Chemically Induced Differentiation of Murine Embryonal Carcinoma in Vivo: Transplantation of Differentiated Tumors

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ABSTRACT

Murine embryonal carcinoma tumors were induced to differentiate in vivo by administration of retinoic acid. Six long-term surviving animals had seven slowly growing tumors which were transplanted s.c. into strain 129 mice. Untreated embryonal carcinomas were transplanted as controls. All of the 16 control transplants grew rapidly and killed their hosts within 25 days. All of the 24 transplants of retinoic acid-differentiated tumor survived. Sixteen experimental transplants originating from five original tumors showed no or slow growth for up to 16 weeks and were found to be histologically benign cystic teratomas. Two original tumors gave rise to eight relatively rapidly growing, histologically malignant tumors. One tumor resulted in four histologically similar solid tumors which resembled chondrosarcomas, and the second tumor gave rise to four histologically similar solid tumors which proved to be a mixture of glioma and chondrosarcoma. Examination of the tumor sources of these latter transplants showed benign cystic teratomas with focal solid, mitotically active cellular areas which were histologically similar to the transplants. These data confirm that retinoic acid-induced differentiation of murine embryonal carcinoma cells results in altered biological potential of these cells and usually the formation of a benign teratoma. However, rarely (about 1 per 2 x 10⁸), the resulting differentiated cells will give rise to rapidly growing, histologically malignant tumors. One can predict such biological propensity when solid, mitotically active areas in the original tumor are found.

INTRODUCTION

Many malignant neoplasms consist of a mixture of cells ranging from relatively poorly differentiated, rapidly dividing stem cells to relatively well-differentiated, slowly dividing and/or postmitotic cells. For example, the keratinized cells, comprising the pearls of a squamous cell carcinoma, do not divide and therefore must be considered biologically benign, even though they are derived from, and are part of, an otherwise malignant tumor (7). In general, tumors with a high proportion of well-differentiated cells are biologically less aggressive than are their poorly differentiated counterparts. Theoretically, if one could stimulate the differentiation of the neoplastic stem cells, one could favorably affect the clinical course of a malignant tumor (6).

We have tested this hypothesis using the murine teratocarcinoma system. This tumor is maintained by rapidly dividing malignant stem cells, termed EC, which can give rise to well-differentiated derivatives of all 3 embryonic germ layers. The vast majority of these derivatives are histologically benign. Murine EC cells can be induced to complete differentiation in tissue culture by the use of several different types of chemical agents, the most potent being RA (3, 13). Recently, we reported EC differentiation that was induced in vivo using a combination of RA and DMA (10). Extensive differentiation resulted in increased survival of the tumor-bearing hosts. When no residual EC cells were left, the mice bore histologically benign teratomas and were long-term survivors. However, if any EC were left, it eventually regrew to kill the host.

In this paper, we extend these observations, using a slightly different method of in vivo induction of differentiation. We also report the results of transplantation of completely differentiated tumors from long-term surviving hosts. The scheme of these experiments is summarized in Table 1. Our results show that differentiation of murine EC cells in vivo, when complete, usually results in the formation of a benign cystic teratoma. However, occasional secondary transformation of differentiated cells can give rise to histologically and biologically malignant differentiated (non-EC) tumors. We describe the origin of 2 such EC-derived malignant differentiated tumors. Further characterization of these lines will be described in the companion paper (11).

MATERIALS AND METHODS

The cloned line of PCC4azal (2) EC cells was used for all experiments. Cells for tumor production were grown in vitro as described previously (12). About 3 x 10⁶ intermediate (69 to 75)- to late (142 to 150)-passage cells harvested from tissue culture flasks were injected s.c. into the right flanks of 6- to 10-week-old male strain 129 mice (The Jackson Laboratory, Bar Harbor, ME). After the tumors were palpable (5- to 15-mm diameter), induction of differentiation with RA (Sigma Chemical Co., St. Louis, MO) dissolved in DMA (Matheson, Coleman, and Bell, Norwood, OH) was started. Stock RA:DMSO solutions (20, 30, and 40 mg/ml) were stored in the dark at -20°. Animals received two 30-μl intratumor injections each day for 4 consecutive days for a total dose of 4.8, 7.2, or 9.6 mg of RA. Control animals received similar injections of DMA only. After this injection schedule was completed, the animals were followed daily for survival. For histological analysis, 2-mm slices were fixed overnight in 70% ethanoglacial acetic acid:30% formaldehyde (20:1:1), postfixed in 70% ethanol, and processed routinely into paraffin. Five-μm sections were stained by the periodic acid-Schiff method.

Long-term survivors were rechallenged with fresh EC in the left flank and sacrificed when tumors were palpable. Grossly viable, solid areas of both RA-treated (differentiated) and untreated (EC-challenge) tumors were minced and surgically implanted s.c. into both flanks of anesthetized mice. The remaining tumor was processed for histology. The animals were followed for tumor growth and host survival. Progressively growing differentiated tumors were retransplanted in a similar fashion and explanted to tissue culture for further studies.

RESULTS

Our previous experiments using a relatively low-dose, 10-day schedule of RA in DMA resulted in 20% long-term survivors, but...
regrowth of EC killed most animals (10). A higher-dose, short-term schedule of RA:DMSO presented problems with systemic toxicity and no increased survival of hosts. DMSO as a vehicle for the RA proved to be less toxic, and all animals except one in the 9.6-mg-RA group survived the treatment. There was still some toxicity as evidenced by erythroderma, decreased activity, and weight loss in the experimental groups, although the mice subsequently recovered. Control mice receiving only DMSO showed no signs of toxicity. The results of RA:DMSO treatment on host survival are summarized in Table 2. Drug treatment resulted in prolonged median survival compared to controls and in at least one long-term survivor (≥98 days) at each dose level. This increased median survival was statistically significant (p < 0.005) by the one-tailed Wilcoxon rank sum test (8) in both groups of Experiment B depicted graphically in Chart 1. Although both the increased median survival and the percentage of long-term survivors clearly increased with increasing dose of RA, this was not significantly different at the 0.05 level.

At autopsy, all control mice had solid, soft, hemorrhagic, necrotic tumors, and histological sections demonstrated typical EC morphology (Fig. 1). Short-term survivors in the experimental groups demonstrated a mixture of well-differentiated tissues and largely necrotic EC. Residual EC had apparently regrown and killed the hosts.

Seven mice from the experimental groups were long-term survivors (summarized in Table 3). Grossly, these RA-treated tumors were largely cystic with focally solid areas. The cysts contained clear to hemorrhagic fluid. Mouse 1-15, in addition to the s.c. tumor, had large cystic i.p. masses. These had arisen not by metastasis, but by inadvertent i.p. injection of EC cells which had been differentiated by systemic distribution of the RA. Histologically, these tumors had a mixture of cytologically benign tissues including cellular, but mature, brain tissue; cysts lined by columnar epithelium and containing periodic acid-Schiff-positive material; and keratin-filled, squamous epithelium-lined cysts (Figs. 2 and 3). Cartilage nodules were infrequent (Fig 4). Three tumors from long-term survivors had small, focal areas of solid, poorly differentiated, mitotically active (non-EC) cells in addition to the predominant benign tissues. The cells had more cytoplasm, and the nuclei had smaller nucleoli and frequent small chromocenters. In Tumor 1-15R, the cells were epithelioid to spindle shaped; in one focus, delicate extracellular matrix was present (Fig. 5). In Tumor 1-15P, the cells were epithelioid with some multinucleated giant cells. In Tumor 2B-19, the cells vaguely resembled glia with small ovoid nuclei, granular cytoplasm, and indistinct cellular borders (Fig. 6).

Approximately 2 weeks prior to sacrifice, 6 of these long-term surviving mice were rechallenged with fresh EC in the opposite flank. All developed progressively growing typical EC tumors. The seventh animal (Mouse 2B-15) was found dead on Day 99. Although he had appeared healthy previously, there was grossly bloody fluid present in some cysts. The tumor was histologically a completely benign, well-differentiated teratoma with no solid, mitotically active tissue and no EC.

Grossly viable, solid areas of both EC and differentiated tumors were transplanted into 2 groups of mice, all of which survived the surgery. The results of these transplants are summarized in Table 4.

All 16 control EC transplants survived, grew rapidly to attain a 1-cm size in 2 weeks or less, and killed their hosts in 14 to 25 days. The seventh animal (Mouse 2B-15) was found dead on Day 99. Although he had appeared healthy previously, there was grossly bloody fluid present in some cysts. The tumor was histologically a completely benign, well-differentiated teratoma with no solid, mitotically active tissue and no EC.

Grossly viable, solid areas of both EC and differentiated tumors were transplanted into 2 groups of mice, all of which survived the surgery. The results of these transplants are summarized in Table 4.
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Table 3
Summary of long-term survivors

Seven long-term surviving mice were obtained from both experiments. All mice were healthy and sacrificed on the day indicated, except 2B-15 which died of tumor hemorrhage. All tumors were present in the s.c. space of the right flank except 1-15IP which arose by inadvertent injection of some EC cells into the peritoneal cavity. Approximately 2 weeks prior to sacrifice, the animals were rechallenged with fresh EC cells in the opposite flank.

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<th>Survival (days)</th>
<th>Final tumor size (mm)</th>
<th>Residual EC</th>
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Table 4
Transplantability of EC and RA-differentiated tumors

At the time of sacrifice, portions of RA-differentiated and EC challenging tumors were minced and surgically transplanted s.c. into strain 129 mice. The mice were followed for tumor growth for up to 16 weeks. All mice receiving control EC tumor cells died within 25 days. Tumors originating from 1-15R and 2B-19R were characterized as a chondrosarcoma and a glioma-chondrosarcoma mixture, respectively. All other transplants of RA-treated tumors survived as benign teratomas. Eight transplants of this line; however, the benign-appearing cysts did not survive further transplantation. This tumor line is lethal for its hosts within 2 months of transplant.

Tumor 1-15R gave rise to four 1-cm tumors in 4 to 5 weeks. Rather than allow the animals to succumb, the tumors were retransplanted s.c., examined histologically, and explanted to tissue culture. The 4 tumors were histologically very similar (Fig. 9) and resembled poorly differentiated chondrosarcomas. A few small histologically benign epithelial cysts were also present. The chondroid phenotype has been maintained in 12 subsequent transplants of this line; however, the benign-appearing cysts did not survive further transplantation. This tumor line is lethal for its hosts within 2 to 3 months after transplantation.

Tumor 2B-19R gave rise to four 1-cm tumors in 7 to 10 weeks. These tumors were also retransplanted, explanted to tissue culture, and examined histologically. These 4 tumors were morphologically similar (Fig. 8) and were determined to be glial in nature. Frozen sections of this tumor as well as tissue culture explants were positive for glial fibrillary acidic protein by indirect immunofluorescence (see Ref. 11). At least 2 of the 4 original transplants from Tumor 2B-19R had an additional cellular component. Microscopic foci of cartilage differentiation were present and, in some subsequent transplants, appear to have overgrown the glial elements. These tumors are lethal for their hosts within 2 to 3 months after transplantation.

Although Tumor 1-15IP had focal solid areas of mitotically active differentiated cells, only benign cystic teratomas were obtained on transplantation. This could have resulted because these cells were, in fact, not transplantable. More likely, however, is the possibility that too few or no such cells were included in the transplanted tissue.

In summary, 16 transplants of 5 completely differentiated tumors gave rise to benign cystic teratomas. Eight transplants of 2 tumors gave rise to 2 types of malignant tumors. One appears to be a chondrosarcoma; the other appears to be a mixture of glioma and chondrosarcoma.

DISCUSSION

These studies reaffirm that murine EC tumors can be induced to differentiate in vivo. Intratumor injections of RA:DMSO appeared to have a dose-related effectiveness in prolonging host survival and causing complete differentiation. One mouse had i.p. tumors that were completely differentiated. Since no RA was

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given i.p., this demonstrates that RA can act systemically to effect differentiation to a significant degree.

Differentiation of EC favorably alters the biological characteristics of the neoplasms. Untreated EC tumors are uniformly lethal, often within a month after transplantation. Partial differentiation with RA:DMSO prolongs survival, but unaffected EC cells eventually regrow to kill their hosts. Complete differentiation results in long-term host survival. Such long-term survival suggests that the EC-derived differentiation neoplasms have become benign teratomas, and histological and transplantation data, in general, support that supposition. However, at least 2 histologically and biologically malignant tumors composed of differentiated (non-EC) cells were identified in these studies. A third tumor was histologically malignant but did not transplant, possibly because of inadequate sampling.

That malignant differentiated tumors were obtained is not too surprising. Secondary malignant change in an otherwise benign teratoma occurs uncommonly in humans. Squamous cell carcinomas, thyroid carcinomas, and melanomas have been described in ovarian teratomas (9). Papaioannou et al. (5), using blastocyst injection of EC cells, obtained several tumors in chimeric animals. Most such tumors had an EC complement as well as differentiated elements, but a few late-appearing fibrosarcomas may have been derived from EC cells after the differentiation events. PYS2, a line of yolk sac epithelium (4), is tumorigenic, although that may be related to its long history in tissue culture.

The question arises as to the frequency of secondary malignant transformation of RA-differentiated tumors. We have histological evidence of 3 malignant tumors of 7 transplanted plus 5 not transplanted (one in this paper and 4 reported previously) (10) for an incidence of 25%. On a cell-to-cell basis, however, the frequency is very low. Assuming an EC cell at 12-μm diameter, knowing the original diameter of the treated tumors, allowing generously (>0.25) for host tissue and necrosis in the tumors, and assuming monoclonal origin of differentiated Tumors 1-15R and 1-15P and bicalon origin of Tumor 2B-19R, one calculates the frequency of secondary transformation at 4 per 9 x 10⁶ cells. Thus, the probability of any given differentiated cell becoming malignant is low, but the large number of cells potentially at risk gives a relatively high rate on a whole-tumor basis. It is not known what effect EC cell line or passage and culture history may have on this frequency.

Modern chemotherapy of human germ cell tumors not uncommonly results in completely differentiated tissues in metastatic sites (1). The question arises as to the biological potential for cancer in these tissues. This murine model of EC differentiation would suggest that histologically benign cystic tumors would behave in a biologically benign manner, although they clearly have a potential for growth by secretion of fluid and slow cell proliferation. In a critical location, such a tumor could cause significant clinical problems, but surgical intervention would have a high probability of cure. On the other hand, solid, mitotically active, differentiated (non-EC) elements are most probably malignant and should be treated as such. Because such tumors are phenotypically different from the parent cell line, one might also expect them to respond differently to therapeutic modalities.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. A typical control tumor showing sheets of undifferentiated EC cells. Periodic acid-Schiff, × 240. *Inset,* cellular detail. Note cleared nuclei with large nucleoli. Periodic acid-Schiff, × 600.

Fig. 2. RA-differentiated primary tumor 1-15R, showing glands lined by columnar epithelium and containing periodic acid-Schiff-positive material with zones of squamous epithelium. Periodic acid-Schiff, × 100.

Fig. 3. Well-developed brain tissue from RA-treated primary tumor 1-15R. Periodic acid-Schiff, × 240.

Fig. 4. Benign cartilage nodule from primary tumor 1-15R. Periodic acid-Schiff, × 600.
Fig. 5. a, solid, cellular area of Tumor 1-15R associated with benign brain tissue. Several mitotic figures are present. Periodic acid-Schiff, x 240. b, higher magnification illustrating delicate extracellular matrix. Nuclei are different from those of EC cells; compare with Fig. 1, inset. Upon transplantation, this tumor gave rise to a chondrosarcoma. Periodic acid-Schiff, x 600.

Fig. 6. a, solid, cellular area of Tumor 2B-19R, which on transplantation gave rise to a mixed glioma-chondrosarcoma. Periodic acid-Schiff, x 240. b, higher magnification showing fibrillar cytoplasm, indistinct cell borders, and nuclei different from those of EC cells. Note mitotic figure. Periodic acid-Schiff, x 600.
Fig. 7. Typical transplant from RA-treated primary tumor devoid of solid cellular areas is composed of benign glands and keratin-filled cysts. The fat and fibrous tissue appear to be host in origin and contain chronic inflammatory cells. All transplants of Tumors 1-15R, 2A-9R, 2A-10R, 2A-11R, and 2B-14R gave rise to such benign structures. Periodic acid-Schiff, × 100.

Fig. 8. Third transplant generation derived from Tumor 2B-19R showing glial area. In addition, chondroid areas were present in later passages. Periodic acid-Schiff, × 270.

Fig. 9. First transplant of Tumor I-15R showing cartilagenous nodules. Periodic acid-Schiff, × 100.
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