fully differentiated as indicated by the Fontana-Masson staining of the melanin in the perinuclear region and by electron microscopy of the cytoplasmic melanosomes in various stages of development. The melanoma cells do not produce virus particles or the major core proteins (i.e., protein with a molecular weight of 30,000) of the feline leukemia virus or the endogenous cat virus RD-114. Retrovirus is also not induced when melanoma cultures are treated with 5-iodo-2'-deoxyuridine or bromodeoxyuridine and cocultivated with susceptible cells. Treatment of cells with Mβ,3β-dibutyryl cyclic adenosine 3':5'-monophosphate or theophyllin, however, accentuates melanin production and increases the number of pigmented cells. The cultured cells develop melanotic tumors in s.c. inoculated newborn cats and athymic nude mice. As far as we can ascertain, this is the first report of a spontaneous malignant melanoma of a cat which provides an important resource for studying cell differentiation in vitro.

INTRODUCTION

Mammalian melanoma cell lines provide a useful resource to study the mechanisms of melanogenesis and to investigate various biological, immunological, and biochemical parameters of cancer. Despite the common occurrence of both hematopoietic and nonhematopoietic tumors in domestic cats, melanomas have been rarely reported (7, 9, 14, 17, 30). In a survey of 3145 cat necropsies, only 3 melanomas were observed (15). Moreover, long-term cultures of many feline tumors have been difficult to establish due to the occasional presence of foamy or other indigenous cytopathic viruses in cat tissues that destroy cell cultures (20).

Over the past decade, we have examined numerous tumors of various histogenic types from diverse cat species (7, 20, 21) and have diagnosed only 2 melanomas during this period. One of these melanomas was cultured in vitro, and several cell lines were established from different metastatic tumors. Cells are fully differentiated in terms of melanin production, and electron microscopy reveals distinct melanosomes of various shapes and sizes in the cytoplasm. The cat melanoma cells do not produce virus particles nor are they positive for feline retrovirus-related major antigens. However, infection of these cultures by the endogenous cat virus RD-114 induces dendritic extensions and neuronal cell morphology (18). Thus, these cells provide a unique system to investigate molecular mechanisms of cell differentiation and cancer. In this article, we describe various pathological, biological, and ultrastructural features of this rare feline tumor.

MATERIALS AND METHODS

Source of Tumor and Procedures. Fresh surgical specimens were obtained from the autopsy of a 9- (?) year-old, gray, long-haired domestic cat. The primary tumor (CT-1413) was a black mass at the base of the tongue that extended into the throat and metastasized to lungs, kidneys, and the heart; other organs were normal. Each of the primary and secondary tumors was processed by standard methods for pathological diagnosis of hematoxylin- and eosin-stained sections. Tumors from various sites were trimmed free of connective tissue, finely minced, and grown in Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 10% fetal bovine serum, 2 mM glutamine, and 50 μg gentamicin per ml (maintenance medium). Antibiotics were added only for the initial establishment of cultures; henceforth, cultures were maintained in antibiotic-free medium at 37°C in a humidified atmosphere with 5% CO2. Subcultures were made with the use of 0.1% trypsin containing 0.01% EDTA, and single-cell clones of tumor cultures were obtained by plating dilutions containing one cell per 0.1 ml of medium in microtiter plates.

For population doubling time, 25,000 cells were plated per 60-sq mm dish, and viable cells from 4 dishes each were counted every 24 hr for 5 days. The zero-time cell number was estimated 24 hr after initial plating, and the population doubling time was determined by plotting increased cell numbers against time. Plating efficiency of the cells was judged by seeding 500 viable cells per 100-mm dish in the maintenance medium and counting colonies after 8 days of undisturbed growth. The plating efficiency was also determined by seeding single-cell suspensions in medium containing 0.3% agar overlaid on top of 1.2% base agar and counting colonies after 9 days. Ultrastructural studies were done on cells fixed in 2.5% cacodylate-buffered glutaraldehyde (pH 7.4), postfixed in 1% chrome osmium, and embedded in Epon Araldite after dehydration. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by electron microscope.

Attempts to Isolate Retrovirus. For virus isolation, melanoma cultures from all 4 sites (i.e., throat, lung, kidney, and heart) were cocultivated separately with human RD (12), human fibrosarcoma (HT-1080) (23), and dog sarcoma (D17) cells (25). These human and dog cell lines have been shown previously to be permissive for replication of both FeLV and endogenous cat retrovirus (RD-114) (13, 20, 22, 23). The supernatant fluids from the cocultures were tested for RT activity at various intervals for up to 60 days.

Two sets of cat melanoma cultures each from lung and throat tumors at passages 5 and 12, respectively, were separately treated with 50 and 100 μg IdUrd or BrdUrd per ml. After 24 and 48 hr, the supernatant fluids from all of the cultures were tested for RT activity, and each group of cells was separately cocultivated with human (RD) and dog (D17) cells.

Infectivity Assays. The cat melanoma cells from the throat and metastatic lung tumors were tested for susceptibility to exogenous
infection with FeLV (GA strain) (7), RaLV (19), BaeEV (31), amphotropic and ecotropic MuLV (MuLV-A and MuLV-E, respectively) (22), and endogenous cat virus RD-114 (13). Indicator cells were plated in media containing 4 μg polybrene per ml and exposed to filtered (0.45-μm pore size) virus as described previously (22, 24). Cultures were tested for virus replication by RT assays and for RD-114 and FeLV p30 antigen expression by the use of specific antisera in an immunofluorescent antibody assay (25). Virus-exposed and unexposed cultures were observed for 6 to 8 weeks for possible morphological alterations.

**Tumorigenicity.** To test the tumorigenic potential of the cat melanoma cells, we inoculated BALB/c athymic nude mice s.c. with cultures from metastatic lung tumor (passage 10) at a density of 1 to 5 × 10⁶ cells per animal. We also tested pathogenicity of these cells in 4 newborn cats by s.c. inoculation of 5 to 10 × 10⁷ cells in randomly distributed areas of the nape and 2 thighs of each kitten.

**RESULTS**

Histologically, the primary (throat, lingual) cat tumor was a widely metastatic malignant melanoma. Despite some areas of necrosis, light microscopy of the hematoxylin- and eosin-stained sections revealed numerous mitoses in the primary tumor of the throat and in the metastatic tumors of lung, heart, and kidney (Fig. 1A). The tumor consisted of densely populated, rounded, and polygonal cells with indistinct and overlapping boundaries of basophilic cytoplasm. The nuclei were large with prominent nucleoli. Although the cytoplasm of some unstrained tumor cells contained darkly pigmented granules, with special Fontana-Mason staining, virtually all of the cells showed pigmentation around the nucleus.

**Establishment and Characterization of Cell Lines.** Long-term cultures were established from primary throat tumor and from metastatic lung and heart tumors (designated CT-1413-T, CT-1413-L, and CT-1413-H, respectively). Cells from all 3 cultures appeared identical in morphology, and no fibroblasts or other cell types were seen in any of these cell lines. In general, the cells adhered loosely to the plastic surface and grew mainly as suspension cultures, except those derived from the lung tumor. We therefore used CT-1413-L cells for most of the experiments, because their adherence to the plastic surface facilitated judgment of cell behavior and morphology in vitro. The cat melanoma cultures have been maintained in vitro for more than 4 years without a lag period initially or in subsequent subculture passages.

When sparsely plated in vitro, the tumor cells (CT-1413-L) attached to the substrate and appeared somewhat spindle shaped with rounded bodies and small bipolar extensions (Fig. 1B). At confluence, however, most of the cells rounded up, some detached from the monolayer and proliferated as aggregates in suspension.

The cat melanoma cells proliferated rapidly with an average population doubling time of 19.5 hr at 37° (Table 1). At lower temperature (33°), the doubling time increased to 29.5 hr; but at higher temperatures, (38-40°), the cell population doubled at the same rate as at 37° (i.e., about 20 hr). However, all 3 cell lines (CT-1413-T, CT-1413-L, and CT-1413-H) were maintained at 37°, unless indicated. The cultures grew to high saturation densities of approximately 5 to 8 × 10⁶ cells per sq cm. The plating efficiency of CT-1413-L tumor, both in soft agar (0.3%) and in liquid culture medium, was higher than that observed for many feline or human tumors. Approximately 80% of the cells formed colonies when plated at low densities (500 cells per 100-mm dish) in liquid medium, and 70% of the cells formed macroscopic colonies within 9 days in 0.3% agar medium on top of agar base layer (Table 1).

A black-brown pigmentation in the perinuclear region of the cells was accentuated when cells reached confluence or were grown as suspension cultures in soft agar (0.3%) or in liquid medium. Hypermelanization was also seen when monolayer cultures were maintained at 38-39°, but cells became hypomelanized at 33°. Treatment of cat melanoma cells with the thymidine analogue IdUrd reduced cell pigmentation, which reappeared after removal of the drug and subsequent subcultures in vitro. However, exposure of melanoma cultures to N⁶(2-butyryl cyclic adenosine 3':5'-monophosphate (1 mM) or theophyllin (1 mM) increased the number of pigmented cells within 48 hr by 5 and 25%, respectively. Since melanin synthesis begins in the melanosomes by the oxidation of tyrosine to dopa in the presence of tyrosinase, we also tested for this enzyme activity in the cell-free extracts (10). Our preliminary results indicated higher levels of tyrosinase activity in melanoma cells compared to the normal cat embryo cells.

Thin-section electron microscopy of the tumor cultures (CT-1413-L) exhibited rounded cells with poorly defined cellular membranes, large nuclei, and prominent nucleoli. The cytoplasm contained distinct melanosomes, Golgi vesicles, abundant free polyribosomes, and only sparse rough endoplasmic reticulum (Fig. 2A). The melanosomes were surrounded by limiting membranes and revealed characteristic internal filamentous structures with granules (Fig. 2B). The shapes and sizes of these organelles varied from oval, ellipsoid to rounded, and they contained different degrees of pigment deposits. Also, the melanosomes were present in various stages of maturation either singly

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**Table 1**

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<tr>
<th>Tumor incidence</th>
<th>Histopathology</th>
<th>Melanin production in vitro</th>
<th>Morphology in vitro</th>
<th>Growth pattern in vitro</th>
<th>Electron microscopy</th>
<th>Chromosomes (passage 3)</th>
<th>Plating efficiency (%)</th>
<th>T₀ (hr)</th>
<th>FOCSA-like cell surface antigen or FeSV-induced (les) protein</th>
</tr>
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<tr>
<td>Rare tumor of domestic cats</td>
<td>Malignant melanoma (throat), metastatic to lung, heart, and kidney</td>
<td>Positive, increased by treatment with cyclic adenosine 3':5'-monophosphate and theophyllin</td>
<td>Rounded and spindle shaped</td>
<td>Grows as monolayer and as suspension culture</td>
<td>Virus particles or Mycoplasma absent. Numerous cytoplasmic melanosomes with black pigmentation</td>
<td>36-43, altered chromosomess</td>
<td>19.5 at 37°</td>
<td>80</td>
<td>Absent</td>
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S. Rasheed, unpublished observations.
or in groups with dense sedimentation of melanin (Fig. 2B). There was no accumulation of melanin in the mitochondria, although pigment was present in membrane-bound lysosomal vacuoles. The tonofilibrils or intracellular bridges were not seen, and only a few microvilli extended from the surface of some cells.

Chromosome analysis of CT-1413-H and CT-1413-L cells at the third and 28th subculture passages, respectively, indicated an altered karyotype of cat chromosomes with modal numbers of 41 and 42. The electrophoretic mobility patterns of lactate dehydrogenase and glucose-6-phosphate dehydrogenase isoenzymes of the melanoma also identified these cells to be of cat origin.

By immunofluorescence staining, the cat melanoma cells did not express the FOCMA using FOCMA-positive natural cat sera (4). Also, the FeSV-induced transformation-specific protein (designated fes) (1, 29) was not present in the cat melanoma, although a normal cellular counterpart of this polypeptide, of about M, 92,000, was identified in these cells (courtesy of Dr. M. Barbacid, National Cancer Institute). Inasmuch as the human and hamster melanoma cell cultures have been shown to display specific membrane receptors for the NGF (5), we tested the cat melanoma cells in a similar NGF receptor binding assay but found none (courtesy of Dr. J. E. Delarco, National Cancer Institute).

The cat melanoma cells did not show retrovirus-like particles or Mycoplasma organisms by electron microscopy nor did they exhibit RT enzyme in their culture fluids or major structural proteins (p30) of FeLV or endogenous cat virus, RD-114, in the cells. Retrovirus was also not induced when these tumor cells were treated with 50 or 100 μg IdUrd or BrdUrd per ml for 24 to 48 hr and each cocultivated separately with human RD or HT-1080 and dog D17 cells. Cultures treated with IdUrd or BrdUrd on those treated and subsequently cocultivated with susceptible human or dog cells did not exhibit RT activity for up to 60 days after the treatment. These cocultures were also negative for FeLV and RD-114 virus p30 antigens by immunofluorescence assays with specific antisera to these proteins.

**Tumorigenicity.** Inoculation s.c. of 1 to 5 × 10⁶ cells (CT-1413-L) in athymic nude BALB/c mice produced large black tumors in 7 to 15 days (Fig. 1C) that appeared to migrate to nearby s.c. sites, including one lymph node. Although the lymph node tumor regressed in 20 to 25 days, the s.c. tumors continued to grow to enormous sizes, and the animals had to be killed. One of these tumors was serially transplanted twice in nude mice, each time producing highly pigmented tumors in 100% of the inoculated animals. We also inoculated 4 newborn kittens with 5 to 10 × 10⁷ CT-1413-L cells under the skin of both the thighs and the nape areas. Within 10 to 15 days, melanotic tumors of about 2 cm in diameter appeared at the inoculation sites of all the cats. These tumors grew progressively, and one nodule appeared in the groin area by 20 to 25 days, although the tumor cells were not directly inoculated in this area. The groin tumor regressed in seven days, but numerous pigmented, grape-sized s.c. tumors in addition to the primary tumors continued to grow. The cats were sacrificed after 45 days.

The histopathology of the transplant tumors was identical to the primary melanoma (Fig. 1D), and the cell lines established from in vivo-induced cat and athymic nude mouse tumors showed morphology and growth properties similar to those of the initially inoculated CT-1413-L cells. The in vitro-grown transplant tumor cells were pigmented and proliferated with approximately the same doubling time as the original cat melanoma cultures (i.e., 20 hr). Moreover, these cells did not show RT activity in the supernatant fluids, indicating absence of replicating retrovirus in the transplant tumors. Further, type C virus was not activated when the in vitro-grown transplant tumor cells from kittens or nude mice were treated with 100 μg IdUrd per ml and subsequently cocultivated with human (RD) or dog (D17) cells.

**Susceptibility of Cells to Virus Infection and Differentiation.** Although detectable levels of endogenous retrovirus activity were not present in any of the 3 cell lines from primary and metastatic tumors (CT-1413-T, -L, and -H), separate exogenous exposure of these cells to various replication competent retroviruses resulted in chronic infection. Thus, the cat melanoma cells were permissive to replication of FeLV, amphotropic MuLV, cat endogenous virus (RD-114), and, to a limited extent, BaEV. As expected, these cells were resistant to replication of ecotropic MuLV and endogenous RaLV and HaLV. The virus-infected and uninfected cultures were morphologically identical, with the exception of cells exposed to RD-114 cat virus. Exposure of melanoma cultures to the RD-114 virus, but not to any other retrovirus, induced neuronal cells with multipolar dendritic extensions in the CT-1413-L cells within 24 hr. These results have been reported elsewhere (18).

**DISCUSSION**

We have established long-term cultures of a malignant melanoma from a domestic cat. The in vitro-derived cultures of the primary and metastatic tumors consist of a fairly homogeneous population of cells that exhibit some morphological resemblance to a melanotic neuroectodermal human tumor described earlier (2). Recently, uveal tumors were induced by intraocular inoculation of a FeSV (GA strain) in kittens, but melanotic cell lines were

<table>
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<th>Normal cell protein</th>
<th>cross-reactive with fes protein</th>
<th>Anchorage independent growth</th>
<th>FeLV and RD-114 p30 antigen</th>
<th>FeLV RNA in cytoplasm</th>
<th>RD-114 virus in cytoplasm</th>
<th>Susceptibility to virus replication</th>
<th>Tumorigenicity</th>
<th>Receptors for NGF</th>
<th>Differentiation</th>
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<td>Present</td>
<td>Absent</td>
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<td>Absent</td>
<td>Present</td>
<td>Present</td>
<td>Absent (preliminary evidence)</td>
<td>Producers in athymic nude mice and newborn kittens</td>
<td>Absent (preliminary evidence)</td>
<td>Produces dendritic extensions upon exposure to RD-114 virus</td>
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<th>Present Forms macroscopic colonies in soft agar</th>
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<th>NGF</th>
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not established from these tumors (28). However, our cell lines were obtained from a naturally occurring cat tumor and do not produce any virus. As far as we can determine, this is the first example of a melanin productive long-term culture of a naturally occurring cat melanoma.

Although tumors of many heterologous species can be transplanted in the nude mice, it has been virtually impossible to induce s.c. tumors in cats by nonviral transformed cat cells. The high malignant potential of these melanoma cells as exhibited by the development of tumors in the inoculated cat is important. Furthermore, because these cells are also differentiated (i.e., melanin productive), they provide a useful tool for investigating important links between oncogenesis and cell differentiation.

Presence of melanosomes in the cytoplasm establishes the melanocytic origin of the cat melanoma cells (Fig. 2, A and B). The hypomelanization of the cat melanoma cells by BrdUrd appears to be similar to the inhibition of hemoglobin production by this drug in the Friend MuLV-infected differentiated cells (16). However, as the level of pigmentation in these cells is influenced by a wide variety of chemicals and other factors including cell density, serum depletion, and temperature and pH changes, the transitory depigmentation by BrdUrd may not be specific to this melanoma or to this drug. Also, the depigmentation does not reflect dedifferentiation, because cultures derived from single-cell clones of apparently depigmented tumor cells (i.e., those that formed whitish rather than black-brown pellets on centrifugation) caused melanotic tumors in the athymic nude mice. This indicates that the transitory reduction in pigmentation is due to the culture conditions or phases in cell cycles and that there is no correlation between pigmentation and tumor induction.

The presence of RD-114 virus-like RNA in the absence of virus production is not an unusual phenomenon, because similar levels of this virus-related RNA in the absence of detectable p30 antigen or type C virus particles are also found in most normal cat embryo and tumor cells tested (27). The cat melanoma cells also lack a FOCMA-like cell surface antigen, albeit other tumor-specific antigens similar to those described for human melanomas (3, 6, 8) may yet be detected. Absence of NGF receptors on the cat melanoma cells suggests that they may possibly produce this factor. However, we failed to induce neurite formation in a human neuroblastoma culture (IMR-32) with the conditioned medium from the cat melanoma cultures.

Levy et al. (11) suggested a possible relationship of type C retrovirus replication to reduced melanin production in melanoma cells. We have not observed any significant visual depigmentation in cells infected separately with various retroviruses compared to the uninfected cultures. The most remarkable change observed in the cell morphology of the cat endogenous virus (RD-114)-infected melanoma cells is the production of a dense network of dendritic extensions and connections with neighboring neuron-like terminally differentiated cells (18). These features are not seen in other mammalian melanoma cells or other retrovirus-infected cat melanoma cells (18). The neuronal cell differentiation of a melanotic cat tumor is particularly important, because during embryogenesis, the neural crest gives rise to both neuronal cells and the melanocytes. Thus, these melanoma cells provide an exclusive system to study the multistage process of oncogenesis and to understand the relationship between cancer and differentiation.

ACKNOWLEDGMENTS

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REFERENCES


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Fig. 1. A, primary melanoma tumor (CT-1413-T). H & E, × 750. B, tissue culture CT-1413-L cells. Phase contrast, × 600. C, athymic nude mouse; melanoma developed within 10 days of s.c. inoculation of tissue culture cells (CT-1413-L). Note the spread of tumor from the nape area to the back. D, histopathology of nude mouse tumor. H & E, × 750.
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Fig. 2. A, ultrathin sections of tissue culture cell (CT-1413-L). Uranyl acetate and lead citrate, × 12,500. B, a portion of pigment-producing cells in culture showing ultrastructures of melanosomes in the cytoplasm. × 40,500.
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