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In Vivo Opossum Xenograft Model for Cancer Research

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Abstract

The inadequacies of athymic mice for research with grafted tumors are well known. *Monodelphis domestica*, the laboratory opossum, has the potential to complement the rodent model because of the immunoincompetent window period during early marsupial development. We injected pups of different ages with mouse melanoma cells and human melanoma, colon, and prostate cancer cells. The results showed that the xenogeneic tumors can grow and metastasize. We also obtained positive results by heterotopically injecting juveniles with mouse melanoma cells. These results establish *Monodelphis* as a natural mammalian model to study the cascade of interactions between xenografted cells and the host system.

Introduction

Maintenance of tumor growth *in vivo* is a valuable tool for cancer research. The most widely used model for research with xenograft tumors is the athymic nude mouse. The usefulness of the murine model, however, is limited by the fact that malignant neoplasms rarely metastasize when transplanted into adult nude mice (1). In addition, the clinical predictability of antitumor drugs screened in the nude mouse model is very low (2). A mammalian model providing a developmental period of immunoincompetence, which allows grafted tumors to naturally establish their neoplastic properties before being recognized and attacked by the host immune system, can complement the mouse model.

*Monodelphis domestica*, a South American marsupial, is small and highly prolific (typical litter size of 8–12; up to 3 litters annually; Ref. 3). Unlike most other marsupials, *Monodelphis* females lack a pouch, and neonates are exposed. Opossums are born at a stage approximating that of 13–15-day fetal rats or 40-day human embryos. Unusual characteristics of the *Monodelphis* immune system, by comparison with that of eutherian mammals, have been documented (4). It was hypothesized that at early developmental stages, the opossum’s incompetent T-cell-mediated self-recognition may lead to immunotolerance to grafted cells (5).

The use of neonatal opossums to grow allografted melanoma cells was successful; allografted UV-induced opossum melanoma cells displayed a capacity for tumor growth and systemic metastasis (5). In addition, Fadem et al. (6, 7) reported successful xenografting of mouse B16 melanoma cells to opossum pups. However, although tumors were induced in suckling young opossums injected at ages of 12 days and beyond, they quickly regressed without demonstrating any invasive or metastatic properties. Apparently, the protocols that Fadem’s group used (5 × 10⁴ cells/animal) were inadequate for sustained xenogeneic tumor growth, invasion, and metastasis. In contrast, Robinson and Dooley (5) reported a high rate of tumor establishment and sustained growth by injecting 1–3-week-old opossum pups with 0.25–1 × 10⁶ allogeneic melanoma cells. We report in this article the results of our studies on the patterns of growth, metastasis, and regression of xenogeneic mouse melanoma and human melanoma, colon, and prostate cancers.

Materials and Methods

Experimental Animals. The animals used in this study were produced in the colony maintained at the Southwest Foundation for Biomedical Research, San Antonio, Texas. All animals were maintained and bred as described previously (3).

Mothers with litters were anesthetized by halothane inhalation (8). Opossum mothers lack a pouch, so when a mother is laid on her dorsal surface, the neonates are fully exposed, each attached to a nipple. A 29-gauge needle attached to a 0.5-ml insulin syringe was used for the injection. After completion of the injection procedure and after the mother was fully awake and ambulatory, the mother was returned to its original cage. The litters were observed weekly for survival and tumor growth. All procedures were preapproved by the Southwest Foundation for Biomedical Research Institutional Animal Care and Use Committee.

Cell Lines. The mouse B16F1 melanoma cells and human HT-29 colon cancer cells used in this study were purchased from the American Type Culture Collection. The B16F1 cells were cultured in DMEM supplemented with 10% FBS, and the HT-29 cells were cultured in McCoy’s 5a medium supplemented with 10% FBS. The human A375 melanoma cells and PC-3p prostate cancer cells were obtained from The University of Texas M. D. Anderson Cancer Center and were cultured in DMEM containing 10% FBS and Ham’s F12K medium containing 10% FBS, respectively.

The body temperature of adult opossums is 32.6°C, and the development of homeothermy of marsupials occurs between birth and weaning (3). To adapt the cancer cells for growth in the *in vivo* marsupial environment, we cultured the cells at 33°C in a humidified 5% CO₂/95% air incubator. The cells were cultured to confluence in 100 × 20-mm culture dishes before harvesting.

Derivation of Mouse B16 Cells from Xenografted Tumors, Chromosome Preparation, and Karyotype Analysis. Two s.c. tumors were excised, mechanically minced, and cultured in DMEM. The viable cells thus derived were cultured to confluence for karyotype analysis.

Freshly fed (24 h before) tumor cells were treated with Colcemid (0.04 μg/ml; Life Technologies, Inc.) for 30 min at 37°C, exposed to a hypotonic solution (KCl, 0.06 M) for 20 min, and fixed for 15 min in a mixture of acetic acid and methanol (1:3 by volume). After being washed thrice with the fixative, cells were dropped onto clean glass slides covered with a film of distilled water and then air-dried. Optimally aged (5–6-day-old) slides were processed for conventional staining and Giemsa banding, following routine laboratory procedures (9). Based on their characteristic Giemsa-banding patterns, the species origin of tumor cells was identified.

Results and Discussion

The cells of each cell line grew well under the modified conditions, reaching confluence 3–5 days after a 1:3–4 subcultivation ratio.
We injected s.c. various doses of mouse B16F1 cells into opossum pups of different ages (day of birth was defined as day 0; Table 1). We injected \(0.25 \times 10^6\) B16F1 cells into the 10 individuals of a 5-day-old litter (litter 1, Table 1) and conducted weekly observations. At week 1 after injection, \(0.25 \times 0.25\)-cm tumors were seen. These tumors measured \(0.75 \times 0.75\) cm 2 weeks after injection (Fig. 1A). One of the tumors continued to grow to \(1.5 \times 1.5\) cm by the time of necropsy at week 4. Despite the size of the tumors, no lymph node or organ metastasis was detected at necropsy.

Ten 13-day-old pups of litter 2 were injected with \(0.5 \times 10^6\) cells. As in litter 1, \(0.25 \times 0.5\)-cm tumors were observed at week 1 and continued to grow to \(1.25 \times 1.25\) cm by week 4. Three pups were euthanized 3 weeks after injection. Two of them each had one \(0.25 \times 0.25\)-cm s.c. tumor, the third had a \(0.5 \times 0.75\)-cm s.c. tumor on each side of the neck region. Axillary lymph nodes of this latter individual were affected on both sides. Additional examination revealed that both lungs contained large numbers of black nodules.

### Table 1. Induction, growth, metastasis, and regression of murine melanoma with B16F1 cells

<table>
<thead>
<tr>
<th>Litter no.</th>
<th>Mother’s ID</th>
<th>Litter size</th>
<th>Injection age (days)</th>
<th>Dose</th>
<th>No. of pups with tumor/total remaining no. of pups (weeks after injection)</th>
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<tbody>
<tr>
<td>1</td>
<td>H6433</td>
<td>10</td>
<td>5</td>
<td>(0.25 \times 10^6)</td>
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<tr>
<td>2</td>
<td>H6466</td>
<td>10</td>
<td>13</td>
<td>(0.5 \times 10^6)</td>
<td>10/10 10/10 7/7 2/2 0/0</td>
</tr>
<tr>
<td>3</td>
<td>H6066</td>
<td>5</td>
<td>7</td>
<td>(0.75 \times 10^6)</td>
<td>5/5 3/3 3/3 2/2 1/1</td>
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<tr>
<td>4</td>
<td>H3575</td>
<td>11</td>
<td>8</td>
<td>(1 \times 10^6)</td>
<td>4/6 3/5 2/4 2/3 1/2</td>
</tr>
<tr>
<td>5</td>
<td>H3767</td>
<td>9</td>
<td>12</td>
<td>(1 \times 10^6)</td>
<td>2/2 0/0</td>
</tr>
<tr>
<td>6</td>
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<td>9</td>
<td>7</td>
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<tr>
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<td>H7304</td>
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<tr>
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<td>56</td>
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<td>63</td>
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<tr>
<td>13</td>
<td>H4962</td>
<td>9</td>
<td>84</td>
<td>(3 \times 10^6)</td>
<td>0/9 0/0</td>
</tr>
</tbody>
</table>

a Tumor regression occurred.
b Reduction in number was attributable to necropsy.
c Metastasis occurred.
d Animal is still alive with tumor.
e Animals were necropsied beyond the age of 17 weeks (see text for details).
after injection, respectively. None of them exhibited observable metastasis.

The 5 7-day-old pups of litter 3 were injected with 0.75 × 10⁶ cells. Tumors, of size 0.25 × 0.25 cm at week 1, grew to 0.75 × 1 cm by week 3. At week 5, 1 of the remaining 2 pups was euthanized. In addition to the primary tumor at the injection site, the tumor had metastasized to the regional lymph nodes, the lungs, and the brain. The tumor on the other animal (0.75 × 1.25 cm) regressed to 0.5 × 0.75 cm after weaning at 2 months. One year after injection, the animal is still alive, and the primary tumor has remained the same size.

The members of litters 4 and 5 were injected with 1 × 10⁶ cells at the age of 8 and 12 days, respectively. These two litters were not examined after injection until weaning age (week 8). Of the 11 pups in litter 4, 6 survived to weaning and 4 exhibited observable tumors (0.5 × 0.5 cm). Systemic pathological examinations of the primary tumor, lymph nodes (inguinal, suprascapular, axillary, cervical), visceral organs, and brain were performed on the 6 animals. Three of the 4 tumor-bearing animals exhibited metastases. One animal (euthanized at week 8) exhibited affected suprascapular, axillary (Fig. 1B), and bronchial lymph nodes. Another animal (euthanized at week 10) demonstrated both lymph node and distant organ metastasis involving not only the lungs (Fig. 1C) and bronchial lymph nodes but also the spleen.

Eight pups of litter 5 survived beyond 17 weeks and 7 of them had tumors (5 were observable). As with litter 4, we performed systemic pathology on the eight animals. Tumor tissues from 2 animals (euthanized at week 26) showed necrosis and mineralization associated with the center of the neoplastic masses, although the tumors appeared at the gross level to be recently established (Fig. 1D). Others were euthanized at weeks 28 (1 animal), 29 (2 animals), 30 (1 animal), and 31 (2 animals), respectively. Four animals showed positive lymph node metastases, including 1 that carried a totally regressed primary tumor. One animal (euthanized at week 30) showed metastasis to the brain meninges (Fig. 1, E–I).

Next, we injected 2 × 10⁶ cells into two litters (6 and 7) consisting of 9 babies each, ages 7 and 11 days, respectively. This dose resulted in overgrowth of tumor in litter 6 and death of 7 pups within 1 week of injection. Necropsies of the 2 surviving pups at 1 week after injection exhibited no observable metastasis. Six of the 9 pups of litter 7 survived 1 week and 4 survived 2 weeks after injection. One of them bearing a tumor of 1.0 × 1.5 cm (euthanized during week 3) showed tumor spread to a cervical lymph node, indicating that metastasis could occur within 3 weeks of injection.

Injection of pups ages 20–84 days with 3 × 10⁶ cells was tested on 6 litters (litters 8, 9, 10, 11, 12, and 13). The 9 20-day-old pups of litter 8 exhibited observable tumors by week 1, with the largest being 0.5 × 0.75 cm (1.0 × 1.5 cm by week 2). Three pups that were euthanized at week 2 showed no observable metastasis. By week 3, the tumors started to regress remarkably; only 3 pups carried observable tumors. We euthanized 2 pups at this stage and found that both carried regressed s.c. black tumor tissue but no metastases. The remaining 4 were euthanized at weeks 5 and 6, respectively. One animal had a black axillary lymph node, and the other 3 had no other tumors. In comparison, tumors were observed on 5 of the 9 27-day-old pups of litter 9 1 week after injection, with the largest measuring 0.75 × 1.0 cm. These tumors started to regress by week 3. All necropsies revealed no metastasis. Litters 10 and 11, each consisting of 5 pups, were injected at the ages of 50 and 56 days, respectively. Tumors quickly disappeared after a brief induction, and no metastasis occurred. Similarly, small 0.25 × 0.5-cm tumors were briefly induced in the 6 63-day-old animals of litter 12, but no tumor was induced in the 9 84-day-old animals (litter 13).

Intracardiac injection mimics the route of cancer cell spread via the blood stream, and this procedure has been used with mice (10). We injected 1 × 10⁶ cells in 10-µl PBS buffer into the heart of a 45-day-old female. The needle was inserted vertically over the pericardial region where the heartbeat was palpated. Upon necropsy 25 days after injection, both lungs were massively affected with mililiary black spots (Fig. 2A), a feature different from the melanomas that had metastasized to the lungs, which were nodular in shape and varied in size (Fig. 1C). No melanomas were detected in the brain or the other organs.

We also performed i.p. injections because this procedure has been widely used in mouse models (11). Three 45-day-old juvenile opossums were injected. At necropsy (1 at 25 and 2 at 36 days after injection), we observed gray-black tumor tissues dispersed in the abdominal cavity and affected mesentery lymph nodes distributed along their routes of drainage (Fig. 2B). Liver and other visceral organs were removed from 1 animal for pathological examination, which revealed no infiltration of tumor cells to any organs. Additionally, we injected another 2 32-day-old pups; necropsy findings (8 and 37 days after injection) were similar to the findings for the former 3 animals.

Tumors xenografted to athymic mice are sometimes cytogenetically transformed to contain host cell chromosomes or chromosomal regions (12). To test the opossum model for this phenomenon, we derived cell lines from each of two xenografted murine melanomas; one was from an animal of litter 9, and the other was from an animal injected with 3 × 10⁶ cells at 50 days of age. These 2 animals were euthanized at 12 and 11 days after injection, respectively. The karyotypes established that both cell lines were of murine origin with no contamination of opossum chromosomes.

Finally, we tested the hypothesis that human cancer cells could grow after injection into neonatal opossums. Human melanoma cells (A375), colon cancer cells (HT-29), and prostate cancer cells (PC-3p) were injected. In each case, tumors were induced (Fig. 3), and metastasis to the lungs (A375), liver and kidneys (PC-3p) was evident from histopathological examinations. The human tumors induced in opossum pups injected ~1 week of age followed a similar growth...
OPOSSUM XENOGRAFT MODEL FOR CANCER RESEARCH

Fig. 3. Human cancers induced in suckling young opossums. A, human melanoma. The A375 cells injected were microscopically amelanotic, and induced tumors also appeared as amelanotic fat-like s.c. tissue. B, human colon cancer (HT-29). Tumors were observable over the left scapular region of both pups, although one is more obvious than the other. C, human prostate cancer (PC-3p). The tumor induced in this 23-day-old pup at the time of necropsy was glandular on gross appearance. A 0.25 × 0.25-cm s.c. tumor over the head was also observed.

pattern, i.e., tumors grew to fairly large size (generally 1 × 1 cm), but by ~4–6 weeks after injection, the tumors started to regress.

Although the ontogeny of the immune system of Monodelphs has not been established, there has been report on that of another marsupial, the brushtail possum, Trichosurus vulpecula, which is born at the same developmental stage as Monodelphis. The brushtail possum is a larger marsupial, and the progeny are weaned at ~25 weeks of age rather than 8 weeks for Monodelphis (13). Therefore, the comparable age of Monodelphis postnatal development is approximately equivalent to 32% of the age of Trichosurus (e.g., a 25-day-old Trichosurus is approximately equivalent developmentally to 8-day-old Monodelphis). Baker et al. (14) reported that the Trichosurus thymus starts to produce T lymphocytes (CD3) soon after birth (2 days old). By day 25, the thymus was populated with CD3-positive T lymphocytes. By day 48, B and T lymphocytes were identified in the spleen and their numbers increased significantly from day 25 to day 100. However, despite the significant increase, the numbers of T and B cells at day 150 were still significantly less than those of the adults. In addition, the T lymphocytes at weaning showed a less active response than at the adult opossum. These findings are consistent with our observations of the process of xenogeneic tumor growth and regression in Monodelphis. Characterization of the specific developmental patterns of the immune system of Monodelphis domestica will provide a more accurate basis for interpretation of our observations.

In summation, our results highlight the potential of Monodelphis as a unique model for research on cancer. The patterns of tumor growth and regression fit a neoplastic transformation model in which a cancerous cell first escapes the normal regulatory mechanisms by not being recognized by the immune system as foreign and later is distinguished from normal cells as a consequence of aberrant expression profiles (15). Additional development of this animal model may provide opportunities to devise therapeutic methods, especially immunotherapeutic methods, for various cancers.

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References

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