TUMORIGENICITY OF INDIAN MUNTJAC DIPLOID CELLS BY THE PROVIRAL INTEGRATION OF SARCOMA GENE OF A MOUSE RETROVIRUS

BY MASAKAZU HATANAKA* AND RICHARD KLEIN‡

From the Laboratory of Molecular Virology and the Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20205; and the Frederick Cancer Research Center, Biological Carcinogenesis Program, Frederick, Maryland 21701

Animal cells cultured after tumor virus infection usually reveal alterations in cell surface, in growth behavior, and in the acquisition of transformed morphology in vitro and tumorigenesis in vivo (1). However, none of them have been proven a causal relationship. For instance, enhanced agglutinabilities by lectins, disappearance of fibronectin proteins, increased proteolysis have been catalogued on changes at the cell surface of the transformed cells (1), and not all of them have been associated with specific heritable changes in the cell genome after viral transformation.

Cells transformed by tumor viruses often result in stable alterations in cellular growth properties in vitro; in particular, the loss of contact inhibition, less dependency on serum concentration, and anchorage independency. Again, not all of the altered cellular growth properties commonly associated with transformed state would be required for neoplastic growth in vivo. Although several reports demonstrated that the tumorigenicity of virus-transformed cells is correlated specifically with anchorage-independent growth in vitro (2, 3), several reports argue against this observation of correlation (4, 5). Also, morphological transformation induced in vitro by the tumor virus does not guarantee tumorigenesis in vivo, or is only coincidentally related to the neoplastic state (4).

In previous studies with Kirsten sarcoma virus-transformed mouse cells, we found that the tumorigenicity in the syngeneic mouse does not necessarily correlate with the transformed morphology of cells, nor with their state of virus production, and concluded that the presence of the sarcoma genome in cells is an essential, but not sufficient, condition of tumorigenesis (6).

Although this inbred mouse system provided much useful information on understanding the gene expression and tumorigenesis of tumor viruses (7–9), it has two major disadvantages for analyses of the mechanism of viral oncogenesis. First, all mouse cells in culture, regardless of whether transformed or nontransformed, became heteroploid in karyotype; this seriously hinders genetic analysis of the mechanism of tumorigenesis caused by tumor viruses. Second, the use of syngeneic systems cannot obviate immune responses to viral, as well as new cellular antigens, which may be

synthesized after tumor virus transformation. Such immune responses may obscure the inherent malignant potential of transformed cells. These two major obstacles have been removed by using the system described in this report.

We succeeded in transforming Indian muntjac deer cells (10) by a murine sarcoma virus, 43-2XV (M. Hatanaka, R. Klein, R. Kominami, T. Oikawa, H. Okabe, N. Tsuchida, E. C. Connors, and A. Carrano. Transformation of Indian muntjac diploid cells by the proviral integration of sarcoma gene of a mouse retrovirus. Manuscript in preparation.). In initial studies we found that clonal isolates of the transformed cell line maintained the diploidal state (2n = 7 in males and 2n = 6 in females); therefore, we isolated a series of cell clones from both male and female muntjac cells transformed by 43-2XV, each of which remained diploid (Hatanaka et al. Manuscript in preparation.). These transformed clonal isolates were then tested for tumorigenicity in athymic nude mice that are not able to develop the thymus as a result of a recessive autosomal mutation and, therefore, are functionally devoid of thymus-derived cell (T cell)-dependent immunity (11, 12). Athymic nude mice are, therefore, unable to reject transplanted tumor cells of allogeneic or xenogeneic origin (13, 14) and thus provide a convenient, immunologically neutral test system for cellular tumorigenicity (15).

This communication presents the variable inherent expressions of tumorigenicity by the clonal isolates of Indian muntjac diploid cells that were transformed by the variable integrations of sarcoma gene of a mouse retrovirus, 43-2XV.

Materials and Methods

Athymic nude mice with BALB/c background were obtained from the Sprague-Dawley Laboratories, Madison, Wis. The transformed clones of Indian muntjac diploid cells, transformed by the proviral integration of sarcoma gene of a mouse retrovirus 43-2XV (Hatanaka et al. Manuscript in preparation.), were tested for their tumorigenicity in nude mice. Cells were harvested by trypsinization and were inoculated subcutaneously into 4- to 6-wk-old athymic nude mice in 0.2 ml of Eagle's minimum essential culture medium. The animals were examined regularly for development of tumors.

Results

Although all the clonal isolates of the transformed muntjac cells revealed typical transformed morphology without any visibly distinguishable characteristics, tumorigenicity in nude mice exhibited four distinct patterns: (a) clones failed to form tumors during an observation period of 45 d after inoculation of 10^7 cells; (b) clones induced tumors which grew very slowly and then regressed; (c) clones produced tumors which grew rapidly but eventually regressed, and (d) clones formed tumors quickly which grew progressively and resulted in death of the tumor-bearing animals.

Fig. 1 shows some of the results obtained from the clonal isolates in each category. Although the results shown in Fig. 1 were obtained with an inoculum of 10^7 cells, tumor formation with 10^6 cells were only successful by the C and D groups.

It is possible that some transformed cells were more vulnerable than other cells to the action of humoral antibodies and the endogenous mouse component, because it is known that nude mice form humoral antibodies to heterologous skin grafts, and it has been shown that these humoral antibodies can induce necrosis in skin allografts when the mice are injected with rabbit complement (16). We tested the effects of the sera of rabbit anti-mouse thymocyte (ATS)[1] and rabbit anti-mouse lymphocyte (ALS)

---

[1] *Abbreviations used in this paper:* ALS, rabbit anti-mouse lymphocytes; ATS, rabbit anti-mouse thymocytes; HA, hemagglutination.
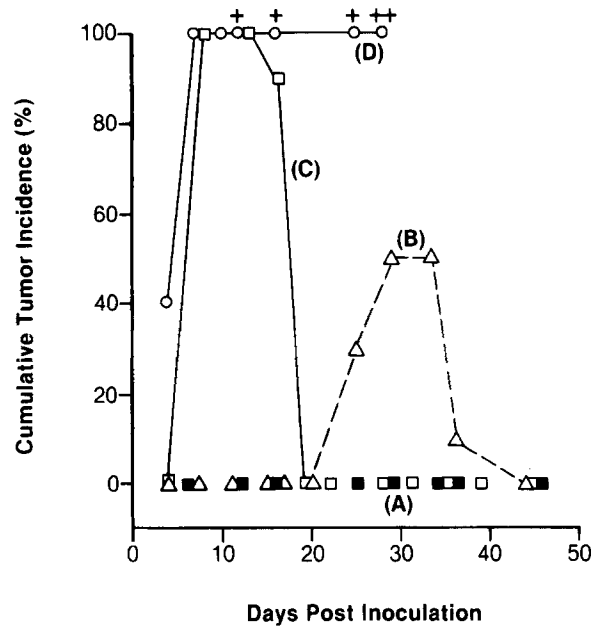
Days Post Inoculation

Fig. 1. Tumor formation by clonal isolates of muntjac diploid cells transformed by 43-2XV in athymic nude mice. 10^7 cells in 0.2 ml of Eagle's medium were inoculated by the subcutaneous route into 4- to 6-week-old athymic nude mice. Each group contained 10–12 animals. Cells were obtained at 8–10 culture passages after cloning. (A) Clone 11-1-23 (■); (B) clone 12-1-57 (△); (C) clone 11-1-08 (□); and (D) clone 12-1-60 (○).

on the regressive tumor formation observed by the transformed Indian muntjac male, 11-1-10. ATS (1:64 by hemagglutination [HA] titer, 1:6,400 by cytolytic test) or ALS (1:128 by HA titer and 1:3,200 cytolytic test) was injected into nude mice on −1, 0, 1, 3, 7, 10, 14, and 19 d after inoculation of the clone 11-1-10 (10^7 cells per mouse). ALS and ATS had no effect on the size or duration of the transient tumorigenesis and failed to prevent regression of the male muntjac transformed cells. Besides conventional reactions of T and B lymphocytes, natural killer cells and macrophage-mediated reactions in nude mice may affect the fate of tumors (by rejection, regression, or progression, depending upon the sensitivity of each cell against these mechanisms of immune surveillance) (17). The Indian muntjac cells, 11-0-00 or 11-1-10 were inoculated first in the right shoulders of nude mice. 8 d later, the cells from the progressive tumor-forming clone 12-1-58 (group D) were inoculated in the left shoulders of the same mice to determine any effects of preinoculation of nontransformed 11-0-00 or transformed but regressive tumor-forming cells, 11-1-10. The procedure of the sequential inoculation did not change the fate of the progressiveness of 12-1-58 clone (data not shown). Thus, whatever the immune mechanism functioning in nude mice, variable expressions of tumorigenesis presented in Fig. 1 must result from different properties of each muntjac clone transformed by the same sarcoma gene.

Within the progressive D group, the tumors derived from the clone 12-1-60 were made up of multiple large cystic cavities filled with exudate without hemorrhage but they ruptured readily with sectioning. The tumors generated by clone 12-1-58, on the other hand, formed massive solid nodules with some areas of necrosis. Histology of all the progressive tumors showed fibrosarcoma with moderate malignancy.

The number of chromosomes was determined for each of the tumors and found to

be the same as that of the original clones, 2n = 7 in males, and 2n = 6 in female cells. Compared to heteroploidy observed in most other tumors, the demonstration of stable diploid tumors from Indian muntjac is intriguing for future work such as identification and isolation of the chromosome responsible for tumorigenesis.

TABLE I

Characteristics of the Transformed Clones

Tumorigenicity group	Isolated clone	Sex	Colony formation in agarose culture	Sarcoma gene
A group (transformed but nontumorigenic)	11-1-16	M	+	+
	11-1-21	M	+	+
	11-1-23	M	+	+
	11-2-00	M	+	+ virus shedding*
B group (slow-growing tumor)	12-1-57	F	+	+
C group (regressive tumor)	11-1-00	M	+	+
	11-1-08	M	+	+
	11-1-10	M	+	+
	80-1-05	M	+	+
	80-1-17	M	+	+
	80-1-26	M	+	+
	11-2-00	M	+	+
	12-1-59	F	+	+
	12-1-51	F	−	+
D group (progressive tumor)	12-1-52‡	F	+	+
	12-1-55	F	+	+
	12-1-58	F	+	+
	12-1-60	F	−	+ virus shedding
	12-1-60T	F	−	+ virus shedding
	12-1-60TE	F	−	− (deleted)§
	12-1-60TH	F	−	+
	12-(1)-01	F	+	+ virus shedding
	12-(1)-02	F	+	+ virus shedding
	12-(1)-04	F	+	+ virus shedding
	12-(1)-06	F	+	+ virus shedding
Uninfected parental group	12-0-01	F	−	−
	12-0-02	F	−	−
	12-0-03	F	−	−
	12-0-04	F	−	−
	12-0-05	F	−	−
	12-0-06	F	−	−
	12-0-07	F	−	−
	11-0-00	M	−	−

The methods for the colony formation in agarose culture and detection of sarcoma gene were described (Hatanaka et al. Manuscript in preparation.).

* The virus shedding clone produce the progeny of 43-2XV.

‡ The clone 12-1-52 (originally diploid) became triploid at the 11th passage after cloning.

§ The clone 12-1-60 contains one copy of the sarcoma gene (Hatanaka et al. Manuscript in preparation.). One of the tumors from the clone was cultured and designated as 12-1-60T. The 12-1-60TE and 12-1-60TH are the subclones of 12-1-60T. The clone 12-1-60TE lost the transformed morphology and the sarcoma gene.

Actually, all the clonal isolates, including transformed but nontumorigenic clones, demonstrated the presence of the sarcoma gene (Hatanaka et al. Manuscript in preparation.). The results suggest that the presence of the sarcoma gene may correlate with the expression of the transformed morphology in cell culture; however, this is not necessarily accompanied by tumorigenic potential when assayed in nude mice (Table I). This data demonstrate that a complexity exists in tumorigenicity caused by the sarcoma gene. The status of the transformed cells shedding the viruses or not producing the viruses (nonproducer) appears irrelevant for tumorigenicity in nude mice, as shown in Table I. This conforms with the previous conclusion obtained from the mouse cell transformed by the same sarcoma gene (6, 7).

Also, the colony formation in agarose does not correlate with the tumorigenicity tested in nude mice (Table I). Despite typical morphological transformation and colony formation in agarose with hypoxanthine aminopterin thymidine + Raji-thymidine kinase negative system, the transformants in the A group failed to form tumors in nude mice at all. On the other hand, the clones 12-1-51 and 12-1-60 formed regressive and progressive tumors, respectively, with negative results of the colony formation in the agarose culture (Table I).

Discussion

The experimentations, including sequential or simultaneous inoculation of cells and the treatments by ATS and ALS, as detailed in the Results, have been designed to exclude immune responses in nude mice as the causes for variable tumorigenic expressions by the sarcoma virus-transformed Indian muntjac cells so that the tumorigenic complexity reflects only the consequence of the interaction between the sarcoma virus cells and host cells. We have found that the clonal isolates of the transformed Indian muntjac cells caused by the integration of the proviral sarcoma gene of a mouse retrovirus, 43-2XV, have four different patterns of tumorigenicity. The variable tumorigenic expressions do not correlate with the in vitro markers commonly observed in the cell transformation, such as growth behaviors, anchorage independency, or the status of virus shedding or nonproducer. The studies of two different host cells, diploid muntjac and heteroploid mouse, both transformed by the same sarcoma gene, support the conclusion (6, 7; Hatanaka et al. Manuscript in preparation; and Results). The transformed Indian muntjac cells with stable diploidy have further advantages when they are used to study the mechanism of viral oncogenesis. Chromosomal aberrations, translocations, or gene dosage effects by abnormal ploidy, are not the factors for the phenotypic differences on tumorigenicity observed in the Indian muntjac study. Thus, variable patterns on tumorigenicity from the same host cells transformed by the same virus may be derived from the variable interactions between the cell and the virus. Although it is premature to speculate, a specific chromosome(s) or a specific site(s) of the integrations of the proviral sarcoma gene may produce a specific expression of tumorigenicity. There are at least two chromosomes and four recombinant sites assigned for the integration of the sarcoma gene into the Indian muntjac gene (Hatanaka et al. Manuscript in preparation.). This hypothesis is strengthened by the use of the cloned host cells with stable diploidy, the clonal isolates of the diploid transformed cell, and the highly sarcomagenic cloned virus, 43-2XV, together with the use of nude mice providing a neutral background for immune responses.

Summary

The transformed clonal isolates of Indian muntjac diploid cells by a mouse sarcoma virus, 43-2XV, were tested for tumorigenicity in athymic nude mice. In spite of the indistinguishable transformed morphology, the tumorigenicity exhibited four different patterns: (a) no tumor formation; (b) slowly growing regressive tumor formation; (c) rapidly growing regressive tumor formation; and (d) rapidly growing progressive tumor formation. This demonstrates that the same diploid host cells transformed by the same virus reveal variable patterns of tumorigenic expression and some transformed host cells lack the tumorigenicity entirely. The findings that there are at least two chromosomes and four recombinant sites assigned for the proviral integrations of the sarcoma gene into the Indian muntjac gene (M. Hatanaka, R. Klein, R. Kominami, T. Oikawa, H. Okabe, N. Tsuchida, E. C. Connors, and A. Carrano. Transformation of Indian muntjac diploid cells by the proviral integration of sarcoma gene of a mouse retrovirus. Manuscript in preparation.) lead us to propose a hypothesis that variable expressions of tumorigenicity under the neutral background of immune responses, may arise from variable integrations of the sarcoma gene into the host chromosome.

References

1. Tooze, J., editor. 1973. The Molecular Biology of Tumour Viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 1.
2. Shin, S.-I., V. H. Freedman, R. Risser, and R. Pollack. 1975. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **72:**4435.
3. Jones, P. A., W. E. Laug, A. Gardner, C. A. Nye, L. M. Fink, and W. F. Benedict. 1976. In vitro correlates of transformation in C3H/10T-1/2 clone 8 mouse cells. *Cancer Res.* **36:**2863.
4. Stiles, C. D., W. Desmond, Jr., G. Sato, and M. H. Saier, Jr. 1975. Failure of human cells transformed by simian virus 40 to form tumors in athymic nude mice. *Proc. Natl. Acad. Sci. U. S. A.* **72:**4971.
5. Leavitt, J. C., B. D. Crawford, J. C. Barrett, and P. O. P. Ts'o. 1977. Regulation of requirements for anchorage-independent growth of Syrian hamster fibroblasts by somatic mutation. *Nature (Lond.).* **269:**63.
6. Hatanaka, M., R. Klein, R. Toni, J. Walker, and R. Gilden. 1973. Mutants of nonproducer cell lines transformed by murine sarcoma viruses. I. Induction, isolation, particle production, and tumorigenicity. *J. Exp. Med.* **138:**356.
7. Hatanaka, M., R. Klein, C. W. Long, and R. Gilden. 1973. Mutants of non-producer cell lines transformed by murine sarcoma virus. II. Relationship of tumorigenicity to presence of viral markers and rescuable sarcoma genome. *J. Exp. Med.* **138:**364.
8. Tsuchida, N., M. Shih, R. V. Gilden, and M. Hatanaka. 1974. Mutants of nonproducer cell lines transformed by murine sarcoma virus. III. Detection and characterization of RNA specific for helper and sarcoma viruses. *J. Exp. Med.* **140:**218.
9. Tsuchida, N., M. S. Shih, R. V. Gilden, and M. Hatanaka. 1974. Sarcoma and helper-specific RNA tumor virus subunits in transformed nonproducer mouse cells activated to produce virus by treatment with bromodeoxyuridine. *J. Virol.* **14:**1262.

10. Wurster, D. H., and K. Benirschke. 1970. Indian muntjac, *Muntiacus muntjak*: a deer with a low diploid chromosome number. *Science (Wash. D. C.).* **168**:1364.

11. Flanagan, S. P. 1966. "Nude", a new hairless gene with pleiotropic effects in the mouse. *Genet. Res.* **8**:295.

12. Pantelouris, E. M. 1968. Absence of thymus in a mouse mutant. *Nature (Lond.).* **217**:370.

13. Rygaard, J., and C. O. Povlsen. 1969. Heterotransplantation of a human malignant tumour to "nude" mice. *Acta Path. Microbiol. Scand.* **77**:758.

14. Povlsen, C. O., P. J. Fialkow, E. Klein, G. Klein, J. Rygaard, and F. Wiener. 1973. Growth and antigenic properties of a biopsy-derived Burkitt's lymphoma in thymus-less (nude) mice. *Int. J. Cancer.* **11**:30.

15. Freedman, V. H., and S.-I. Shin. 1974. Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell.* **3**:355.

16. Koene, R. A. P., P. G. G. Gerlag, J. J. Jansen, J. F. H. Hageman, and P. G. A. B. Wijdeveld. 1974. Rejection of skin grafts in the nude mouse. *Nature (Lond.).* **251**:69.

17. Haller, O., M. Hansson, R. Kiessling, and H. Wigzell. 1977. Role of non-conventional natural killer cells in resistance against syngeneic tumour cells in vivo. *Nature (Lond.).* **270**: 609.