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Malignant Melanoma in Ultraviolet Irradiated Laboratory Opossums: Initiation in Suckling Young, Metastasis in Adults, and Xenograft Behavior in Nude Mice[1]

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ABSTRACT

Litters of suckling young of the laboratory opossum (*Monodelphis domestica*) were irradiated with UV light from sunlamps with a spectral emission peak at 302 nm (UVB) to induce melanocytic nevi. Total doses of 0.87–5.0 kJ/m^2 were divided equally among up to 14 exposures during the 19 days from birth. Of 358 sucklings exposed, 217 survived to weaning, and 22 (10%) possessed a nevus when shaved and examined at or after weaning. Affected animals were then exposed 3 times/week to 125 J/m^2 of UVB for up to 45 weeks to promote progression to malignancy. Nevi of 8 of the 20 chronically-exposed animals progressed to malignant melanoma with metastases to lymph node(s). Cell cultures were prepared from affected nodes to confirm that pigmented nodal cells were metastatic melanomas. One established cell line (TD15L) contained highly pigmented, dendritic, malignant melanoma cells. These cells, injected s.c. as xenogeneic grafts into athymic nude mice, remained viable in the subcutis and were moderately tumorigenic in the dermis. UVR exposure of *Monodelphis* sucklings is a novel, effective, and proficient way of initiating melanocytic lesions for studies on susceptibility and progression to melanoma, and the cell lines derived from these melanomas will provide promising new reagents for chemotherapy and immunotherapy investigations.

INTRODUCTION

The relationship between human cutaneous melanoma and exposure to the UV component in sunlight is supported by strong evidence (1–3) but there is little understanding of the precise mechanisms of melanoma initiation, the biologically effective radiation dose, or the preclinical events in the latent period of this disease (4). More is known about the molecular mechanisms involved at clinical stages leading to frank metastasis, but many basic issues associated with melanoma progression and invasion have yet to be resolved (5). Given the difficulties associated with human studies, there has been a long-standing interest in the development of animal models to study not only the origins of melanoma but its complex progression, treatment, and prevention. Each of the widely used model species [rodent (primarily mouse), pig (Sinclair swine) and fish (*Xiphophorus*)] has some value in specific areas for comparative studies of human melanoma but none provides a satisfactory match of the genetic, biochemical, and pathological characteristics of the human disease (6).

The potential of the laboratory opossum (*Monodelphis domestica*) as a useful melanoma model became clear following the dermatological and photobiological studies of Dr. R. D. Ley *et al.* (7, 8). One attractive aspect of the model is that unique among the mammalian species thus far examined, benign and malignant melanomas may be induced by chronic UVR exposure alone, without the concomitant application of chemical carcinogens (9, 10). Using a similar protocol for shaving and exposing adult animals, we sought to develop *Monodelphis* as a new animal model for examining various aspects of the genetics and chemotherapy of melanocytic nevi and melanoma (as well as other skin and eye neoplasias known to occur in this species) (11–13). In our initial study of 137 adults chronically exposed to UVR[3] for up to 40 weeks or more, melanocytic nevi were induced in 19 (14%). None of the nevi progressed to malignant melanoma. A follow-up study involved 97 animals that were introduced to the same irradiation regimen at an earlier (weanling) stage (8–10 weeks after birth). Widespread freckling was observed but animals with melanocytic nevi were extremely rare. A single animal, however, developed malignant melanoma with presumptive metastasis to the spleen (14).

In an attempt to investigate further the question of differential age-related susceptibility to formation of melanocytic lesions, neonates were targeted as the initial stage for UVR exposure. Several features of the early postnatal development of *Monodelphis* made this approach feasible. Adult females (unlike most female marsupials) lack a pouch, so that sucklings firmly attached to a teat are fully exposed on the ventral surface of the mother and entire litters can be exposed to UVR over their dorsolateral regions. With a maximum litter size of 13 (mean = 7), many individuals can be irradiated repeatedly for almost 3 weeks before periodic detachment from the nipple normally begins. Also, at this early stage of development, hair growth is rudimentary so the thin, partially differentiated skin requires no shaving.

In this paper, we provide the first descriptions of melanocytic lesions of weanlings that were exposed to UVR as suckling young, and on the progression of many of these lesions to melanomas after continued UVR exposure of affected animals. We also report on the characteristics of novel cell lines derived from melanoma cells of lymph nodes in culture and in athymic nude mice. Finally, we give an assessment of the potential of opossum melanoma cell lines for investigations in genetics, immunology, and chemotherapy.

MATERIALS AND METHODS

Animals and Irradiation Procedures. All animals were produced and maintained at the SFBR in conditions similar to those used for laboratory rodents. Details of the husbandry and care of animals in the SFBR colony have been described previously (15, 16). All of the experimental procedures described in this report received prior approval from the SFBR Animal Research Committee.

Litters of 6 or more qualified for entry to the irradiation protocol. Suckling young received their first dose of UVR ~12 h after birth (Fig. 1*a*) or up to 5 days later. Each naked suckling is attached continuously, for about 19 days after birth (Fig. 1*b*), to 1 of the 13 teats of the dam in a well defined ventral abdominal field (17). In the absence of a pouch that in most marsupials protects the young, entire litters aged up to 19 days were exposed to UVR by placing the dam in a standard polypropylene or polycarbonate mouse cage with the floor replaced by wire mesh. The cage was fixed 26 cm above an FS40 fluorescent sunlamp source (National Biological Corporation, Twinsburg, OH) with an emission spectral peak of 302 nm. The dam was free to move around the floor of the cage horizontally, but her vertical movements were inhibited by the floor of a second cage placed inside the exposure cage. In these constrained maternal conditions, the dorsal and lateral surfaces of the pendulous young were assumed to receive approximately equal doses of UVR. Of the 43

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[3] The abbreviations used are: UVR, UV radiation; SFBR, Southwest Foundation for Biomedical Research.

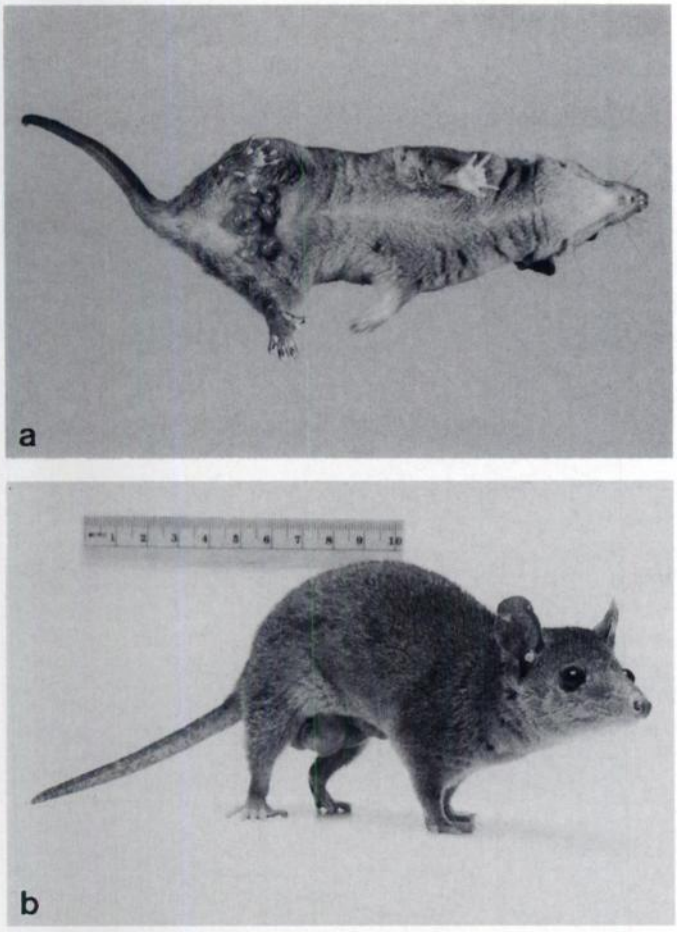

Fig. 1. *Monodelphis* females with litters of attached suckling young representing the earliest (*a*) and latest (*b*) stages exposed to UVR. (*a*) Litter <12 h postpartum, photographed from below through a glass plate on which the dam was walking. (*b*) Litter at 18 days, attached to the abdominal surface of the dam. *Ruler*, scale (in cm).

exposed litters, 13 received doses of approximately 125 J/m^2 of UVB every other day, for up to 19 days after birth, with a maximum total dose of 1.12 kJ/m^2. In an attempt to determine an optimal protocol for lesion induction, groups among the remaining 30 litters received different total doses, based on the number of exposures and the dose per exposure, up to a total dose of 5.0 kJ/m^2. Irradiations were carried out inside a chamber with the light source on the floor and a wooden frame supporting the cages. Dose rates were monitored with an Optronic Model 730 spectroradiometer (Optronic Laboratories Inc., Orlando, FL). From the time of entry into the irradiation protocol, females with litters were housed in rooms illuminated with red fluorescent lamps (General Electric, F40R). Red light is outside the wavelength range known to activate a photolyase which repairs pyrimidine dimers induced by UVR in *Monodelphis* (18).

All animals exposed as sucklings were shaved and examined for melanocytic nevi at weaning (2 months postpartum). Assuming that further exposure might promote lesion growth and tumorigenesis, animals with nevi were introduced to an established adult and juvenile irradiation protocol (13, 14) of 3 exposures/week for up to 45 weeks unless euthanasia was required or unscheduled death occurred. A group of 30 unaffected weanlings was held for up to 4 months before being shaved and examined again for the presence of nevi. All affected animals that were irradiated during postweanling stages were necropsied near the end of the irradiation protocol or up to 15 weeks later.

Lesion Morphology and Microanatomy. At the time of initial identification, the shape and size of each melanocytic lesion were recorded (maximum and minimum diameters), together with details of color, margin integrity, surface texture, elevation, and location [left or right of the midline, and anterior (neck), middle (lumbar), or posterior (rump)]. The overall state of the dorsal skin and the presence of other types of lesions were also recorded. Checks of all of these features were made every 3 weeks until the death of the animal. Following euthanasia in excess CO_2, animals were photographed and necropsied. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, stained routinely with hematoxylin and eosin, and when appropriate, with Masson-Fontana (with and without bleach) or Gomori's iron stain.

Cell Lines. Lymph node biopsies were removed from two affected *Monodelphis* (B7841 and B9870) to generate cell lines for growth *in vitro*. The melanotic cell lines were established in culture at 33°C in 5% CO_2 from fresh, finely-minced biopsy material in medium containing Iscove's modified Dulbecco's medium, 16% fetal bovine serum, 5 μg/ml insulin-5 μg/ml transferrin-5 ng/ml selenite, 2 mM L-glutamine, 2 mM $CaCl_2$, and 0.2% Pen-Strep (19), plus 1 nM cholera toxin and 12 nM 12-*O*-tetradecanoylphorbol-13-acetate. To enhance explant attachment, minimal volumes of medium were used (3–5 ml/10-cm plate).

Athymic Nude Mice. The melanocytic cell lines were tested *in vivo* for evidence of the tumorigenic phenotype by s.c. injection of 1×10^6 cells into the dorsal flanks of adult female athymic NCr-*nu* nude mice (NCI and Taconic Labs) to detect whether malignant tumors could be formed. Control human and murine malignant melanoma cell lines have been tested s.c. under similar conditions and were found to be tumorigenic in NCr-*nu* mice (19, 20). The nude mice were maintained in "quasi-sterile" conditions within a laminar flow hood in autoclaved microisolators containing shavings, rodent chow, and water. After 30, 60, 90, and 120 days, the mice were examined externally; after the last examination they were euthanized with CO_2 and photographed. The dorsal skin was surgically excised to determine the extent of tumor cell growth in intradermal and s.c. tissue before examination of internal body sites.

RESULTS

Suckling Survival and Incidence of Melanocytic Nevi. A summary of litter and individual survival numbers at weaning, following UVR exposure, is presented in Table 1. Of the 43 litters (358 individuals) entering the irradiation protocol, 11 (26%) failed to survive to weaning, a further 20 (46%) lost 1 or more individuals, while the remaining 12 (28%) were brought to weaning without loss. Only at the highest total doses was marked erythema observed, and the survival rate of these affected young was similar to that of young exposed to the lowest (nonerythemal) doses. Loss of entire litters occurred over the full range of exposure protocols. An overall loss of 39% (141 of 358) of individuals between birth and weaning is within the range of losses recorded for the *Monodelphis* colony at large.

Of the 217 weaned individuals, 20 possessed a single melanocytic nevus when first shaved and examined at or soon after weaning. Two more were observed among animals shaved and examined up to 4 months later. When first observed, lesions were usually black (rarely dark brown), round to oval, flat, regular in outline, and 2–7 mm in maximum diameter. Nevi were found in animals exposed to a variety of doses including the lowest and highest total doses used. They occurred with similar frequencies in males and females and were equally distributed on the right and left sides of the body. More were located in the anterior and middle of the body than in the posterior region, probably because the strongly recurved hindquarters were protected from exposure to the UVR.

Malignant Melanoma in Adults. At necropsy, after up to 45 weeks of chronic UVR exposures, 8 adults were provisionally diagnosed from gross observation as having malignant melanoma with

Table 1 *Loss and survival of litters and individuals at weaning after UVR exposures during the first 19 days after birth*

Litters		Individuals	
No. introduced	Outcome	No. lost	No. at weaning
11	Entirely lost	78	0
20	Partial loss	63	112
12	No loss	0	105
Totals 43		141	217

Table 2 *Summary of suckling exposures and weanling lesion location in eight animals that subsequently developed malignant melanoma*

Animal ID	First suckling exposure[a]	No. of exposures	Total dose (kJ/m^2)	Nevus first observed[b]	Nevus location[c]
B7841	4	8	1.00	W+4	AR
B7851	3	9	1.12	W	MR
B7899	4	8	1.00	W	ML
B7917	3	9	1.69	W	ML
B7928	2	9	1.69	W	PL
B8123	3	7	2.19	W	AR
B8264	4	8	5.00	W	AR
B9870	1	9	1.10	W+1	AL

[a] Days after birth.
[b] W, at weaning; +, months postweaning.
[c] A, anterior; M, middle; P, posterior; R, right; L, left.

metastases to 1 or more lymph nodes. Histopathology later confirmed all of the diagnoses. Details of the exposure history of these 8 animals when they were sucklings, together with age and location of melanocytic nevi when first identified, are given in Table 2.

Growth curves for the eight lesions progressing to melanoma from the time of their discovery until necropsy are shown in Fig. 2 as two groups of four which overlap at the earliest stages but later exhibit different growth rates. In four animals euthanized before the end of the planned irradiation protocol because of the advancing condition of skin or eye neoplasias or for diagnostic purposes, lesion growth was rapid and sustained. In the other four individuals necropsied up to 15 weeks after the end of the irradiation protocol, little or no growth occurred over long periods and none of them reached the size of those in the first group.

The typical morphological and histopathological features of malignant melanoma in *Monodelphis* are represented in Fig. 3. All of the skin lesions were distinctly raised, frequently nodular, usually asymmetrical with irregular margins, uniformly black, and more or less shiny. Regional lymph nodes were black to dark gray and enlarged in the immediate lymph drainage areas. No metastatic lesions were seen at necropsy in any locations other than lymph nodes. Histologically, the primary sites of all malignant melanomas were in the dermis, with three of the eight extending through the subjacent muscle and along the fascial planes. The epidermis was not involved except for rare, minimal pigment transfer into epithelial cells. There was thus no junctional activity or epithelial ulceration. Malignant melanoma cells in the skin were locally infiltrative of nerves, vessels, and lymphatics. In both skin and lymph nodes, melanoma cells were round to oval or polygonal, with abundant cytoplasm and round to oval nuclei with single, small nucleoli. Mitotic figures were rare and no inflammatory cell response was elicited by the neoplasms. The metastatic foci in the lymph nodes were located in the medullary and subcapsular sinuses and extended into the lymphatic parenchyma and capsular tissue.

Morphology and Behavior of Cultured Melanoma Cells. To confirm that the pigmented cells present in the lymph nodes of UVR-treated *Monodelphis* were metastatic melanomas, cell cultures were prepared from affected lymph nodes. Nodes containing presumptive pigmented melanoma cells were removed from two animals (B7841 and B9870), minced, and cultured *in vitro*. The microscopic phenotype of the cultured cells derived from the left suprascapular lymph node of B7841 was particularly striking and distinct from benign melanocytic cell lines reported previously (19). The TD15L cell line contained pigmented, dendritic malignant melanoma cells which were frequently bipolar. This phenotype resembles that of human and rodent melanocytes and, in some instances, human primary cutaneous melanomas. Two lines were derived from TD15L by ring cloning (TD15L1 and TD15L2) to reduce potential genetic heterogeneity. Both the L1 and L2 lines also exhibited the pigmented dendritic melanoma morphology and minimal substrate adherence (Fig. 4*a*). They were thus easily removed mechanically without the need for trypsin digestion, which is necessary for other cutaneous lesion-derived cell lines. Furthermore, cell pellets from both of these lines are black. These properties (pigmentation and dendricity) are consistent with metastatic malignant melanoma. The TD18L cell line (derived from a node of B9870) was also pigmented in culture but exhibited a more epithelial and adherent morphology (data not shown).

Cell suspensions were injected s.c. into athymic nude mice to assess whether lines L1 and L2 were capable of producing tumors. The cells did not grow into palpable tumors in the subcutis (*i.e.*, they were not highly proliferative) as would be expected for human or mouse malignant melanoma cell lines (6, 20). However, s.c. pigmentation was visible externally from the day of injection until necropsy. The cells clearly retained their viability, since pigmented melanoma cells could be cultured out of the mouse skin at later times. Four months after s.c. injection, the L1 and L2 lines showed evidence of proliferation within the dermis of nude mice at the site of the needle tract for s.c. injection (Fig. 4*b*). All intradermal lesions were densely pigmented. Some lesions were elevated and proliferation at this site resembled the histology of UVR-induced melanocytic nevi in *Monodelphis* dorsal skin, thus confirming the suspected tumorigenic potential (albeit mild) of these *Monodelphis* melanoma cell lines.

DISCUSSION

Marsupials (metatherians) are clearly distinguished from placental (eutherian) mammals by the immaturity of their young at birth (21). *Monodelphis* neonates are equivalent in many respects to 4–6-week-old human embryos (15, 22) and skin morphogenesis and differentiation are far from complete. By 19 days postpartum (the age at which UVR exposures of sucklings were discontinued), hair growth has begun (pelage hairs have not erupted), the epidermis is more keratinized, and the dermis more collagenized than in neonates but much less

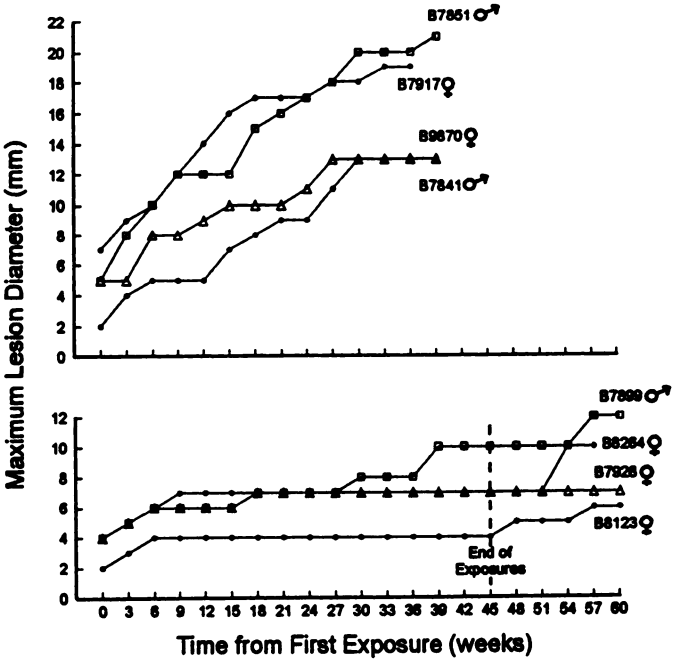

Fig. 2. Growth curves for each of the eight melanocytic lesions initiated in sucklings and confirmed as malignant melanoma at necropsy after chronic postweanling exposure. First exposure (week 0) was given on the day each lesion was first obseved. Maximum diameter of each lesion was recorded at 3 weekly intervals. For clarity, curves of faster growing lesions are shown above and slower growing lesions below.

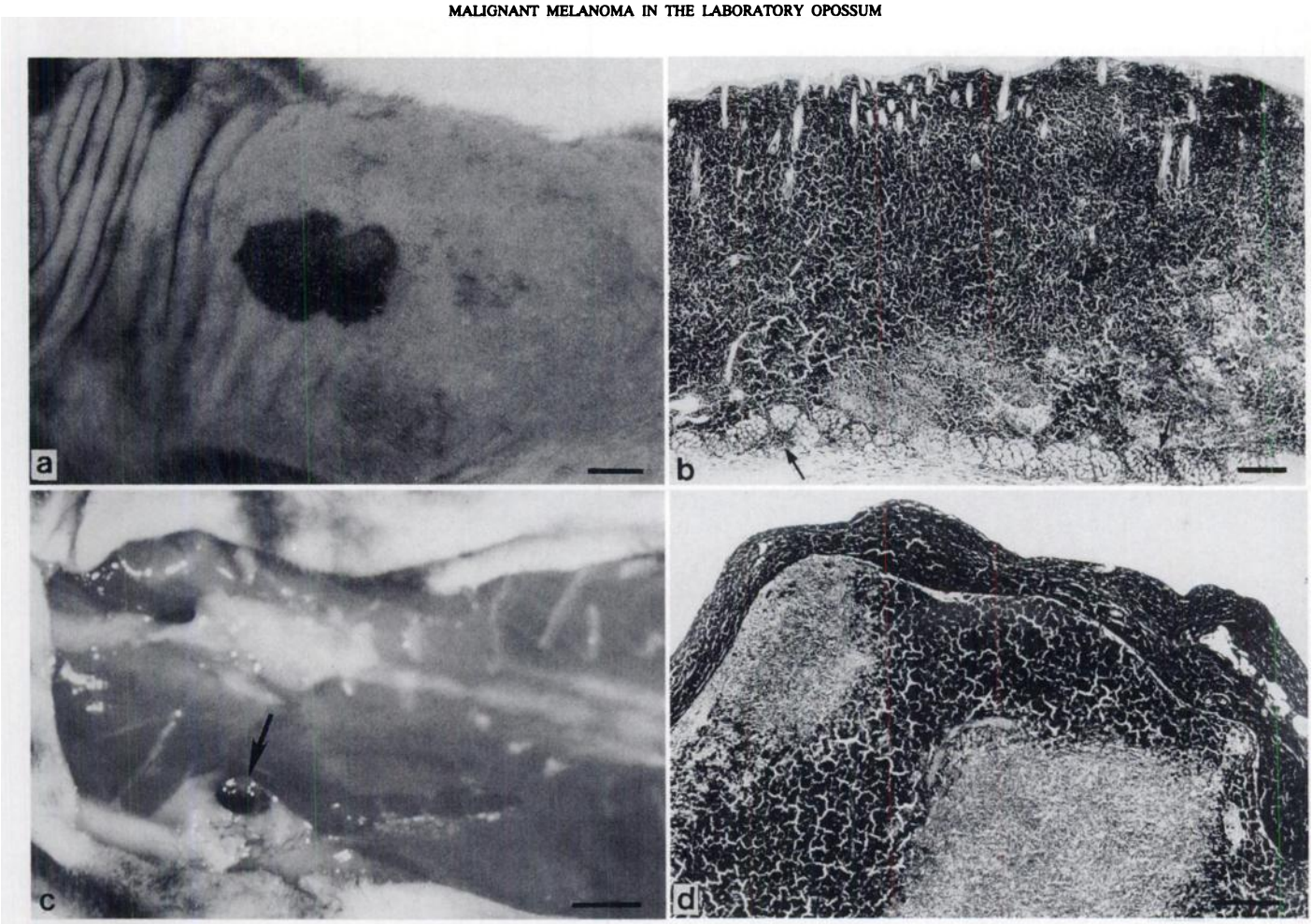

Fig. 3. Examples of gross and histological appearance of malignant melanoma in *Monodelphis*. (*a*) Black, raised skin melanoma with irregular margin (B7917). *Bar*, 5 mm. (*b*) Low power view of skin melanoma (B7899) showing vertical and lateral extension of pigmented cells in the enlarged dermis, with penetration through the subdermal skeletal muscle layer (*arrows*) into the underlying fascia. H&E; *bar*, 100 μm. (*c*) Enlarged suprascapular lymph node (*arrow*) with foci of pigmented, metastatic melanoma cells (B7917). *Bar*, 5 mm. (*d*) Low power view of lymph node (B7899) showing metastatic melanoma cells infiltrating the capsule and the subcapsular and medullary parenchyma and sinuses. H&E; *bar*, 100 μm.

so than in the exceptionally thick and tough skin of juveniles and adults. Skin growth and development may affect sensitivity to UVR damage. Under similar radiation protocols, melanocytic nevi were induced in sucklings with much lower total doses than those required for lesion induction in older animals. For example, the lowest total dose of UVR that led to nevus initiation in a suckling was 0.87 kJ/m^2, compared with the lowest total dose of 4.5 kJ/m^2 for nevus development in an adult.

Localized immunosuppressive effects of UVR in mouse skin augment melanoma development (23). Conversely, immune surveillance has been shown to be an important factor in melanoma regression in humans, probably via a cellular and/or humoral response to melanoma associated antigens expressed by autologous tumor cells (24, 25). Although the developmental profile for humoral and cellular immunity in *Monodelphis* is poorly understood, it is known that newborns are agammaglobulinemic (26), that the thymus does not appear to be a fully functional gland at birth (27), and that mouse melanoma cells injected 1–3 weeks after birth proliferate for 3–5 weeks before regressing (28, 29). Clearly, *Monodelphis* sucklings, like the early young of the related Virginia opossum *Didelphis virginiana* (30–32), have underdeveloped immune competence for part or all of the developmental window equivalent to the UVR exposure period. It is therefore plausible to suggest that this may play a role in the susceptibility of sucklings to radiation-induced skin damage. Whether a specific period in suckling development is differentially susceptible to lesion formation has yet to be determined.

Exposure of sucklings is a novel, effective, and proficient means of initiating melanocytic lesions for investigations targeting nevus progression to metastatic melanoma. In our earlier experiments, none of the melanocytic lesions produced after exposures of 151 adults, and only one lesion among 97 animals exposed from the weanling stage, progressed to metastatic melanoma. However, in the present study of the animals that already possessed lesions at the weanling stage, 40% (*i.e.*, 8 of 20 with suckling-induced lesions) progressed to metastatic melanomas after chronic exposure as juveniles and adults. This represents a conservative assessment. One animal not included as affected was clinically diagnosed as possessing a typical cutaneous malignant melanoma but died unexpectedly in its cage and adequate confirmatory histopathology could not be carried out. Another animal was excluded because of an equivocal diagnosis. Histopathology of nevi and malignant melanomas of the skin and lymph nodes are essentially the same as those already described in animals exposed only as adults (10, 13, 33). We are examining the effect of eliminating or at least shortening the postweanling exposure period, not only to reduce the time, labor intensity, and therefore expense involved in the experimental production of malignant melanoma, but also to prevent an undesirable side effect of exposing adults, namely, the development of aggressive corneal tumors which may occur as a result of chronic exposure (34, 35). Furthermore, unlike animals exposed only from the weanling stage, those with nevi induced as sucklings sometimes developed other skin lesions such as hyperkeratoses, fibrosarcomas, and hemangiomas late in the chronic exposure regimen.

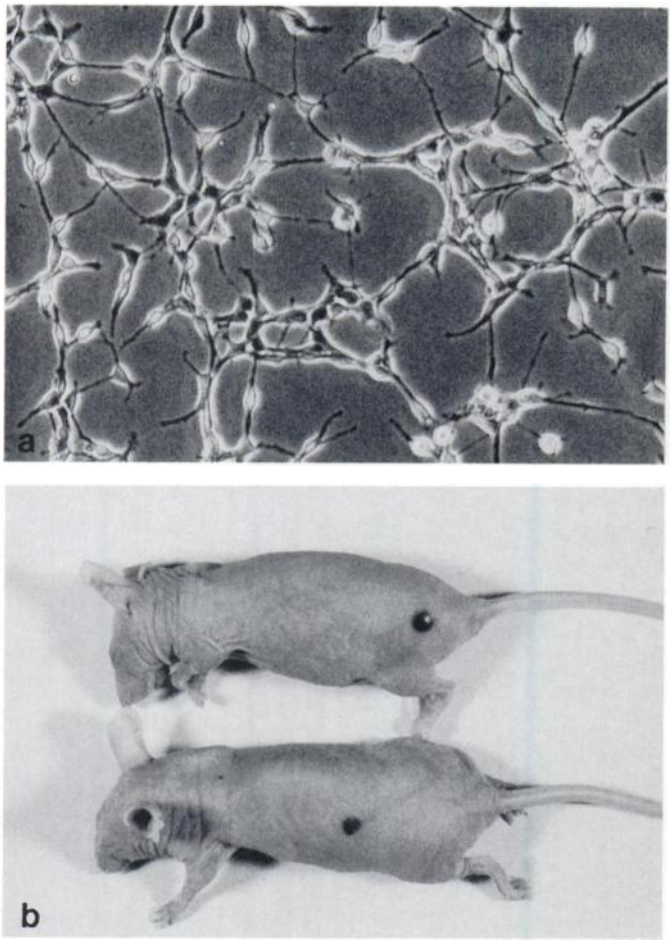

Fig. 4. *Monodelphis* cell line TD15 L1 *in vitro* (*a*) and *in vivo* (*b*). (*a*) Photomicrograph (phase contrast) of the malignant melanoma cell line derived from a lymph node of B7841 which contained pigmented metastatic cells. Length of micrograph, ⅔ mm. (*b*) Black, raised cutaneous tumors in athymic nude mice injected s.c. (4 months prior) with TD15 L1 cells.

The potential utility of the *Monodelphis* model extends from formal mendelian genetics and cytogenetics to cellular and molecular biology. The end point phenotype of susceptibility to UVR-induced nevi can be observed before sexual maturity. Unlike animals first exposed as adults, which are generally too old to mate by the time nevi develop, affected individuals produced by suckling exposure can be used for breeding purposes when they are sexually mature (3–4 months after weaning). If susceptibility is heritable, the establishment of susceptibile stocks is feasible and practical for genetic studies and provides a new strategy aimed at increasing lesion frequency.

Cell lines derived *in vitro* from affected *Monodelphis* lymph nodes provide definitive evidence of malignancy in this model. The two clonal derivatives of parent cell line TD15L (L1 and L2) exhibit a highly dendritic and pigmented phenotype *in vitro*. Both of these properties are consistent with melanotic metastatic melanoma present in the original lymph node (taken from an irradiated animal) from which the cell lines were derived. These results represent the first published demonstration that the pigmented cells within the lymph nodes are *bona fide* malignant melanoma cells. Had the lymph nodes merely contained melanophages (lymphocytes or macrophages, for example, that had phagocytosed melanosomes), they would not grow *in vitro* with these characteristics.

Further evidence in support of the malignant/metastatic phenotype is provided by xenogeneic grafting in athymic nude mice. When TD15 L1 or L2 were injected s.c. into immunoincompetent nude mice, the cells did not proliferate in the subcutis, although they remained viable for months. However, the cells did colonize the dermis at the site of the needle injection. This result has been interpreted to mean that the *Monodelphis* melanoma cells exhibit a dermal tropism and perhaps a temperature preference. A mean body temperature for *Monodelphis* of 32.6°C (36) is significantly lower than the body temperature of nude mice and led to the selection of 33°C as the preferred temperature for *in vitro* culture of *Monodelphis* cells. Perhaps the lack of growth of *Monodelphis* melanoma cells s.c. in nude mice was due to suboptimal (elevated) body temperature. These results further demonstrate that *Monodelphis* malignant melanoma cells can proliferate, are moderately tumorigenic, and produce pigment in nude mice.

Several features of *Monodelphis* malignant melanoma cell lines make them attractive as experimental model systems for melanoma studies: (*a*) they are derived from tumors that were initiated and promoted by UVR alone; (*b*) they retain the ability to produce pigment [most human metastatic melanoma cell lines now available lack pigment, as do cell lines derived from tumors that develop spontaneously in transgeneic mouse melanoma models (37–38)]; and (*c*) preliminary studies on cell line TD15L indicate that it is karyotypically stable. These characteristics suggest that TD15L and other cell lines derived from melanomas of *Monodelphis* should be useful new reagents for the discovery and development of antimelanoma therapies, including conventional chemotherapeutic studies of cytotoxic and cytostatic activity *in vitro*. The potential of *Monodelphis* suckling young as models for examining growth and development of melanoma xenografts (28, 29) also raises the possibility of successful allogeneic grafting of metastatic cell lines in this species for use in *in vivo* chemotherapy trials and for testing immunomodulatory therapies.

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