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CONTENTS

Use of Genetic Markers to Study Cellular Origin and Development of Tumors in Human Females

PHILIP J. FIALKOW

Electron Spin Resonance Studies of Carcinogenesis

HAROLD M. SWARTZ

Some Biochemical Aspects of the Relationship between the Tumor and the Host

V. S. SHAPOT

Nuclear Proteins and the Cell Cycle

GARY STEIN AND RENATO BASERGA

ONCOGENICITY AND CELL TRANSFORMATION BY PAPOVAVIRUS SV40: THE ROLE OF THE VIRAL GENOME[1]

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I. Introduction

Simian virus 40 (SV40) has undergone intensive study by many investigators in the decade following its discovery (Sweet and Hilleman, 1960). It is one of the most, if not the most, well-understood model tumor virus containing deoxyribonucleic acid (DNA).

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A number of attributes have encouraged the selection of SV40 as the viral agent for so many studies: it can be readily propagated and accurately assayed in tissue culture; it can transform cells *in vitro* as well as induce tumors *in vivo;* it has a limited content of genetic information; and its nucleic acid can be isolated in an infectious form. It is a member of the papovavirus group (Melnick, 1962) and the family Papovaviridae (International Committee on Nomenclature of Viruses, 1971).

Several recent reviews have covered different aspects of SV40 tumorigenesis (Rapp and Melnick, 1966; Black, 1968; Deichman, 1969; Rapp, 1967, 1969), so this chapter will not attempt to recapitulate all the recorded facts about the virus. Rather, we have posed questions pertinent to the phenomenon of oncogenesis and surveyed the literature for possible answers. The subjects considered, among others, are: (*a*) the role of complete and defective viral genomes in oncogenesis and transformation; (*b*) the antigenic changes which accompany transformation and their relationship to each other; (*c*) the virogenic and genotypic changes in transformed cells; (*d*) the failure to recover virus from tumor cells; (*e*) the requirement for a persisting viral genome to ensure the maintenance of the transformed state, and (*f*) the factors which affect malignancy of transformed cells.

Obviously, all the answers to the above questions are not yet known, but some intriguing clues have been obtained. We have attempted to evaluate critically the available knowledge of SV40 oncogenesis and to assess the implications for carcinogenesis by DNA-tumor viruses as a whole.

II. Oncogenic Potential of SV40 *in Vivo*

A. Role of the Host Animal

The oncogenic potential of SV40 has been demonstrated only in hamsters (Eddy *et al.*, 1962; Girardi *et al.*, 1962; Ashkenazi and Melnick, 1963; Black and Rowe, 1964). Efforts to induce tumors by SV40 in mice, guinea pigs, rats, rabbits, and monkeys have been unsuccessful (Eddy, 1964).

The latent period of tumor induction by SV40 in hamsters ranges from 3 months to more than a year, depending upon the concentration of virus employed and the age of the animal at the time of inoculation. The incidence of tumors in animals inoculated as newborns is usually over 90%, but it is variable in older animals. Girardi *et al.* (1963) reported that 23% of hamsters inoculated at 1 month of age ultimately developed tumors with an average latent period of 360 days. In other studies,

tumors failed to appear in hamsters inoculated at 3 weeks of age, although some hamsters inoculated at 4 months of age developed tumors when held for long periods (Allison *et al.*, 1967).

Newborn hamsters are susceptible to the oncogenic effects of SV40 whether inoculated by the subcutaneous, intracerebral, intraperitoneal, or intrathoracic route. However, no tumors were observed when newborn hamsters were inoculated by the oral and intranasal routes (Eddy, 1964). Of the preceding methods of inoculation, the subcutaneous route is the one of choice since neoplasms are visible and can be scored soon after appearance. There seems to be some correlation between the concentration of the virus inoculum and the latent period for tumor development when animals are injected by the subcutaneous route (Eddy *et al.*, 1962). DNA isolated from SV40-infected monkey cells is also oncogenic in these animals (Boiron *et al.*, 1965).

Histologically, SV40-induced tumors in subcutaneous tissues, lungs, and kidneys have been designated as undifferentiated sarcomas, although some fibrosarcomas have been reported (Eddy, 1964). Intracerebral inoculation of the virus into newborn hamsters resulted in the development of ependymomas (Kirschstein and Gerber, 1962). Induction of ependymomas by SV40 in mastomys has also been reported (Rabson *et al.*, 1962) although no evidence was presented that the observed neoplasms were related to SV40.

X-Irradiation of adult animals prior to virus inoculation enhances their susceptibility to tumor induction (Allison *et al.*, 1967). Also, adult animals inoculated in the cheek pouch with SV40 were found to develop tumors after a latent period of only 97 days, as compared with a latent period of 490 days when animals of the same age were inoculated by the subcutaneous route. All tumors which developed were SV40 tumors based on the presence of SV40 transplantation antigen (Allison *et al.*, 1967). The study indicated that potentially malignant cells can remain dormant in the animal for a long period of time and that depression of the immune response of the host will result in the growth of such "dormant" cells.

Tumor induction in mice by SV40 has not been demonstrated, even by the use of immunosuppressed animals (Tevethia, 1971). The virus can infect mouse cells rather easily, as shown by (*a*) transformation experiments *in vitro* using 3T3 mouse cells (Black and Rowe, 1963b; Todaro and Green, 1964); (*b*) the elevated enzyme levels and increased DNA synthesis following infection of mouse kidney cells (Kit *et al.*, 1966b), and (*c*) the ability of the virus to induce SV40 specific transplantation immunity in mice (Kit *et al.*, 1969; Wesslén, 1970). It appears

that a mouse cell may possess many of the characteristics of transformed cells after infection with SV40 and yet not be able to grow as a tumor cell.

B. Role of Defective Viral Genomes

Viral defectiveness may be divided into two broad categories—conditional and nonconditional. Conditionally defective viruses are unable to complete the cycle of replication under certain conditions, such as in a nonpermissive host cell, in the absence of a helper virus, or at an elevated temperature [temperature-sensitive (ts) mutants]. In contrast, nonconditionally defective viruses are not able under any known conditions to complete a replicative cycle.

In this section, the significance in oncogenesis is considered of both conditionally defective helper-dependent viruses as well as other viral preparations which may, or may not, be nonconditionally defective. The defective viruses may be conveniently subdivided into those which are produced by some exogenous treatment of the virus stock, such as irradiation, and those which occur naturally (Table I).

TABLE I

Types of Defective SV40 Genomes Shown to Be Oncogenic

A. Produced by exogenous treatment
1. Irradiated virus
2. Hydroxylamine-inactivated virus
B. Naturally occurring
1. T-antigen-inducing defective particles
2. PARA(defective SV40)-adenovirus hybrid population

Defendi and Jensen (1967) demonstrated that, after inactivation by ultraviolet or by gamma radiation, SV40 not only retained its oncogenicity for newborn hamsters, but actually exhibited an enhanced tumor-producing capability when compared to that of the untreated virus. Most of the virus in the irradiated samples had been inactivated as determined by infectivity assays. Similar results were reported by Altstein *et al.* (1967a) using hydroxylamine-inactivated virus.

The authors postulated that their results could be explained if the oncogenic potential of SV40 is actually due to the presence of defective particles in virus stocks. After inactivation of the virus preparation, the concentration of these hypothetical defective particles would increase, and these, in turn, would be responsible for the observed enhancement in oncogenicity. However, defective virions may not be the only explanation for oncogenicity because of some other unexplained observations: (*a*) complete virus genomes can be recovered from some transformed cells (Section V,A), (*b*) nononcogenic variants of defective SV40

(PARA) have been described (see below) and (*c*) inactivated virus preparations that exhibit increased transforming capacity *in vivo* do not do so *in vitro* (Section III,B). One other possible explanation offered for enhanced levels of oncogenicity is that tumors produced by defective viruses may lack tumor-specific transplantation antigens (TSTA), the absence of which allows the tumors to grow without being influenced by the hosts' immune mechanisms. However, no experimental evidence has been provided to support this claim.

Naturally occurring SV40 particles which are defective for the synthesis of late viral proteins and infectious virus, but which are able to induce the synthesis of SV40 tumor (T) antigen, have been described (Sauer *et al.*, 1967; Altstein *et al.*, 1967b; Uchida *et al.*, 1968). The concentration of defective particles in preparations of SV40 has been shown to increase after serial passage using undiluted inocula (Uchida *et al.*, 1966). The naturally occurring defective particles were oncogenic in newborn hamsters (Uchida and Watanabe, 1969). The tumors induced by the defective viruses contained SV40 T-antigen.

A defective SV40 genome (PARA) carried by an unusual strain of human adenovirus type 7 has been described (Huebner *et al.*, 1964; Rowe and Baum, 1964; Rapp *et al.*, 1964c). For a current review of the properties of PARA-adenovirus populations, see Rapp (1971). The PARA genome is defective in that it cannot code for SV40 coat protein; it is encased in adenovirus capsids supplied by helper adenovirions present in the mixed population (Boéye *et al.*, 1966; Butel and Rapp, 1966a; Rowe and Baum, 1965). PARA-adenovirus 7 produces SV40 type tumors in newborn hamsters (Huebner *et al.*, 1964; Rapp *et al.*, 1966a), induces the synthesis of SV40 T-antigen *in vitro* (Rowe and Baum, 1964; Rapp *et al.*, 1964c), and induces SV40 TSTA in weanling hamsters (Rapp *et al.*, 1966b, 1967a). The discovery and subsequent characterization of PARA provided convincing evidence that late functions associated with the synthesis of capsid proteins and maturation of progeny virions are not required for the induction of SV40 T-antigen, TSTA, or oncogenicity.

The tumors induced by the original PARA-adenovirus 7 population had latent periods similar to those induced by parental SV40, but the oncogenic potential varied following "transcapsidation" (Rapp *et al.*, 1965b) to a series of different human adenoviruses (Rapp *et al.*, 1968). PARA particles which had been transcapsidated to adenovirus types 14, 16, and 21 were nononcogenic, whereas transcapsidant PARA-adenovirus population types 1, 2, 3, 5, and 6 were oncogenic in newborn hamsters. However, both the nononcogenic and oncogenic PARA-adenovirus populations were capable of inducing SV40-specific transplantation immunity in weanling animals. When PARA was transcapsidated from

the nononcogenic adenovirus 21 population to adenovirus 6, it remained nononcogenic, whereas PARA from the oncogenic adenovirus 6 population retained its oncogenic potential upon transcapsidation to adenovirus type 21 (Rapp *et al.*, 1970). These results suggested that variants of PARA which differed in oncogenicity were present in the original population of PARA-adenovirus 7. Such variants were then isolated from the parental PARA-adenovirus 7 population by two successive plaque purifications in monkey kidney cells; 20 of the 112 cloned lines of virus tested were shown to be nononcogenic (Rapp *et al.*, 1969). The nononcogenic variants of PARA were capable of transforming hamster cells *in vitro* (Butel *et al.*, 1971c; Rapp and Duff, 1971); however, the transformed cell lines which were established varied in transplantability (Butel *et al.*, 1971c).

When PARA was transcapsidated from adenovirus type 7 to the highly oncogenic Huie strain of adenovirus type 12, it produced SV40 tumors in newborn hamsters with a short latent period of only 5–7 weeks (Butel *et al.*, 1971a). All the early-appearing tumors induced by PARA-adenovirus 12 contained SV40 T-antigen. Parental SV40 and PARA-adenovirus 7 did not induce any tumors within the same time period. Artificial mixtures of adenovirus 12 and SV40 or adenovirus 12 and PARA-adenovirus 7 did not induce any tumors containing SV40 T-antigen within 5–7 weeks. Early SV40 tumors induced by PARA-adenovirus 12 contained SV40 TSTA, ruling out the absence of antigen as the cause of the early appearance of the SV40 tumors. One possible explanation for the results with PARA-adenovirus 12 was that recombination had occurred between a portion of the adenovirus 7 region of the PARA genome and a portion of the adenovirus 12 genome. Such recombinant particles might contain the adenovirus 12 marker(s) responsible for the rapid appearance of tumors, and, in the process of tumor development, the linked SV40 information would be expressed. There is no direct proof yet available, however, that the postulated recombinational event actually occurred.

Defective viruses can be very useful tools for determining viral functions necessary for oncogenicity. They can be replicated with the aid of a helper virus which, if it is nononcogenic, does not interfere with subsequent tests *in vivo*. With a conditionally defective virus which may have lost part of the parental genome, the system is not complicated by any possibility of leakiness or reversion on the part of the mutant. In addition, strict conditions do not have to be imposed to ensure defectiveness as is the case, for example, with temperature-sensitive mutants. With the *ts* mutants, unfortunately, the body temperature of the intact animal required for oncogenicity studies is frequently that of the permissive temperature.

C. Factors Affecting Viral Oncogenicity

As described above, if SV40 is inoculated into hamsters within 24 hours of birth, tumors will develop in a majority of the animals with latent periods ranging from 3 to 6 months. Without exception, all SV40-induced tumors are antigenic and contain TSTA at the cell surface (see Section IV,B).

Polyoma virus can induce the development of tumors in certain strains of mice if inoculated into newborns. However, C57Bl, strain A, and C3H/Lw mice are resistant to polyoma virus oncogenesis (Law, 1969). If the resistant strains were made immunologically deficient by thymectomy at birth, they became susceptible to polyoma virus tumor induction. Similar results were obtained when the resistant strains were treated with antilymphocytic serum. The immunologic capacity of the thymectomized mice and the resistance to polyoma virus oncogenesis could be restored by intravenous injection of syngeneic spleen or lymph node cells from intact animals.

The role of immunocompetence in resistance to SV40 oncogenesis in hamsters was demonstrated by Allison *et al.* (1967), when they showed that tumor development in hamsters was dependent upon the age of the animal at the time of virus inoculation. SV40 produced tumors in adult animals only after X-irradiation of the host. Tumors also developed when virus was inoculated into the cheek pouch of animals (an immunologically privileged site). These studies suggested the following sequence of events upon inoculation of the virus into an adult animal: (*1*) cells are transformed, (*2*) TSTA at the transformed cell surface sensitizes the host, and (*3*) potential tumor cells are eliminated. This sequence of events was first postulated by Habel (1962) and is supported by data in the report by Tevethia *et al.* (1968c). Antihamster thymocyte serum, a potent suppressor of cellular immunity, prevented the sensitization of animals to SV40 TSTA when administered to hamsters during the period of virus immunization. The failure to become sensitized to TSTA was demonstrated by the fact that treated animals were unable to reject a challenge of SV40-transformed cells. In addition, spontaneous regression of primary tumors induced by SV40 in hamsters has been observed (Tevethia *et al.*, 1968a; Deichman, 1969).

The appearance of primary tumors in hamsters inoculated as newborns requires discussion in view of the fact that SV40 tumors have long latent periods and contain TSTA. The development of immunologic tolerance to SV40 TSTA can be ruled out, since SV40 oncogenesis can be blocked by immunization of the animals during the latent period with either virus or virus-transformed cells (Goldner *et al.*, 1964; Girardi, 1965; Tevethia *et al.*, 1968b). Such immunization, however, is ineffective

in thymectomized animals (Girardi and Roosa, 1967). Hamsters undergoing viral oncogenesis do not become sensitized to SV40 TSTA before the appearance of palpable tumors (Deichman and Kluchareva, 1964). However, animals bearing tumors become sensitized and can reject a challenge of SV40-transformed cells at a site distant from the primary tumor (Lausch and Rapp, 1971), which may explain the absence of widespread metastases in tumor-bearing animals.

There are several possible mechanisms to explain the growth of primary SV40 tumors:

1. A transformed focus in a newborn animal may be established before development of immunocompetence. If the transformed focus reaches a certain critical size, it may sensitize the host without being rejected. However, this mechanism is unlikely since SV40 tumors have long latent periods (100–200 days) and the animals become immunocompetent long before the appearance of palpable tumors (Friedman and Goldner, 1970a). Also, infection of animals with SV40 does not lead to generalized immunosuppression (Friedman and Goldner, 1970b).

2. The amount of TSTA present in the developing focus of transformed cells might not be enough to sensitize the host. By the time the concentration of TSTA becomes sufficiently high to sensitize the host, the tumor might already have grown to a size such that it cannot be rejected by the immune lymphocytes. In support of this, Deichman and Kluchareva (1964) demonstrated that hamsters inoculated with SV40 as newborns were as susceptible to challenge with SV40-transformed cells as control animals. The fact that SV40 oncogenesis can be prevented by immunization of hamsters during the latent period with either SV40 or transformed cells indicates a block at the efferent level of the immunological arc. Insufficient TSTA to sensitize the animals undergoing viral oncogenesis can be ruled out since only a single inoculation of virus during the latent period is enough to prevent tumor appearance.

Deichman (1969) advanced a hypothesis to explain the difference between newborn and adult animals with respect to the development of transformed foci. She proposed that the development of TSTA in virus-infected cells and the transformation of the cells are separate functions. She postulated that TSTA is synthesized in cells of both newborn and adult animals infected with SV40. In newborn animals, however, TSTA would disappear from most of the cells perhaps because of the fast rate of cell division; the number of cells which ultimately transform would not be large enough to sensitize the host. In contrast, in adult animals TSTA would persist long enough to sensitize the host. In support of this hypothesis, Kluchareva *et al.* (1967) showed that hamster cells infected

with SV40 *in vitro* developed SV40 TSTA which could immunize a host.

3. Blocking antibody (K. E. Hellström and Hellström, 1970) may be responsible for the growth of primary SV40 tumors. Such antibody molecules bind to TSTA and protect the antigenic sites from the action of immune lymphocytes. The possibility that blocking antibodies are responsible for the growth of primary tumors before they become palpable is unlikely, for the following reasons: (*a*) Blocking antibodies have been demonstrated only in tumor-bearing animals and cannot be detected before the development of the tumor or after the tumor is surgically removed. (*b*) Animals undergoing SV40 oncogenesis behave as relatively nonsensitized animals before the appearance of palpable tumors. Blocking antibodies may play an important role, however, in guarding the tumor cells from the action of immune lymphocytes once the animal has been sensitized.

4. The failure to demonstrate immunity to SV40 TSTA in hamsters undergoing SV40 oncogenesis may be explained by the immobilization of the immunoblasts in the lymph nodes draining the tumor site (Alexander *et al.*, 1969). The release of the immunoblasts was achieved either by surgical removal of the tumor or by immunization of the host with tumor cells. These results may explain why SV40 oncogenesis can be prevented by immunization of the host during the latent period.

Although it is well established that SV40 can induce oncogenesis *in vivo*, the role of the viral genome in the acquisition of malignant potential by cells transformed *in vitro* is not known. Transformed hamster cells can usually be readily transplanted in adult animals. In contrast, mouse cells transformed by SV40 can be transplanted in syngeneic animals only with great difficulty, frequently requiring depression of the immune response of the host (Takemoto *et al.*, 1968a). Prolonged cultivation *in vitro* of the transformed cells is also often necessary before a successful transplant *in vivo* can be achieved (Kit *et al.*, 1969; Wesslén, 1970).

Enders and Diamandopoulos (1969) observed that hamster heart cells transformed by SV40 became highly transplantable after passage *in vivo*. Sixteen clones were isolated from the original population of transformed cells and a wide variation was found in the transplantability of the clonal lines. Some clones were highly transplantable whereas others failed to produce tumors unless more than a million cells were inoculated per animal. When the lines of apparent low transplantability were passaged *in vivo*, the oncogenic potential of the cells increased markedly. These studies indicated that cells of high oncogenicity may be selected *in vivo* and that cellular mutations may be responsible for the increase in oncogenic potential. The authors concluded that the viral genome did

not appear to be responsible for the increase in transplantability of the transformed cells.

Variation in the transplantability of hamster lung and kidney cells transformed by various PARA-adenovirus hybrid populations has been observed by Butel *et al.* (1971c). Virus clones which appeared to be nononcogenic in newborn hamsters were able to transform hamster cells *in vitro*, but the transplantability in weanling hamsters of early passages of the transformed cells varied greatly. Some of the cell lines were readily transplantable whereas other lines transformed by the same clone of virus were either not transplantable or could be transplanted only with difficulty.

The mechanism responsible for the observed differences in transplantability of transformed cells is not known. However, immunological factors which may operate at either the level of the host or that of the tumor cell may be important. All cells transformed by SV40 contain TSTA which mediates the development of specific resistance against transplantation of the transformed cells. Most lines of SV40-transformed mouse cells are not transplantable in the syngeneic host, but can be transplanted in immunosuppressed animals (Takemoto *et al.*, 1968a; Tevethia, 1971) indicating the role of immunological surveillance on the part of the host. This hypothesis is supported by the observation that an animal bearing a progressively growing SV40-transformed cell transplant is immune to a second transplant of the same cells at a distant site. This concomitant immunity is viral-specific (Lausch and Rapp, 1971).

However, SV40-transformed cells can become immunoresistant and are then capable of growing in immune animals. Deichman and Kluchareva (1966) reported the loss of SV40 TSTA from cells taken from metastatic tumors. SV40-immunized animals were unable to reject tumor cells derived from lung metastases. However, these cells were not tested for their capacity to immunize hamsters against a challenge of an immunosensitive cell line to definitely prove they lacked SV40 TSTA. There is the possibility that the cells from the lung metastases were simply immunoresistant in view of the fact that Tevethia *et al.* (1971) have isolated immunoresistant variants from a population of SV40-transformed cells. These variants are not rejected by SV40-immunized animals but can immunize hamsters against a challenge of immunosensitive tumor cells, showing that the immunoresistant cells do contain TSTA. Major factors that may contribute to immunoresistance include (*a*) a faster rate of cell growth *in vivo*, (*b*) lower concentrations of TSTA, (*c*) masking of TSTA by mucopolysaccharides, and (*d*) interference by blocking antibodies.

Beyond these reports, which suggest that immunological factors may

play a role, very little is known about the event(s) which determine the malignancy of a transformed cell. One factor which may affect the transplantability of transformed cells may be surface changes other than SV40 TSTA. These changes may be reflected in either increased or decreased transplantability of tumor or transformed cells. Deichman and Kluchareva (1966) reported that SV40-transformed cells, when grown *in vitro* in the presence of sera from SV40 tumor-bearing animals, became immunoresistant. This change was not observed when the cells were grown in the presence of sera from hamsters which had rejected SV40 tumors or from animals bearing polyoma tumors. These findings strongly suggest the presence of antigens which can act as TSTA but are capable of "modulation." Conclusive evidence favoring this hypothesis is still lacking.

III. Transformation of Mammalian Cells *in Vitro* by SV40

Viral transformation has been defined as an induced inheritable change in the properties of a cell, accompanied by the loss of regulatory controls of cell growth. The criteria for transformation of cells generally include the following (Enders, 1965): (*a*) loss of contact inhibition, (*b*) altered morphology, (*c*) increased growth rate, (*d*) increased capacity to persist in serial subcultures, (*e*) chromosomal abnormalities, (*f*) increased resistance to reinfection by the transforming virus, (*g*) emergence of new antigens, and (*h*) capacity to form neoplasms. Black (1968) recently summarized the details of various SV40–host cell transformation systems. Consequently, only certain features of transformation by SV40 will be emphasized at this time to provide a background for subsequent sections of the chapter.

A. Transformation of Permissive and Nonpermissive Cells by SV40

As discussed above, SV40 is maximally oncogenic *in vivo* in newborn hamsters. The narrow host range of the virus has been widely extended by *in vitro* transformation experiments. In addition to transformation of hamster cells in culture (Black and Rowe, 1963a; Rabson and Kirschstein, 1962; Ashkenazi and Melnick, 1963; Shein *et al.*, 1963), it has also been reported that SV40 can transform cells of human (Koprowski *et al.*, 1962; Ashkenazi and Melnick, 1963; Shein and Enders, 1962), mouse (Black and Rowe, 1963b; Todaro and Green, 1964; Kit *et al.*, 1966b), rabbit (Black and Rowe, 1963b), rat (Diderholm *et al.*, 1966), bovine (Diderholm *et al.*, 1965), guinea pig (Diderholm *et al.*, 1966), and monkey (Fernandes and Moorhead, 1965; Koprowski *et al.*, 1967; Wallace, 1967; Margalith *et al.*, 1969; Shiroki and Shimojo, 1970) origin.