Loss or Persistence of the Differentiated State of Simian Virus 40-induced Hamster Tumor Cells before and after Serial Passage in Culture

George Th. Diamandopoulos, Mary Harsanyi Miller, Mary-Frances McLane, and Priscilla G. Evans

Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

SUMMARY

The transformed cells that arise from among the hamster epithelial and mesenchymal cells exposed to SV40 in vitro are, as a rule, fibroblastoid and pleomorphic rather than epithelioid. Moreover, the neoplasms that these transformed cells induce in the allogeneic host are spindle cell sarcomas and pleomorphic sarcomas rather than carcinomas. Since this phenomenon may result from cellular dedifferentiation in culture, to the extent that the anaplastic morphology and lack of specialized function can no longer suggest the cell of origin, we investigated the fate of the differentiated state of cells of three types of SV40-induced hamster tumors before and after serial passage in vitro. The tumors evaluated were three reticulum cell sarcomas, three osteogenic sarcomas, and two lymphosarcomas of B-cell origin. Our data demonstrate that reticulum cell sarcoma cells lose their morphological differentiation soon after the original tumors are dissociated into cell suspensions but preserve their phagocytic activity throughout their in vitro passage. Osteogenic sarcoma cells lose their differentiated phenotype and their capacity to form osteoid during but not before their serial passage in culture. Lymphosarcoma cells preserve their lymphoid morphology and their ability to produce immunoglobulin even after many in vitro passages. These results indicate that, in many types of SV40-induced tumors, neoplastic cell dedifferentiation, following serial passage in culture, is responsible to a great extent for the emergence of new cell phenotypes lacking in morphological and functional features characteristic of the cells originally transformed by SV40.

INTRODUCTION

When hamster epithelial and mesenchymal cells of embryonic, newborn, weanling, or adult origin are exposed to the oncogenic DNA SV40 in vitro, the cells among them that transform are, as a rule, fibroblastoid and pleomorphic rather than epithelioid (5, 10). Moreover, the tumors that these transformed cells induce upon implantation into the allogeneic host are spindle cell sarcomas and pleomorphic sarcomas rather than carcinomas (5, 10). In order to account for these findings, 4 possible explanations may be considered: (a) SV40 may possess a neoplastic morphogenetic potential that is under the control of its viral genome. Although this may be true both in vitro and in vivo for oncogenic adenoviruses (24), it was not shown to be the case for SV40, particularly in the in vivo system (6, 7). (b) SV40 may exhibit an exclusive tropism for infection and transformation of mesenchymal cells. This seems unlikely since SV40 has, on rare occasions, transformed epithelial cells that upon implantation into allogeneic animals gave rise to carcinomas (9, 10). (c) although both epithelial and mesenchymal cells can be transformed by SV40 in vitro, the cultural conditions used may favor the establishment of mesenchymal transformed cells only. This possibility also seems unlikely since the use of growth media that encourage survival of epithelial cells while suppressing the growth of mesenchymal cells does not result in the establishment of predominantly epithelial cell transformants (8). (d) although epithelial and mesenchymal cells survive in culture following their transformation, both types of cells may de-differentiate to such an extent that their anaplastic morphology and lack of specialized function cannot be characterized by their cell of origin. We have chosen to investigate this last possibility since no evidence is available to the contrary. We have used cells of 3 different histological types of malignant neoplasms: reticulum cell sarcoma, osteogenic sarcoma, and lymphosarcoma, all of which were induced in hamsters (6, 7) by SV40. Our data indicate that, in many types of SV40-induced tumors, neoplastic cell dedifferentiation before or after serial passage in culture is the factor primarily responsible for the anaplastic cell phenotype and the lack of specialized function encountered in such tumors.

MATERIALS AND METHODS

The experimental design is summarized in Chart 1. Tumors. Three reticulum cell sarcomas, 3 osteogenic sarcomas, and 2 lymphosarcomas (1 lymphoblastic and 1 lymphocytic) were used in this study. The tumors were induced in Syrian golden hamsters following the i.v. inoculation of SV40 into 3-week-old male weanling animals (6, 7). Both the lymphoblastic and the lymphocytic types of lymphosarcoma cells were shown to contain membrane-bound immunoglobulin that was exclusively of the 7S\textsubscript{v} class (2), a finding that established their bone marrow or B-cell origin.
Chart 1. Summary of the experimental design. RCS, reticulum cell sarcomas; OGS, osteogenic sarcomas; LS, lymphosarcomas; P-O, passage 0 (cells after tumor dissociation but before in vitro passage); P-10, 10th in vitro passage.

**Cells.** A monodisperse suspension of one of the 3 reticulum cell sarcomas and the 3 osteogenic sarcomas was prepared by stirring small fragments of the tumor in 0.25% trypsin solution for 3 hr. Small fragments of the 2 lymphosarcomas were mechanically dissociated into a cell suspension in culture medium using a glass homogenizer with a loose-fitting glass pestle (19).

**Cell Cultures.** Cells derived from the reticulum cell sarcoma and the osteogenic sarcoma were serially passaged in vitro at weekly intervals by dividing the trypsinized cells in a ratio of 1:2, using Puck's 10-10 growth medium (9). Serial passage in vitro of the lymphosarcoma cells (G. Th. Diamandopoulos, unpublished data) was done twice weekly, using RPMI-1640 growth medium supplemented with 15% heat-inactivated fetal calf serum and antibiotics, in the presence or absence of 2-mercaptoethanol, a substance that enhances survival of lymphoid cells in culture (1).

**Cytological Evaluation.** Cells of the 3 reticulum cell sarcomas and the 3 osteogenic sarcomas were grown in tissue culture chamber-slides (Lab-Tek Products, Division Miles Laboratories, Inc., Naperville, Ill.) at their 10th in vitro passage. Two and 3 days after initiation of the cell cultures, the growth medium was removed, and the cells were rinsed with Hanks' balanced salt solution, fixed in Bouin's fixative, and stained with eosin only. In all cell cultures, the monocytoid cells, i.e., before in vitro growth and at their 10th serial passage in culture. Each suspension was implanted into 6 3-week-old weanling male Syrian golden hamsters, in the form of 1 ml of growth medium containing 1 × 10⁶ cells per inoculation site. While cell suspensions of all the neoplasms were implanted in both CP², cell suspensions of the 2 lymphosarcomas were also inoculated at i.m. and s.c. sites.

**Histological Evaluation.** Two, 4, and 8 weeks after tumor cell implantation, hamsters bearing tumors were anesthetized, killed, and necropsied. While part of the tumor was used for imprint preparations (11), the remaining portion and the animal's organs were fixed in Bouin's solution, sectioned, and stained with hematoxylin and eosin for subsequent microscopic examination. Some of the air-dried tumor imprints were stained with Wright's and counterstained with Giemsa. The remaining imprints were fixed in acetone at room temperature for 5 min, placed in screw-capped jars containing Drierite (anhydrous CaSO₄), and stored at −70°C for later use in indirect immunofluorescence tests. Each tumor induced by any of the 16 types of cell inocula, representing the 8 neoplasms before and after in vitro passage, was evaluated microscopically and its cytological and histological features were compared and contrasted with those of all other tumors similarly derived.

**Immunofluorescence Technique.** The indirect immunofluorescence test was used for localizing the intranuclear SV40 T-antigen (22) in tumor cells and for identifying antibody to the SV40 T-antigen in sera of animals bearing these tumors. Surface Ig was detected in living lymphosarcoma cells by the membrane immunofluorescence technique, as previously described (2).

**RESULTS**

**Morphological and Functional Characteristics of the Reticulum Cell Sarcoma Cells before and after Serial Passage in Culture.** Monodisperse cell suspensions derived by trypsinization of each of the 3 reticulum cell sarcomas were either grown in culture or directly implanted into animals.

**Morphology in Vitro.** Cells grown in culture showed pleomorphism, nuclear hyperchromasia, and tumor giant cell formation. With further subcultivation, these cytological characteristics became less pronounced so that by the 10th in vitro passage of the 3 lines consisted almost exclusively of monocytoid cells, while the other 2 lines contained, in addition to the monocytoid cells, a small number of cells exhibiting fibroblastic or stellate features. In all cell cultures many mitoses could be easily found, a few appearing abnormal.

**Function in Vitro.** The phagocytic activity of the cells of the 3 reticulum cell sarcoma lines and the 3 osteogenic sarcoma lines was evaluated soon after tumor-cell dissociation and at their 10th in vitro passage by exposing them to growth medium containing carbon black. Twenty-four hr after exposure to the carbon particles, the monocytoid cells present in all 3 reticulum cell sarcoma lines were filled with carbon. The amount of carbon per cell was so large that it

---

² The abbreviations used are: CP, cheek pouch; T-antigen, tumor antigen.
obscured the nucleus. The small number of stellate cells that were found in 2 of the 3 lines accumulated within their cytoplasm a much smaller number of carbon particles, allowing for easy visualization of their nuclei. Cells of the 3 osteogenic sarcoma lines that were used as negative controls showed minimal or no uptake of carbon particles under identical experimental conditions.

**Morphology in Vivo.** Since each of the 3 original tumors consisted predominantly of cells with monocytoid features, it was diagnosed as a reticulum cell sarcoma (Fig. 1). All the tumors induced by cells derived from the trypsinization of these tumors consisted of pleomorphic and giant cells (Fig. 2), features that suggested the diagnosis of pleomorphic sarcoma. On the other hand, all the neoplasms that arose from cells of the 10th in vitro passage of each of the 3 lines were composed predominantly of elongated cells and, to a lesser extent, of pleomorphic cells, characteristics diagnostic of spindle cell sarcoma (Fig. 3) and of pleomorphic sarcoma, respectively.

**Morphological and Functional Characteristics of the Osteogenic Sarcoma Cells before and after Serial Passage in Culture.** As was the case with the reticulum cell sarcomas, osteogenic sarcoma cells before and after serial passage in culture or directly implanted into animals.

**Morphology in Vitro.** Cells exhibited minimal pleomorphism and moderate nuclear hyperchromasia early in their growth in culture. With subsequent in vitro passages, however, the pleomorphism became more pronounced and was accompanied by the appearance of a large number of tumor giant cells. Many mitotic figures could be seen, a few of which were aberrant.

**Morphology in Vivo.** Each of the 3 original bone tumors was selected from many similar ones (7) because grossly it was very hard and microscopically it exhibited the classical features of a well-differentiated osteogenic sarcoma composed of neoplastic cells producing large amounts of osteoid (Fig. 4). All of the tumors induced by cells derived from the trypsinization of the original tumors were also well-differentiated osteogenic sarcomas (Fig. 5). The amount of osteoid present in these tumors was equal to that found in the original bone tumors. However, all the tumors that arose from cells of the 10th in vitro passage of each of the 3 cell lines were either spindle cell sarcomas (Fig. 6) or pleomorphic sarcomas containing tumor giant cells. In one-third of these tumors, a rare microscopic focus of osteosarcoma with osteoid formation was found.

**Function in Vivo.** Since collagen fibers or calcium apatite microcrystals, which are considered the specific morphological hallmarks of bone-forming mesenchymal cells, cannot always be demonstrated by electron microscopy in osteosarcoma cells cultured in vitro (18), the functional capacity of the osteosarcoma cells was evaluated in vivo only. As mentioned already, all of the tumors induced by cells derived from the trypsinization of the original osteogenic sarcomas produced osteoid, which demonstrated that the cells had preserved their functional differentiated state. After 10 in vitro passages, however, the osteogenic sarcoma cells could induce almost exclusively spindle cell sarcomas and pleomorphic sarcomas, a finding that signified loss of functional capacity, at least as far as osteoid formation is concerned.

**Morphological and Functional Characteristics of the Lymphoblastic and the Lymphocytic Lymphosarcoma Cells before and after Serial Passage in Culture.** Monodisperse cell suspensions derived by mechanical dissociation of each of the 2 lymphosarcomas were either grown in culture or directly implanted into the animals.

**Morphology in Vitro.** Ten cell lines of the lymphoblastic lymphosarcoma and 4 cell lines of the lymphocytic lymphosarcoma were established in culture. The cells of all 14 lines grew in suspension without adhering to the glass of the bottles in which they were being propagated, while preserving their spherical lymphoid morphology when observed in the fresh unfixed state. When cytocentrifuge preparations were stained and examined under the microscope, the lymphoblastic lymphosarcoma cells exhibited a large, lacy nucleus with prominent nucleoli and scant cytoplasm. Although the majority of the cells contained a single nucleus, there were a few larger cells containing 2, 3, or even more nuclei. There was also a small number of cells that were of giant size containing a large hyperchromatic nucleus usually with a distorted shape. Many mitotic figures could be seen, a few of which were abnormal. The lymphocytic lymphosarcoma cells, on the other hand, exhibited striking uniformity in size and morphology. While the majority of them were generally small, there were a few that were larger or even of giant size. In stained preparations the cell nuclei appeared to be deeply purplish, being composed of dense aggregates of chromatin. Nucleoli could be found with difficulty. Most of the cells contained a narrow rim of light blue cytoplasm. In a few, particularly in the larger and the giant cells, the cytoplasm was quite abundant. Mitoses could be seen rarely.

**Function in Vitro.** Both the lymphoblastic and the lymphocytic lymphosarcoma cells derived from the dissociation of the original tumors and from the various cell lines at their 10th in vitro passage were incubated with fluorescein-labeled anti-lg at 4° and examined under the microscope using dark-field optics and UV light. They all exhibited distinct pinpoint areas of fluorescence throughout the plasma membrane and a rim of fluorescence around the periphery. As the cells were warmed to 37°, most of them lost their diffuse fluorescence and developed instead many bright spots. In a few cells, the spots gradually coalesced into 1 area of the cell, forming a cap. With time, this area of concentrated fluorescence became smaller and brighter, and in a rare cell it appeared to be internalized. The majority of the giant lymphosarcoma cells maintained a patchy surface fluorescence throughout the observation period. In general, the redistribution of lg-anti-lg complexes in the lymphosarcoma cells was less pronounced than that of normal hamster lymphocytes (G. Th. Diamandopoulos, M. H. Miller, P. G. Evans, and M-F. McLane, unpublished data).

**Morphology in Vivo.** One of the 2 original lymphosarcomas was of the lymphoblastic type (Fig. 7). All of the tumors induced by cells derived either from its dissociation (Fig. 8) or from the 10th in vitro passage (Fig. 9) of any 1 of the 10 cell lines established in culture were cytologically and histologically similar to the original tumor. Namely, each neo-
DISCUSSION

Data presented in this communication and summarized in Table 1 demonstrate that after in vitro passage the reticulum cell sarcomas lost their morphology but preserved their phagocytic function, the osteogenic sarcomas lost their morphology and their ability to produce osteoid, and the lymphosarcomas preserved their lymphoid morphology and their capacity to form immunoglobulin. Although these results could indicate that the cultural conditions used, acting as selective pressures, favored the establishment and proliferation of fibroblastoid and pleomorphic cells at the expense of the better differentiated and functionally more specialized elements, this interpretation seems unlikely. As mentioned already, each of the 3 original bone tumors was selected on the basis of its well-differentiated state, being composed almost exclusively of neoplastic cells that produce osteoid. No fibroblastoid or stellate cells could be identified in the sections of the tumors examined microscopically that could account for the establishment of these elements following in vitro cultivation. Admittedly, the absence of fibroblastoid and pleomorphic cells in the reticulum cell sarcomas was more difficult to establish with any degree of certainty. A more likely explanation, therefore, for the emergence of cells that lack specialized structure and function after in vitro passage is that the differentiated parenchymal cells of the original reticulum cell sarcomas and osteogenic sarcomas but not of the lymphosarcomas dedifferentiated to the extent that they appeared to be fibroblastoid and pleomorphic.

Loss of morphological differentiation of neoplastic cells has been observed in polyoma-induced salivary gland tumors (4). When the epithelium of salivary gland rudiments is transformed neoplastically in vitro by polyoma virus, it induces on implantation into syngeneic hosts, tumors consisting of spindle-shaped or spherical cells that characterize tumors of mesenchymal origin. This finding was interpreted to indicate that many of the polyoma-induced tumors exhibiting connective tissue features actually arise from epithelial cells that have lost their morphological characteristics following their neoplastic transformation.

Loss of functional differentiation of neoplastic cells has also been observed. For example, the ability of mammalian fibroblasts transformed by polyoma virus or by SV40 (13) to synthesize collagen is inhibited even under conditions that permit maximal amounts of collagen formation. Moreover, when chick embryo fibroblasts are transformed by Rous sarcoma virus, their collagen production is reduced by 90% within 72 hr as a result of their failure to synthesize collagen polypeptides (17). Nontransforming Rous-associated viruses, on the other hand, do not inhibit collagen synthesis.

Although little is known (20, 23, 25) of the factors that determine loss or persistence of the morphological and functional differentiated state of neoplastic cells before and after their serial passage in culture, one may speculate that on the basis of the present data it appears more likely that, in the SV40 hamster cell system the intrinsic nature of the cell rendered neoplastic by the virus rather than the viral genome determines the expression of the cell's differentiated state. Namely, since loss of cellular differentiation either before or after serial passage in culture was not accompanied by a loss of the SV40 T-antigen, it follows that the presence within the cell of the SV40 genome does not play a decisive role in the persistence of the differentiated state. Most probably, reticulum cells which are known to possess totipotential properties could easily dedifferentiate to a more primitive morphology while preserving their functional differentiation as manifested by phagocytic properties (16, 21). Osteocytes, on the other hand, which may not have committed themselves irreversibly to the bone-forming pathway of differentiation, could preserve their differentiated state at first but lose it when passed in vitro, most likely because of cultural conditions not favoring the persistence of an osteoid-producing cell phenotype. Finally, lymphoblastic and lymphocytic cells that were committed irreversibly to the lymphoid pathway of differentiation preserved their morphological and functional characteristics after their neoplastic transformation and following cultivation in vitro.

The above interpretation does not exclude the possibility that in other virus-cell systems, the genome of the oncogenic virus rather than the transformable target cell plays the decisive role in the expression of the neoplastic differentiated state. In fact, there is evidence from in vitro studies (12, 15) indicating that in some instances the viral genome...
can prevent neoplastic cell differentiation from taking place. Namely, when chick embryo myoblasts grown in culture are transformed by a temperature-sensitive mutant of Rous sarcoma virus they lose their ability to differentiate, i.e., to form myotubes, at the permissive temperature of 36°C, as the normal myoblasts do, but they are capable of differentiation if transformation is blocked by a shift to the nonpermissive temperature of 41°C. When these cultures are cooled again to the permissive temperature of 36°C, the myotubes vacuolate and degenerate within 72 hr. Creatine kinase levels, which signify extent of myoblast fusion, parallel the formation and degeneration of myotubes during these temperature shifts (15).

In conclusion, these findings indicate that in many SV40-induced hamster tumors dedifferentiation of the neoplastic cells, before or after serial passage in culture, could explain the phenomenon of the emergence of new cell phenotypes that lack the morphological and functional features characteristic of the original cells that were transformed by SV40.

## ADDENDUM

The 10 cell lines of lymphoblastic lymphosarcoma and the 4 cell lines of lymphocytic lymphosarcoma were reevaluated at their 55th serial passage in culture. The cells of all 14 lines retained their respective lymphoblastic or lymphocytic morphology, remained positive for surface immunoglobulin, lymphocytic lymphosarcoma were reevaluated at their 55th serial passage in culture. The phenomenon of the emergence of new cell phenotypes can prevent neoplastic cell differentiation from taking place. Namely, when chick embryo myoblasts grown in culture are transformed by a temperature-sensitive mutant of Rous sarcoma virus they lose their ability to differentiate, i.e., to form myotubes, at the permissive temperature of 36°C, as the normal myoblasts do, but they are capable of differentiation if transformation is blocked by a shift to the nonpermissive temperature of 41°C. When these cultures are cooled again to the permissive temperature of 36°C, the myotubes vacuolate and degenerate within 72 hr. Creatine kinase levels, which signify extent of myoblast fusion, parallel the formation and degeneration of myotubes during these temperature shifts (15).

In conclusion, these findings indicate that in many SV40-induced hamster tumors dedifferentiation of the neoplastic cells, before or after serial passage in culture, could explain the phenomenon of the emergence of new cell phenotypes that lack the morphological and functional features characteristic of the original cells that were transformed by SV40.

## REFERENCES

21. Ralph, P., and Nakoiniz, I. Phagocytosis and Cytolysis by a Macrophage...
G. Th. Diamandopoulos et al.


Fig. 1. Section of 1 of the 3 original reticulum cell sarcomas, Case GD-310, consisting of cells with monocytoid features. H & E, × 500.

Fig. 2. Section of a pleomorphic sarcoma induced by cells derived from the dissociation of part of the reticulum cell sarcoma of Fig. 1. H & E, × 500.

Fig. 3. Section of a spindle cell sarcoma induced by cells derived from part of the reticulum cell sarcoma of Fig. 1 and grown in culture for 10 serial passages. H & E, × 500.

Fig. 4. Section of 1 of the original 3 well-differentiated osteogenic sarcomas, Case GD-437, exhibiting excessive osteoid formation by the osteosarcoma cells. H & E, × 500.

Fig. 5. Section of a well-differentiated osteogenic sarcoma induced by cells derived from the dissociation of part of the osteogenic sarcoma of Fig. 4. H & E, × 500.

Fig. 6. Section of a spindle cell sarcoma induced by cells derived from part of the osteogenic sarcoma of Fig. 4 and grown in culture for 10 serial passages. H & E, × 500.

Fig. 7. Section of the original lymphoblastic lymphosarcoma, Case GD-36, consisting of primitive lymphoid cells with large nuclei, prominent nucleoli, and scant cytoplasm. H & E, × 500.

Fig. 8. Section of a lymphoblastic lymphosarcoma induced by cells derived from the dissociation of part of the lymphoblastic lymphosarcoma of Fig. 7. H & E, × 500.

Fig. 9. Section of a lymphoblastic lymphosarcoma induced by cells derived from part of the lymphoblastic lymphosarcoma of Fig. 7 and grown in culture for 10 serial passages. H & E, × 500.
Loss or Persistence of the Differentiated State of Simian Virus 40-induced Hamster Tumor Cells before and after Serial Passage in Culture

George Th. Diamandopoulos, Mary Harsanyi Miller, Mary-Frances McLane, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/36/9_Part_1/3171

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.