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Biochemical Properties of Media Conditioned by Simian Virus 40-induced Hamster Tumor Cells: Correlation with Distinct Cell Phenotypes but not with Oncogenicity

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ABSTRACT

Hamster cells, transformed *in vitro* by SV40, have been reported to secrete an unidentified factor(s) that inhibits thymidine uptake (TU) by various normal cell types, including activated lymphocytes. It has been postulated that this apparent antiproliferative effect may play an *in vivo* role in the high tumorigenic capacity of SV40-transformed hamster cells. In keeping with this hypothesis, Adenovirus type 2-transformed hamster cells, which are only weakly tumorigenic, do not inhibit TU by indicator cells *in vitro*. To study the biological relevance of this phenomenon, we assayed 11 cell lines derived from different fibrosarcomas, induced in Syrian hamsters by SV40, for their ability to inhibit TU by normal rabbit kidney indicator cells. In contrast to cells transformed *in vitro* by SV40, media conditioned by 6 of 11 tumor-derived cell lines did not inhibit TU. Our results do not support the hypothesis that an antiproliferative factor secreted by SV40-transformed cells promotes the tumor-inducing capacity of these cells. Furthermore, inhibition of TU does not appear to be due to the production of a specific antimitotic peptide, but rather to other biochemical properties of the media conditioned by transformed cells. Finally, these biochemical properties do appear to correlate with specific morphological and growth characteristics of the tumor cells, but probably as an effect and not a cause.

INTRODUCTION

SV40- and Ad2[2]-transformed hamster embryo cells readily induce tumors in immunoincompetent syngeneic hamsters, but only SV40-transformed cells induce tumors in adult syngeneic and allogeneic hamsters (1). This observation suggests that interactions between DNA virus-transformed cells and the host immune response may be critical in determining whether transformed cells are rejected by the host or evolve into progressive tumors. However, the reasons for the differences in the oncogenic potential of Ad2- and SV40-transformed cells remain elusive (1).

Akagi *et al.* (2) reported that conditioned media from two independently derived lines of hamster embryo cells transformed *in vitro* by SV40 (SV40HE) inhibit the growth of normal, untransformed rodent cells (as determined by inhibition of TU in NRK indicator cells stimulated with mitogens), while medium conditioned by hamster cells transformed *in vitro* by Ad2 stimulates the growth of the same cells. When medium conditioned by SV40HE-1 was concentrated, dialyzed extensively, and fractionated by gel filtration high pressure liquid chromatography, the inhibitory activity eluted within a molecular weight range of 25,000–40,000 (2). This partially purified inhibitory activity blocked TU by hamster spleen T- and B-lymphocytes activated with concanavalin A or pokeweed mitogen (2). The results suggested that the secretion of a putative mitogenic inhibitor(s) by SV40-transformed hamster cells might contribute to the very aggressive tumorigenic phenotype of these cells by interfering *in vivo* with the mobilization of an effective immune response at the site of tumor growth (2). On the other hand, the weakly tumorigenic phenotype of Ad2-transformed cells might be related to the lack of production of this factor(s). These data suggested the possibility that control of the host immune response by factors secreted by tumor cells may be critical in regulating tumor progression.

To define more clearly the role of these factors in SV40 tumorigenesis, we have addressed several questions. First, Akagi *et al.* (2) studied only cells transformed *in vitro* by SV40. If the production of this putative mitogenic inhibitor(s) is related to the highly oncogenic phenotype of SV40, this factor(s) should also be produced by cells derived from tumors induced *in vivo* by SV40 virus. To address this question, we examined the antimitotic activity of media conditioned by cells derived from 11 different fibrosarcomas that had been induced in Syrian hamsters by the s.c. injection of SV40 (3–5).

SV40 oncogenicity is determined by the SV40 early proteins, large T antigen and small t antigen. While the large T antigen is required for transformation under all conditions tested (6), deletions in the small t antigen gene of SV40 virus do not prevent transformation of dividing cells but are associated with significantly prolonged tumor incubation periods following s.c. injection of newborn hamsters (3–5). To investigate the possibility of a correlation between production of mitogenic inhibitor(s) and tumor latency, cells derived from fibrosarcomas induced by the small t deletion mutants dl883, dl884, and dl890 of SV40 (4, 5) were included in this study. To test the hypothesis that production of a putative mitogenic inhibitor(s) might interfere with the host immune system, we studied whether the ability of SV40-transformed cells to induce tumors in allogeneic hosts was correlated with the biochemical characteristics of media conditioned by these cells.

MATERIALS AND METHODS

Animal Studies. The SV40-induced tumor cell lines used were derived from fibrosarcomas that developed following s.c. injection (between the scapulae) of newborn Syrian hamsters with SV40 wild type or SV40 small t deletion mutants (dl883, dl884, or dl890). The tumor induction studies and the establishment of the SV40-induced tumor cell lines have been reported (3–5). The cell lines SV40HT-1, -2, and -3, were derived from 3 tumors of the 9 induced in a study in which 10 LSH hamsters were injected s.c. with 10^6 to 10^8 plaque-forming units/ml of SV40 (3). The other lines were derived from 8 tumors of the 119 induced in a study in which 372 newborn outbred Syrian hamsters were injected s.c. with 2×10^8 plaque-forming units of purified SV40 or SV40 small t deletion mutants (dl883, dl884, or dl890) as described (4,5). Tumor latency was determined by observing injected animals twice weekly for tumor formation for a period of 1 year. Once observed (tumor latency reported in Table 1), tumors were allowed to develop (no tumor regression was observed) for 6 weeks or until they reached

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[2] The abbreviations used are: Ad2, Adenovirus type 2; DM, defined medium; CDM, conditioned DM; FBS, fetal bovine serum; NRK, normal rat kidney; dThd, thymidine; TU, thymidine uptake; MR, mitogenic response.

Table 1 *Tumor latency and inhibition of TU*

Cell line	Inducing virus	Tumor latency (wk)	Inhibition of TU[a]
Ad2HE-1	Ad2-adenoid 6	NA	–
SV40HE-1	SV40 wt 776	2[b]	++
SV40HT-1	SV40 wt 776	13	–
SV40HT-2	SV40 wt 776	20	+
SV40HT-3	SV40 wt 776	21	++
830-FS-9	SV40 wt 830	34	–
830-FS-11	SV40 wt 830	11	–
830-FS-166	SV40 wt 830	41	++
883-FS-5	SV40 dl 883	32	+
883-FS-6	SV40 dl 883	34	–
884-FS-20	SV40 dl 884	45	–
890-FS-2	SV40 dl 890	34	++
890-FS-6	SV40 dl 890	38	–

[a] –, negative; +, intermediate; ++, positive; NA, not applicable. Ad2HE-1 is not oncogenic in adult hamsters.

[b] The short tumor latency observed for SV40HE-1 when injected in adult LSH animals is due to the injection of 10^7 *in vitro*-transformed cells and not of free virus.

40 to 60 mm, whichever came first. Tumor cell lines were established by treating minced tumor tissue with 1 mg/ml collagenase and allowing the cells to grow in tissue culture flasks. The cell lines derived from these tumors and used in this study were shown to be mycoplasma free by the Gen-Probe Mycoplasma TC rapid detection system (Gen-Probe, Inc., San Diego, CA).

Tumor Transplantation Studies. The LSH and CB strains of inbred Syrian hamsters used for tumor transplantation studies have been inbred for more than 50 brother-sister matings and differ by at least one strong histocompatibility locus (7, 8). Tumor cells (10^7) were injected s.c. in either adult LSH or CB hamsters. Inoculated animals were checked for tumor development for 12 weeks.

Conditioned Media and Mitogenic Assay. Briefly, following the procedure of Akagi *et al.* (2), tumor cells cultured in Dulbecco's modified Eagle's medium [GIBCO Laboratories, Grand Island, NY] with 5% FBS until 95% confluent, were overlaid with serum-free DM [a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (Whittaker M. A. Bioproducts, Walkersville, MD)]. After 2 or 3 days, the CDM (derived from viable cell cultures as shown by trypan blue exclusion) were collected and diluted with DM in several proportions (1:10, 2:10, 3:10, 4:10); 0.5 ml of these mixtures with 1% FBS, 10 ng/ml of epidermal growth factor (Sigma, St. Louis, MO), and [^{3}H]-thymidine (2–5 μCi/ml; New England Nuclear, Wilmington, DE) was overlaid onto quiescent indicator NRK cells in 16-mm dishes (approximately 2–3 × 10^5 cells/dish). The cells were incubated for 26–28 h, washed with phosphate-buffered saline, fixed with methanol, and solubilized with 1.0 M NaOH; DNA was precipitated on glass filters with 5% trichloroacetic acid, and radioactivity was determined in a scintillation counter (2). The MR was then calculated:

$$MR = 100 \times \frac{\text{cpm in CDM} - \text{cpm in DM}}{(\text{cpm in 1\% FBS} + 10\ \text{ng/ml EGF}) - \text{cpm in DM}}$$

where cpm is the average trichloroacetic acid-precipitable radioactive cpm on replicate filters. The controls for the mitogenic assay included cells incubated in DM without CDM or serum (MR = 0%) and cells incubated in 1% FBS + 10 ng/ml epidermal growth factor without CDM (MR = 100%). Since Akagi *et al.* (2) have shown that the ability of SV40 CDM to inhibit TU by NRK cells or lymphocytes is comparable, we used NRK indicator cells in these experiments because of the large number of assays necessary for screening.

RESULTS AND DISCUSSION

Production of Antimitotic Activity by Tumor Cells. To evaluate the possible role of antimitotic factors in SV40 tumorigenesis, we asked whether CDM from cell lines derived from tumors induced *in vivo* by SV40 expressed an antimitotic activity. We also investigated the possible correlation of antimitotic activity and tumor latency. To accomplish this, following the same experimental procedures as Akagi *et al.* (2), we studied the antimitotic activity (as expressed by the inhibition of TU in NRK indicator cells stimulated with mitogens) of CDM from cells derived from SV40-induced fibrosarcomas which appeared with varying latencies.

We examined the ability to inhibit TU of serum-free media conditioned by 6 cell lines derived from fibrosarcomas induced by wild type SV40 and 5 cell lines derived from SV40 small t deletion mutant (dl883, dl884, dl890)-induced fibrosarcomas. All of these cell lines were derived from tumors induced in different Syrian hamsters and they differed substantially in tumor latency (Table 1). The *in vitro*-transformed cell lines SV40HE-1 and Ad2HE-1 (2, 9) were used as positive and negative controls, respectively. The inhibition of TU by CDM from these two control cell lines is shown in Fig. 1, *A* and *B*. As reported previously (2), we found that when increasing amounts of CDM from SV40HE-1 were added to NRK cells in the presence of 1% FBS and 10 ng of EGF, the TU of these normal target cells was decreased. In contrast, there was little or no decrease in TU in NRK cells when CDM from Ad2HE-1 was used. The apparent inhibitory response evoked by the SV40HE-1 CDM appeared to be dependent on the length of the conditioning period, since the inhibitory activity increased with a longer conditioning time.

The TU of NRK cells in the presence of CDM from the SV40 tumor-derived cell lines was then measured and compared to that of the control cell lines. All 11 tumor-derived cell lines were studied in the confluent stage of growth, and serum-free media were conditioned for 2 and 3 days; results of one such experiment with two lines are shown in Fig. 1, *A* and *B*. CDM from the tumor cell line 830-FS-166 appeared to inhibit TU, although perhaps somewhat less than SV40HE-1. In contrast, CDM from another tumor cell line, 884-FS-20, failed to inhibit TU.

In Fig. 1*C* we show the inhibition of TU by all 11 tumor cell

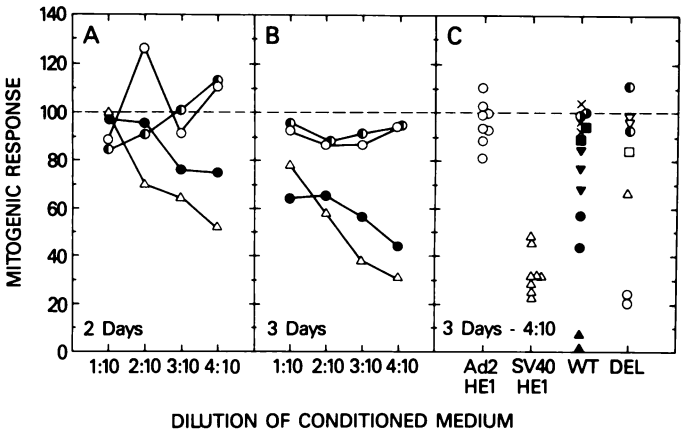

Fig. 1. Inhibition of TU by conditioned media from control cell lines and from 11 different SV40 tumor-derived cell lines. *Abscissa: A* and *B*, dilution of the conditioned medium used in the assay (see "Materials and Methods"); *C*, type of cells tested (*WT*, wild type SV40-derived; *DEL*, small t deletion mutant-derived). *Ordinate*, mitogenic response. *A* and *B*, concentration dependence of inhibition of the mitogenic response with conditioned medium collected after 2 days of conditioning (*A*) or 3 days of conditioning (*B*). *C*, the inhibition of the mitogenic response with day 3 CDMs at the highest concentration (CDM:DM, 4:10); these data were taken from experiments in which the concentration dependence and time of conditioning were determined as shown in *A* and *B*. The indicator cells were NRK. The mitogenic response was determined in DM with 1% FBS and epidermal growth factor (10 ng/ml). In *A* and *B*, cell lines used were: ○, Ad2HE-1; △, SV40HE-1; ●, 830-FS-166; ◐, 884-FS-20. In *C*, wild type SV40-derived tumor cell lines were: ×, SV40HT-1; ▼, SV40HT-2; ▲, SV40HT-3; ◑, 830-FS-9; ■, 830-FS-11; ●, 830-FS-166; small t deletion mutant-derived tumor cell lines were: △, 883-FS-5; ▽, 883-FS-6; ◐, 884-FS-20; ○, 890-FS-2; □, 890-FS-6.

lines and the two control lines studied. Here we present only the inhibition of TU by media conditioned for 3 days at the highest concentration used in these assays (4:10 dilution, CDM:DM; see "Materials and Methods"), since at this concentration we observed maximal inhibition of TU. Media conditioned by three cell lines, wt830-FS-166, SV40HT-3, and dl890-FS-2, were able to inhibit TU at least as much as SV40HE-1. Two cell lines, dl883-FS-5 and SV40HT-2, produced lower but still detectable inhibition of TU, and six cell lines, wt830-FS-9, wt830-FS-11, SV40HT-1, dl883-FS-6, dl884-FS-20, and dl890-FS-6, failed to inhibit TU.

Because some SV40 tumor-derived cell lines do not inhibit TU at all, this characteristic does not seem to play a major role in determining the tumor-inducing capacity of SV40-transformed cells. In addition, because no correlation was found between the ability to inhibit TU and the latency of SV40-induced tumors (Table 1), this characteristic does not seem to be involved in determining tumor latency. Furthermore, there was no correlation between inhibition of TU and the integrity of the small t antigen gene of SV40.

Transplantation of Tumors. SV40HE-1 and several other SV40-transformed hamster cell lines induce tumors with the same high efficiency in both syngeneic and allogeneic adult animals, while Ad2-transformed hamster cells are nontumorigenic in both syngeneic and allogeneic adult hamsters (1). The results of Akagi *et al.* (2) suggested the possibility that the production of an antimitotic factor(s) which inhibits lymphocyte proliferation at the site of tumor growth by SV40-transformed cells could be playing a role in the ability of SV40-transformed cells to induce tumors in allogeneic animals. We evaluated two tumor-derived cell lines, one of which (SV40HT-1) did not inhibit TU by indicator cells and the other (SV40HT-2) of which did inhibit TU, for their ability to form tumors after transplantation into syngeneic LSH hamsters and allogeneic CB hamsters (see "Materials and Methods"). Both cell lines produced tumors efficiently in both newborn and adult syngeneic as well as allogeneic hamsters. Thus, the different biochemical properties of SV40-derived CDM do not appear to correlate with differences in tumor induction in either syngeneic or allogeneic hosts.

Characterization of the Inhibitory Activity. During these studies we used the same assay developed by Akagi *et al.* (2); however, from our experiments we were confronted with the absence of any apparent correlation between inhibition of TU and SV40 tumorigenicity as originally suggested (2). On the other hand our data indicated that some SV40 lines had indeed the ability to inhibit TU. To understand whether the different characteristics of the SV40 CDM had some biological significance, we tried to identify the factor(s) responsible for the inhibition of TU. First, we noted that all of the inhibitory activity measurable in crude CDM, obtained from both *in vitro*- and *in vivo*-derived SV40 lines, passed through an Amicon YM2 filter with a M_r 1000 cutoff and was also eluted from a Sephadex G-25 column in the vicinity predicted for dThd. These data indicated that the inhibitory activity was of low molecular weight. We noted that the low molecular weight inhibitory activity that elutes from a Sephadex G-25 column in the vicinity of dThd has a UV absorption spectrum with maxima around 205 and 265 nm, consistent with the presence of nucleosides. Finally, we found that the assay developed by Akagi *et al.* (2) to measure TU is very sensitive to the presence of unlabeled dThd and/or other nucleosides in the CDM. When we adjusted the assay conditions to prevent this problem (*i.e.*, increasing the total dThd concentration in the assay to 200 μM), we no longer observed an apparent inhibition of mitogenesis by CDM from the SV40-transformed cells which were shown previously, by the unmodified assay, to have inhibitory activity. Consistent with this finding, CDM that appeared to have inhibitory activity for NRK cells by the original [^{3}H]dThd uptake assay did not detectably inhibit the growth of NRK cells as determined by counting cells. These results indicated that the inhibitory activity assayed in the crude CDM was dThd or some other factor(s) that competes with [^{3}H]dThd for uptake. We were unable to find any additional inhibitory activity at a higher molecular weight as observed by Akagi *et al.* (2) even in the SV40HE-1 cell line (positive control). A reexamination of the SV40HE-1 cell line from which the M_r 25,000–40,000 inhibitory activity had been originally prepared revealed that the line was contaminated with *Mycoplasma*. This same cell line had originally been tested by a commercial testing service and reported to be free of *Mycoplasma*. Reconstruction experiments confirmed that only SV40HE-1 cells contaminated with *Mycoplasma* produced a M_r 25,000–40,000 inhibitory activity. This inhibitor was not present in media conditioned by uncontaminated SV40HE-1 or Ad2HE-1, nor was it secreted by *Mycoplasma*-contaminated Ad2HE-1. This inhibitory activity was initially of interest since it reversibly arrested NRK cells in G_1, but it did not block TU when added to S-phase cells. However, the origin of this high molecular weight inhibitory activity remains unknown.

Growth Characteristics and Morphology of Tumor Cells. During this study we noted that the SV40 tumor-derived cells that did not inhibit TU seemed to have growth characteristics different from the cells that did. The cell lines that inhibit TU grew to confluence and could be maintained for several days at confluence; cell lines that did not inhibit TU had a tendency to pile up, slough off, and die before reaching confluency (Table 2).

Furthermore, the 11 tumor cell lines differed in their morphologies in tissue culture and there appeared to be a correlation between their morphological characteristics and their ability to inhibit TU in NRK cells. The cell lines which inhibited TU had a typical spindle-shaped flat fibroblastic appearance (Fig. 2*a*). The cell lines which did not produce inhibitory activity comprised a mixture of smaller, less adherent, spindle-shaped, stellate, and round cells showing a criss-cross growth pattern (Fig. 2*b*). The two cell lines that produced lower but detectable inhibition of TU were intermediate in terms of morphology.

Sief *et al.* (10) reported similar morphological differences between *in vitro*-transformed (SV40HE-1-like) and *in vivo*-derived (883-FS-6-like) SV40 rat cells. However, since at least in the hamster system, SV40 tumor-derived cells may have either the "SV40-like" or the "Ad2-like" morphology, the dif-

Table 2 *Growth characteristics of SV40 tumor lines*

Viable cells (2×10^6; trypan blue exclusion) were plated in 25-cm^2 flasks with 10 ml of Dulbecco's modified Eagle's medium containing 5% FBS and scored at the indicated time.

Cell line	Days after plating		
	3	6	9
SV40HT-1	5.3[a]	2.2	0
SV40HT-3[b]	2.5	6.7	8.5
830-FS-9	6.3	1.0	0
830-FS-11	10.8	1.3	0
830-FS-166[b]	9.0	15.3	16.8
883-FS-6	10.0	1.1	0
884-FS-20	3.2	0.8	0
890-FS-2[b]	10.5	9.0	1.2
890-FS-6	9.2	1.9	0

[a] Number of viable cells $\times 10^6$.
[b] Cell lines that inhibit TU.

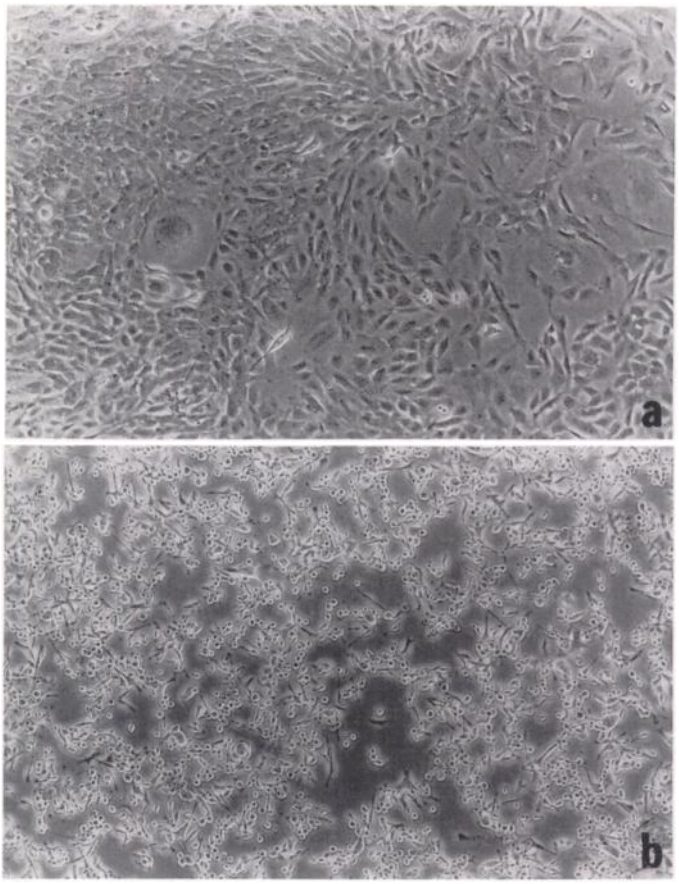

Fig. 2. Morphology of the SV40 tumor-derived cell lines. *a*, cell line SV40HT-3, typical fibroblastic morphology characteristic of all of the cell lines studied that do inhibit TU. *b*, cell line 883-FS-6, an almost undifferentiated cell morphology, characteristic of all of the SV40-induced tumor-derived cell lines that do not inhibit TU. Original magnification, × 200.

ferent appearance in culture of these cells should not be regarded as the expression of a more malignant *versus* a less malignant phenotype, or a characteristic of tumor cells *versus* tissue culture-transformed cells as suggested previously (10).

Because the cell lines derived from SV40-induced tumors that inhibit TU have a fibroblast-like morphology and can be maintained at confluence for several days, as with the cell lines transformed by SV40 *in vitro*, the biochemical characteristics of the SV40-derived CDM seem to be associated with a particular cell phenotype rather than with the expression of the SV40 genome *per se*. It is known that cells excrete large amounts of nucleosides after DNA replication is blocked by certain agents (11). Therefore, because the different growth characteristics of these SV40 cells did correlate with their ability to inhibit TU, we speculate that when cell growth slows, cells may excrete excess nucleosides. If only cells that enter a state of growth arrest secrete nucleosides, this might easily account for the different biochemical characteristics of the CDM studied. In keeping with this hypothesis, cell lines that do not inhibit TU seem to lose growth control; cells from these lines pile up, slough, and die. The observed differences among the tumor cell lines may reflect differences in the type of cell initially transformed by the virus, or they may be due to differences in the way the virus interacts with the host cell to bring about the transformation event.

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