ABSTRACT

Adenovirus 2 (Ad2)- and simian virus 40 (SV40)-transformed hamster embryo cells differ markedly in a number of phenotypic properties including their potential for inducing tumors in hamsters. Both Ad2- and SV40-transformed cells are immortalized and readily induce tumors in immunoincompetent newborn syngeneic hamsters, but only SV40-transformed cells are highly oncogenic in both adult syngeneic and allogeneic immunocompetent hamsters. The reasons for the difference in the oncogenic potential of the Ad2- and SV40-transformed phenotypes remain elusive. However, recent studies with transforming growth factors (TGFs) indicate that these factors play an important role in determining many phenotypic characteristics of transformed cells. To determine whether TGFs secreted by Ad2- and SV40-transformed hamster embryo cells differ, we have examined the ability of media conditioned by these two transformed cell phenotypes to modulate thymidine uptake in quiescent, untransformed cells. We found that both transformed phenotypes secrete very similar TGFα-like mitogenic factors which inhibit binding of 125I-labeled epidermal growth factor to its receptor. Our results also show that SV40-transformed cells, but not Ad2-transformed cells, secrete a powerful mitogenic inhibitor (MI). The MI secreted by SV40-transformed cells is inhibitory for several transformed and untransformed cell types and exerts a cytostatic, not cytolytic, action on untransformed primary hamster embryo cells. MI elutes from size exclusion high-performance liquid chromatography columns with a molecular weight of 24,000. Although MI has about the same molecular weight as TGFβ, it differs from TGFβ in two important respects: it is heat labile and it has a different target specificity for antimitogenic activity. The MI secreted by SV40-transformed cells also inhibits thymidine uptake by concanavalin A-stimulated spleen lymphocytes. This finding suggests that MI might contribute to the extreme oncogenicity of SV40-transformed cells by inhibiting mobilization of immune effector cells at the site of tumor cell proliferation.

INTRODUCTION

SHE cells transformed by Ad2 or SV40 differ markedly in their ability to induce tumors in immunocompetent hamsters (1, 2). Ad2-transformed SHE cells are small, highly undifferentiated cells that have a round or cuboidal morphology. They accumulate relatively low levels of fibronectin (3) and exhibit anchorage-independent growth in soft agar. These cells readily induce tumors in syngeneic newborn immunocompetent hamsters but rarely do so in immunocompetent syngeneic adult hamsters and never in allogeneic hamsters (2, 4). In contrast, SV40-transformed SHE cells closely resemble untransformed SHE cells. They are large and fibroblastic and accumulate substantial amounts of fibronectin (1). Nevertheless, these cells grow well in soft agar and readily induce tumors in immunocompetent syngeneic as well as allogeneic hamsters (2, 4).

Several proposals have been put forward to account for differences in the tumorigenic potentials of the highly oncogenic (Ad2 and SV40) and nononcogenic (Ad2 and Ad5) DNA viruses. It has been suggested that cells transformed by nononcogenic viruses express highly immunogenic tumor-specific transplantation antigens which induce a strong immune response (5). Other investigators have suggested that expression of Ad12-transforming genes, but not Ad5-transforming genes, causes down-regulation of histocompatibility antigens, thus permitting Ad12-transformed cells to elude detection by the host’s immune system (6–9). Finally, there is evidence indicating that cells transformed by SV40 are relatively resistant to in vitro lysis by natural killer cells and activated macrophages, while cells transformed by Ad2 are very sensitive to nonspecific immune effector cells (2, 3, 10).

Despite these findings, there is no agreement that any of the proposed immunological mechanisms is, in itself, sufficient to account for the striking difference in the oncogenic potential of hamster cells transformed by highly oncogenic and nononcogenic DNA viruses (3–5, 11–16). Nonetheless, immune resistance is essential if immortalized cells are to develop into tumors, and the absence of an explanation which completely accounts for the tumorigenic differences between cells transformed by different DNA viruses has led us to look for other mechanisms. Both Ad2- and SV40-transformed cells have been reported to secrete TGFs that compete with EGF and induce anchorage-independent growth in untransformed cells (17–19). Could TGFs play a role in influencing the tumorigenic phenotypes of hamster cells transformed by DNA viruses?

The role of TGFs in influencing the tumorigenic potential of the cells that secrete them has been the subject of much inquiry in recent years (20–22). This work has led to the identification of two principal types of TGFs. TGFα is a small peptide secreted by many transformed mammalian cells. This factor has a molecular weight of 6000 and has considerable homology with EGF. TGFα competes with EGF for binding to the EGF receptor, and it is mitogenic for cells that express EGF receptors. It has been proposed that TGFα acts in an autocrine fashion to stimulate proliferation of the cells that secrete this factor, both in vitro and in vivo (23–25). Larger TGFα-like growth factors with molecular weights greater than 6000 have also been reported to be secreted by transformed cells as well as by embryonic tissue (26–29).

The second major type of TGF, TGFβ, appears to play a much more complex role in regulating cell growth. The principal source of TGFβ is normal human platelets and it is found in other normal tissues as well as transformed cells (20, 22). This peptide comprises two identical subunits which are held together by disulfide bonds, giving it a molecular weight of about 25,000 (22, 30). TGFβ induces morphological changes and anchorage-independent growth in untransformed cells, and it inhibits the cytolytic activity of natural killer cells in vitro.
(31). TGFβ inhibits proliferation of many cell types including T and B lymphocytes (18, 30, 32-35); it is probably identical to the GI secreted by BSC-1 cells (35). TGFβ induces angiogenesis (36), and it promotes differentiation in human bronchial epithelial cells (30, 37). Thus, this factor may influence both cell proliferation and differentiation, depending on the target cell and the actions of other growth factors.

Although it is likely that TGFs function in regulating transformed cell proliferation in vivo as well as in vitro, their role in determining the tumorigenic potential of the cells that secrete them is not clear. In the work reported here, we have studied mitogenic and antimitogenic growth factors secreted by Ad2- and SV40-transformed SHE cells with the objective of determining whether TGFs might influence any of the disparate phenotypic properties, including oncogenicity, exhibited by these two transformed cell phenotypes.

MATERIALS AND METHODS

Cell Lines. Untransformed LSH SHE cells were prepared by mincing and trypsinizing whole 14-day-old hamster embryos. Transformed cells were established by infecting SHE cells with UV-inactivated SV40 (strain 777) or Ad2 (prototype Ad2); each line was established from a single focus of transformed cells grown on plastic dishes under serum-free (1). Cell lines were carried in DMEM containing 5% FBS. Frozen cell stocks, shown to be free of mycoplasma, were maintained, and cell cultures were frequently started from frozen stocks to minimize contamination. Frozen stocks of mitogenic indicator cells were also maintained. These were SHE cells, normal rat kidney cells (NRK-49F, ATCC CRL 1570), and mouse AKR-2B cells (a gift from Susan Bates, National Cancer Institute). Spleen lymphocytes were obtained from adult hamsters (8-10 months old). The spleens were removed aseptically, minced, pressed through 60-mesh wire screens, and resuspended in RPMI 1640 medium (2% FBS). The cells were washed twice with medium and counted.

Ad2HE1, Ad2HE3, Ad2HE6, SV40HE1, and SV40HE3 are transformed cell lines established from individual foci of SHE cells infected with Ad2 or SV40. Hybrid cell lines were formed by fusing SV40HE1 and Ad2HE1 (38) and cloning hybrid foci. The S‘A’ clonal cell lines have both SV40 and Ad2 tumor antigens but express the Ad2-transformed cell phenotype (i.e., nontumorigenic in immune competent syngeneic hamsters). The S‘A’ clonal cell lines contain only SV40 tumor antigens and express the SV40-transformed cell phenotype (i.e., highly tumorigenic in immune competent allogeneic hamsters). Ad5HKc>gj.2 were established from hamster kidney cells transfected with Ad2 or SV40. Cultures of transformed SHE cells, which differ in a number of phenotypic properties including oncogenic potential, might also differ in mitogenic and antimitogenic growth factors secreted by Ad2- and SV40-transformed SHE cells.

Mitogenic Assay. Frozen indicator cells were thawed and incubated in DMEM (5% FBS) until 90-95% confluent (about 3 days). The serum-containing media were aspirated and the cell sheets were washed with serum-free DMEM. The washed cells were overlaid with DMEM and incubated for 1-2 h. The DMEM was aspirated and the cells were overlaid with serum-free DMEM (a 1:1 mixture of DMEM and Ham’s F 12). After various periods (usually 3 days), the CDM was decanted and centrifuged at 2000 x g for 10 min. The supernatants were decanted and stored at 4°C. For studies with lymphocytes, we conditioned RPMI medium as indicated. The incubation mixture contained insulin (5 ng/ml, Sigma), transferrin (10 ng/ml, Sigma), crystalline bovine serum albumin (1 mg/ml, Sigma), and [3H]thymidine (2-5 µCi/ml; New England Nuclear). The total volume was 0.5 ml. Where indicated, serum mitogens (either 3% FBS or 1% FBS with EGF (10 ng/ml, Sigma)) were added to the incubation mixture. The cells were incubated 26-28 h, washed with PBS, and fixed with methanol. The fixed cells were solubilized with 1.0 M NaOH; DNA was precipitated on glass filters with 5% trichloroacetic acid, and radioactivity was determined in a scintillation counter. The MR was then calculated:

\[
MR = 100 \times \frac{[cpm \text{ in CDM} - cpm \text{ in DM}]}{[cpm \text{ in 10% FBS} - cpm \text{ in DM}]} \]

where cpm is the average trichloroacetic acid-precipitable radioactive counts/minute on multiple filters. The data are presented as percentage of MR with a standard deviation for multiple (2-4) samples. The controls for mitogenic assays included cells incubated in DM without CDM or serum (MR = 0%) and cells incubated in 10% FBS without CDM (MR = 100%). Preliminary studies on the kinetics of serum-stimulated SHE cells showed that thymidine uptake began at 8-10 h and was essentially complete at 28-30 h depending on the serum concentration. Similar studies showed that maximum stimulation was achieved with 10-15% FBS.

The mitogenic response of spleen cells was measured as follows. Approximately 2 x 10^6 cells/well were pipetted into 96-well plates. Conditioned RPMI medium was added and diluted to a concentration of 50% with RPMI medium (2% FBS); the cells were then stimulated with con A (2 µg/ml, Sigma). The total volume was 250 µl. The cells were incubated for 48 h and [3H]thymidine (4 µCi/ml) was added. Incubation was continued for 4 h and the cells were collected on glass filters with a Skatron cell harvester. The thymidine uptake of quadruplicate samples was determined in a scintillation counter.

Concentrated CDM. CDM, contained in dialysis tubes impermeable to molecules of molecular weight > 3000, was concentrated 10-fold using polyethylene glycol (molecular weight = 20,000). The concentrated CDM was extensively dialyzed against distilled water and lyophilized. The lyophilized CDM was extracted several times with small volumes of 0.5 M NaCl and centrifuged at 15,000 x g for 15 min. The total volume of the extracted supernatants was 1/1000 of the original volume of CDM.

HPLC Fractionation. Concentrated CDM (100 µl) containing 1-2 mg of protein was layered onto a size-exclusion HPLC column (Beckman 600-mm analytical Spherogel-TSK 2000-SW) and eluted with microfiltered PBS (0.5 M NaCl) at a flow rate of 1 ml/min. Approximately 60 0.5-m1 fractions were collected and assayed for protein content by measuring the absorbance at 280 nm. The mitogenic activity of each fraction (10-µl aliquots) was determined on NRK cells, either without serum mitogens or with 1% FBS and EGF (10 ng/ml). The molecular weights of the eluted fractions were estimated from the elution positions of proteins of known molecular weights.

The fractions were assayed for EGF competition on NRK cells as follows: Cells were cultured overnight in growth medium, washed free of serum, and incubated for 2 h with 10-µl aliquots of fractions in binding buffer [PBS with 1% bovine serum albumin, 5 mM MgCl2, and [125I]EGF (1000 cpm/well)]. The cells were washed with binding buffer and trypsinized, and radioactivity was determined in a gamma counter. The amount of EGF-competing material was estimated from standard curves obtained by competing [125I]EGF with authentic EGF. The data are presented as ng equivalents of EGF.

RESULTS

Mitogenic Activity of Media Conditioned by Ad2- or SV40-transformed SHE Cells. To determine whether Ad2- and SV40-transformed SHE cells, which differ in a number of phenotypic properties including oncogenic potential, might also differ in the kinds of TGFs they secrete, we have examined the mitogenic activity of serum-free media conditioned by several cell lines independently transformed by Ad2 or SV40. Cultures of trans-
formed cells were grown to near confluence in serum-containing media. The monolayers were washed free of serum and overlaid with serum-free DM. The DM was conditioned (CDM) by incubation with the transformed cells for various periods of time, and the mitogenic activity of the CDMs was tested by measuring thymidine uptake in primary untransformed SHE cells or NRK cells.

In preliminary experiments (data not shown), we studied the mitogenic response evoked by media conditioned by seven hybrid cell clones derived by fusing SV40HE1 with Ad2HE1, Ad2HE3, and Ad2HE6 as well as the parental nonhybrid cell lines. In these experiments CDMs from Ad2-transformed cells and hybrid cells which expressed the Ad2 phenotype (see footnotes to Table 1) stimulated much more thymidine uptake in quiescent NRK cells than did CDMs from cells having the SV40-transformed phenotype. We subsequently found that an even more dramatic difference between media conditioned by the two transformed cell phenotypes could be demonstrated using quiescent SHE cells stimulated with serum mitogens in the presence of conditioned media. The mitogenic response (percentage of thymidine uptake induced by CDM compared to that induced by 10% FBS) of SHE cells to media conditioned by seven transformed cell lines is shown in Table 1. The data indicate the mitogenic response in CDM alone (diluted 1:1 with DM), the mitogenic response in 50% CDM with serum mitogens, and the mitogenic response with serum mitogens without CDM. The last column shows the mitogenic index, which indicates whether the CDM acts synergistically with serum mitogens (mitogenic index >1) or whether the CDM is inhibitory (mitogenic index <1). Although there is variation in the data, which could be due in part to variation in cell number, it is clear that, when quiescent SHE cells are stimulated by FBS in the presence of CDM, the response of the indicator cells to the two kinds of CDMs is always different no matter what the cell number might be. While Ad2-CDM acts synergistically with FBS in eliciting a mitogenic response (mitogenic index >1), SV40-CDM is inhibitory (mitogenic index <1). CDMs from somatic cell hybrids which have the Ad2-phenotype (S1'A1*) (3, 14, 38) also act synergistically with FBS, while CDMs from hybrids of the SV40-phenotype (S1+A1~) are inhibitory (mitogenic index <1). SV40-CDM is inhibitory (mitogenic index <1). Although there is variation in the data, which could be due in part to variation in cell number, it is clear that, when quiescent SHE cells are stimulated by FBS in the presence of CDM, the response of the indicator cells to the two kinds of CDMs is always different no matter what the cell number might be. While Ad2-CDM acts synergistically with FBS in eliciting a mitogenic response (mitogenic index >1), SV40-CDM is inhibitory (mitogenic index <1). CDMs from somatic cell hybrids which have the Ad2-phenotype (S1'A1*) (3, 14, 38) also act synergistically with FBS, while CDMs from hybrids of the SV40-phenotype (S1'A1~) are inhibitory. The mitogenic indices of CDMs from Ad5HPKs7al and Ad5HK9a12 (39) are also greater than one. Together these results suggest that Ad2-, Ad5-, and SV40-CDMs all contain mitogenic factors. Moreover Ad2- and Ad5-CDMs contain factors that act synergistically with serum in inducing a mitogenic response, while SV40-CDMs contain factors that inhibit the response of SHE cells to serum mitogens.

In other experiments we measured the ability of Ad2- and SV40-CDMs to influence growth of subconfluent SHE cells in the presence of serum mitogens. We found that SV40-CDM suppressed cell growth, while Ad2-CDM enhanced cell growth compared to cells grown with serum mitogens in the absence of CDMs. These effects were observed for as long as 72 h after addition of CDM; however, CDM treatment had no apparent effect on cell morphology during this period (data not shown). Thus there was a good correlation between the ability of CDMs to regulate cell growth and to influence thymidine uptake by indicator cells.

To determine whether the mitogenic activity of CDMs might be influenced by the age of the transformed cell culture or the length of the conditioning period, a series of CDMs were prepared from cultures of Ad2HE1 and SV40HE1 cells which had been cultured for 1, 2, or 3 days with 5% FBS, at which point cultures of different ages were washed free of serum, and serum-free media were conditioned for 1, 2, or 3 days. The results of this experiment are shown in Fig. 1, in which it is seen that CDMs prepared from fresh cultures of both types of transformed cells, and conditioned for short periods, exhibit mitogenic indices >1. As the age of the culture (with a concomitant increase in cell density) and the periods of conditioning increase, the two kinds of CDMs elicit quite different responses—the mitogenic index of Ad2-CDM increases while that of SV40-CDM sharply decreases. This result indicates that as SV40HE1 cultures approach confluence, they secrete a mitogenic inhibitor(s). It should be noted that this effect is not due to cell density alone since the cell densities of the Ad2HE cultures are usually much greater than those of SV40HE cultures. However, after 3 days, SV40HE cultures are generally confluent, while the smaller, Ad2HE cells grow in clusters, frequently leaving empty spaces on the culture flask.

Since longer periods of conditioning appeared to enhance the stimulatory or inhibitory response of the two types of conditioned media, we compared media conditioned for 3 days by Ad2HE1, Ad2HE3, SV40HE1, and SV40HE3 (Table 1); all

<table>
<thead>
<tr>
<th>Cells used to prepare CDM*</th>
<th>With CDM, no serum mitogens (Cs)</th>
<th>With CDM and serum mitogens (Cs)</th>
<th>With serum mitogens, without CDM (S)</th>
<th>Mitogenic index (Cs/C + S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad2HE1</td>
<td>25.2 ± 3.5</td>
<td>116.1 ± 24.7</td>
<td>56.2 ± 2.4</td>
<td>1.43</td>
</tr>
<tr>
<td>S1'A1't +</td>
<td>7.6 ± 3.9</td>
<td>120.6 ± 17.8</td>
<td>57.7 ± 0.8</td>
<td>1.85</td>
</tr>
<tr>
<td>Ad5HK9a12</td>
<td>3.1 ± 0.4</td>
<td>102.8 ± 0.5</td>
<td>71.0 ± 8.7</td>
<td>1.35</td>
</tr>
<tr>
<td>Ad2HE3</td>
<td>5.0 ± 0.4</td>
<td>103.2 ± 5.1</td>
<td>71.3 ± 7.7</td>
<td>0.93</td>
</tr>
<tr>
<td>SV40HE1</td>
<td>18.3 ± 1.2</td>
<td>63.0 ± 3.0</td>
<td>49.4 ± 3.7</td>
<td>0.61</td>
</tr>
<tr>
<td>S1'A1't +</td>
<td>36.6 ± 1.2</td>
<td>55.6 ± 6.5</td>
<td>65.2 ± 6.2</td>
<td>0.56</td>
</tr>
<tr>
<td>S1'A1't +</td>
<td>5.0 ± 0.4</td>
<td>42.0 ± 7.3</td>
<td>63.7 ± 7.5</td>
<td>0.56</td>
</tr>
</tbody>
</table>

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* Ad2HE1, Ad2HE3, SV40HE1, and SV40HE3 are transformed cell lines established from individual foci of SHE cells infected with Ad2 or SV40 (see "Materials and Methods"). S1'A1't +, S1'A1't +, and S1'A1't + are cloned hybrid cell lines formed by fusing SV40HE1 and Ad2HE1 (38). The S1'A1't +, clonal cell line had both SV40 and Ad2 tumor antigens but expressed the Ad2-transformed cell phenotype (i.e., nonmalignant in immunocompetent syngeneic hamsters). The S1'A1't + and S1'A1't + clonal cell lines contained only SV40 tumor antigens and expressed the SV40-transformed cell phenotype (i.e., highly tumorigenic in immunocompetent syngeneic hamsters). Ad5HPKs7al and Ad5HK9a12 were established from hamster kidney cells transplanted with the left 16% (Xhol, fragment C) of the Ad5 genome (39).

CDMs were prepared by incubating DM for 24 h (upper panel) or 72 h (lower panel) with the indicated cell lines. The mitogenic response of SHE cells to CDMs (diluted 1:1 with DM) was determined with (Cs) or without serum mitogens (C). The control was the mitogenic response of SHE cells with serum mitogens without CDM. The data indicate the percentage of thymidine uptake induced by CDM compared to the response of control (S) stimulated much more thymidine uptake in primary untransformed SHE cells or NRK cells. The last column shows the mitogenic index, which indicates whether the CDM is mitogenic (index >1) or antimitogenic (index <1).
four cell lines were transformed independently by either Ad2 or SV40 (1). It is evident that, qualitatively, the mitogenic response elicited by conditioned media is a characteristic of the transforming virus.

In Fig. 2 we show that the synergistic action of Ad2-CDMs and the inhibitory action of SV40-CDMs on serum-stimulated indicator cells are concentration dependent. It should be noted that the action of Ad2HE3 CDM is very weak compared to that of Ad2HE1 and Ad2HE6. This result indicates that the TGFs secreted by Ad2HE3 which are mitogenic (Table 1B) do not significantly influence the response of SHE cells to serum mitogens.

To determine whether the effects of CDMS on the mitogenic response of SHE cells to serum mitogens are reversible, we pretreated cells for 24 and 48 h with CDMS, removed the CDMs, and measured the mitogenic response of the pretreated cells to serum mitogens (Table 2). It is evident that SHE cells pretreated with DM or CDM respond equally to serum mitogens. This result indicates that the inhibitory action of SV40-CDM is cytostatic rather than cytolysic. Moreover, these data show that the synergistic factors in Ad2-CDM probably do not exert their influence by irreversibly changing the competence state of the indicator cells; the factors must be present to exert their synergistic action.

It seemed possible that SV40-CDMs might contain mitogens that act in the presence of serum but that their effect is masked by an inhibitor which exerts a dominant influence on mitogenesis. To examine this question we measured the mitogenic response of SHE cells to mixtures of Ad2-CDM and SV40-CDM. This experiment showed that SV40-CDM is no less inhibitory when the concentration of Ad2-CDM is twice the concentration of SV40-CDM than when no Ad2-CDM is present (data not shown).

HPLC Fractionation of Ad2- and SV40-CDMs. To examine this question in more detail, we concentrated CDMS from Ad2HE1 and SV40HE1 1000-fold and fractionated them by size exclusion HPLC (see “Materials and Methods”). The mitogenic activities of aliquots of the eluted fractions were analyzed on NRK cells stimulated by serum mitogens (Fig. 3). It is evident that both CDMS contain approximately the same mitogens having molecular weights ranging from less than 6,000 to greater than 68,000, as estimated from the elution positions of protein markers. However, the SV40-CDM also contained a powerful antimitogen that elutes with a molecular weight of approximately 24,000. Since SV40-CDM appears to contain the same mitogens as Ad2-CDM, it is evident that the antimitogen secreted by SV40-transformed cells is dominant in determining the response of the indicator cells to SV40-CDMS.

The same fractions were also assayed for EGF competition (Fig. 3). Again it appears that both types of CDMS exhibit almost identical elution profiles. Each CDM has two major peaks of EGF-like factors that elute at molecular weights of about 7,000–8,000 and 18,000–20,000.

Conditioned medium from untransformed SHE cells was also concentrated and fractionated on HPLC. SHE CDM showed considerably less mitogenic activity than that observed with CDMS from transformed cells. Moreover, it contained neither of the peaks of EGF-competitive activity seen in the CDMS from the two transformed cell types; however, SHE-CDM did show a peak of EGF-competitive activity which eluted at molecular weights of 40,000–50,000 (data not shown).

The fractions containing the MI (fractions 34–36 of SV40HE1-CDM, Fig. 3) were pooled and tested on various cell types in the presence of serum mitogens (Table 3). It is evident that MI is inhibitory for both Ad2HE1 and SV40HE1 as well as for the two untransformed indicator cells.

Table 2 Mitogenic response of SHE cells pretreated with various CDMS for 24 or 48 h

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>58.5 ± 10.2</td>
<td>66.5 ± 16.4</td>
</tr>
<tr>
<td>1% FBS</td>
<td>70.4 ± 16.3</td>
<td>84.8 ± 17.7</td>
</tr>
<tr>
<td>60% Ad2HE1-CDM</td>
<td>92.8 ± 16.6</td>
<td>102.8 ± 21.3</td>
</tr>
<tr>
<td>60% Ad2HE6-CDM</td>
<td>113.2 ± 35.7</td>
<td>131.2 ± 35.7</td>
</tr>
<tr>
<td>60% SV40HE1-CDM</td>
<td>113.2 ± 35.7</td>
<td>131.2 ± 35.7</td>
</tr>
<tr>
<td>60% SV40HE3-CDM</td>
<td>113.2 ± 35.7</td>
<td>131.2 ± 35.7</td>
</tr>
</tbody>
</table>

* Mitogenic response of SHE cells pretreated with CDMS (conditioned for 3 days) was determined in DM supplemented with 1% FBS and EGF (10 ng/ml) (see “Materials and Methods”).

* CDMS were prepared by incubating the various cell lines with DM for 3 days. See footnotes to Table 1 for a definition of the cell lines.

* NT, no treatment.
GROWTH FACTORS IN Ad2- AND SV40-TRANSFORMED HAMSTER CELLS

TGFβ has been shown to inhibit the mitogenic response of a number of cell types (20, 22, 30, 40, 41), and MI appears to be about the same size as TGFβ. It seemed possible that MI might in fact be a hamster TGFβ. We therefore compared MI with TGFβ from porcine platelets (purchased from R&D Systems, Inc.). In Fig. 4 we show the mitogenic response of AKR 2B cells and NRK cells in the presence of MI and porcine TGFβ. It is evident that, while MI is inhibitory to both indicator cells, only AKR 2B cells are sensitive to the antimitogenic activity of TGFβ. We also compared the stability of MI and TGFβ in PBS heated to 56°C for 30 min. Unlike TGFβ, MI loses 40–50% of its inhibitory activity on heating. Although MI is not a purified protein, it appears to differ from TGFβ in two important respects: heat lability and target specificity for antimitogenic activity.

Role of Ad2- and SV40-TGFs in Oncogenicity. Together, these data suggest that the TGFs secreted by both Ad2- and SV40-transformed cells could act in an autocrine fashion to stimulate the proliferation of these cells in vivo. The antimitogen, MI, secreted by the SV40-transformed cells, may act to regulate the growth of these cells in vitro, but in vivo MI may act to modulate the growth of host cells as well (32). For example, if MI acted to inhibit the proliferation of immune effector cells, it could interfere with the mobilization of these cells at the site of tumor cell proliferation. Not only must transformed cells be able to proliferate in vivo, but they also must be able to resist the host’s immune defenses if they are to develop into tumors. Given the fact that SV40-transformed cells are highly tumorigenic in immunocompetent syngeneic and allogeneic hamsters and Ad2-transformed cells are not, it is possible that the antimitogen secreted by SV40-transformed cells could play an important role in determining the tumorigenicity of these cells.

To approach this question we have tested the effect of our CDMs on con A-stimulated spleen lymphocytes. The results of these experiments are shown in Table 4, in which it can be seen that the response of con A-stimulated lymphocytes to the five CDMs is much the same as that of fibroblasts (Fig. 2); Ad2-CDMs stimulate thymidine uptake and SV40-CDMs inhibit that uptake. This finding supports the notion that the mitogenic inhibitor secreted by SV40-transformed cells may contribute to the tumorigenic potential of these cells by inhibiting mobilization of immune effector cells in vivo.

**DISCUSSION**

Ad2- and SV40-transformed hamster embryo cells differ markedly in a number of phenotypic properties. The most distinguishing characteristic of these two transformed phenotypes lies in their potential for inducing tumors in hamsters. Both are highly oncogenic in newborn hamsters; however, SV40-transformed cells, but not Ad2-transformed cells, readily induce tumors in immunocompetent syngeneic and allogeneic hamsters (4).

Ad2-transformed SHE cells appear to be highly undifferentiated and accumulate much less fibronectin compared to the untransformed cells from which they were derived. In contrast,
SV40-transformed cells closely resemble untransformed SHE cells in cytomorphology and fibronectin content; they appear to be highly differentiated (3). Thus, it is somewhat paradoxical that the SV40-transformed cells are the more oncogenic.

If any transformed cell is to develop into a tumor in immunocompetent animals, it must, of course, withstand recognition and destruction by immune effector cells. As discussed in the "Introduction," the immunological reasons for the differences in the oncogenic potentials of Ad2- and SV40-transformed phenotypes remain elusive. However, recent studies with TGFs indicate that these factors play an important role in determining the phenotypic characteristics of transformed cells (19, 21). With this in mind, we have examined the ability of media conditioned by Ad2- and SV40-transformed SHE cells to modulate thymidine uptake in a number of cell types with the objective of determining whether TGFs might influence some of the disparate phenotypic properties of these transformed cells. In the work reported here, we show that both transformed phenotypes secrete very similar mitogenic factors. Our results also show that SV40-transformed cells, but not cells transformed by the "nononcogenic" adenovirus serotypes 2 and 5, secrete a powerful antimitogen.

We have compared the mitogenic activity of CDMs from three cell lines independently transformed by Ad2. The response of untransformed primary SHE cells to these three CDMs is qualitatively similar but differs quantitatively. Since the cell densities of the conditioning cultures were very similar, it is likely that the quantitative difference in these CDMs arises from phenotypic differences in Ad2-transformed cells. The mitogenic response induced in indicator cells by CDMs prepared from both types of transformed cells is probably due, in part, to the TGF-α-like factors which are present in both CDMs (Fig. 3). However, both CDMs also appear to contain mitogenic factors which do not compete with EGF.

In addition to factors that stimulate quiescent cells to incorporate thymidine, Ad2-CDMs (and possibly SV40-CDMs) appear to contain factors which enhance thymidine incorporation by serum-stimulated SHE cells. We also found that Ad2-CDM is 2-3 times more mitogenic for NRK cells than it is for SHE cells (data not shown). However, the synergistic effect of Ad2-CDM with serum mitogens is much reduced in NRK cells compared to SHE cells. The mechanism of the synergistic response of SHE cells to Ad2-CDM and serum mitogens is not clear. It apparently does not result from inducing a competence state in the indicator cells, since pretreatment of SHE cells with Ad2-CDM does not enhance the response of these cells to serum mitogens in the absence of Ad2-CDM.

In contrast to the mitogenic action of Ad2-CDM, SV40-CDM strongly inhibits thymidine uptake in SHE indicator cells stimulated by serum mitogens. This inhibitory response is also reversible. SHE cells treated with SV40-CDMs for as long as 48 h respond just as readily to serum mitogens as do SHE cells incubated for the same period in DM alone or DM with 1% FBS. Thus, the inhibitory action of SV40-CDM is cytostatic rather than cytolytic.

HPLC fractionation of SV40-CDM has revealed that the inhibitory response is mediated by a factor (MI, Fig. 3) that elutes with a molecular weight of 24,000. In molecular weight and inhibitory activity, MI appears to be similar to TGFβ (GI), a well-characterized growth regulatory peptide which exerts a strong inhibitory action on the proliferation of many cell types in monolayer culture (20, 22, 30, 34). Although HPLC-purified MI (Fig. 3) is not homogeneous by sodium dodecyl sulfate gel electrophoresis, it appears to differ from TGFβ in heat sensitivity and target-cell specificity for mitogenic inhibition (Fig. 4). A more precise characterization of MI will require purification to homogeneity in sufficient quantity to permit a complete chemical, immunological, and functional analysis.

The association of SV40 transformation with the MI seems unequivocal, given that these data are based on studies of five independently derived transformed cell lines, three clones of two of these lines, and two additional clones of a sixth transformant. Are differences seen between the adenovirus and SV40 transformants due to the differences between the viruses or the cells that have been transformed, i.e., do the two viruses transform two different cell types with two different phenotypes, or do the two viruses transform the same cell type but induce alternative phenotypes by differently affecting the expression of cellular genes? There is considerable evidence to suggest that a particular transformed phenotype results from differential activation and deactivation of cellular genes by different viral gene regulators within the same cell. In earlier work (3, 10, 38) we showed that hybrid cells formed by fusing SV40-transformed hamster cells with Ad2-transformed hamster cells have an Ad2 phenotype if they express Ad2 antigens, even though they also express SV40 antigens (S*A+); if they express only SV40 antigens (S*A-), the hybrid cells retain the SV40 phenotype. This observation indicates that expression of the Ad2 transforming genes suppresses the SV40 phenotype in hybrid cells. Thus, these results suggest that the two viruses may transform the same cell type; but by differentially regulating cell genes, they induce alternative phenotypes in this same cell, and the dramatic phenotypic differences between Ad2- (or Ad5-) and SV40-transformed cells are due to differences in the cellular genes which are activated or deactivated by the viral gene regulators.

We now conclude that one of these phenotypic differences is that the MI gene, which is not expressed in untransformed hamster cells, is activated by SV40 gene regulators and expressed by Ad2 gene regulators.

Neither the mechanism of the inhibitory action of MI nor its role as a growth regulatory factor is entirely clear. However, since it appears to be secreted at a greater rate as the SV40-transformed cells approach confluence (Fig. 1), it is possible that the function of MI is to modulate cell growth as the culture medium is depleted of nutrients. Although the precise role of MI as a growth regulator for SV40-transformed cells is not clear, it is reasonable to ask whether it might contribute to the phenotypic properties of the cells that secrete it. The most important difference between Ad2- and SV40-transformed SHE cells is the ability of the latter to develop into tumors in immunocompetent hamsters. It is possible that MI, like TGFβ (GI), acts as a differentiating agent that maintains SV40-transformed cells, which closely resemble untransformed cells, in a partially differentiated state. Thus, SV40-transformed cells, while immortalized, might, like normal untransformed cells, resist the cytotoxic action of the immune effector cells of the host.

It is also possible that MI inhibits proliferation of the immune effector cells of the host. It is known that TGFβ (GI) inhibits both T- and B-lymphocyte proliferation, and it appears to inhibit the cytolytic action of NK cells in vitro (34, 42, 43). If immune effector cells cannot proliferate, their mobilization at the site of tumor growth would be greatly impaired. To approach the answer to this question, we have tested the effect of Ad2- and SV40-CDMs on con A-stimulated hamster spleen lymphocytes based on the finding that the mitogenic action of con A is specific for T lymphocytes (44). We found that thymidine uptake by spleen lymphocytes, like that of SHE cells, is usually enhanced by Ad2-CDMs and is always strongly inhibited by SV40-CDMs. This result suggests that the MI secreted by SV40-transformed cells could contribute to the ability of
these cells to develop tumors by inhibiting proliferation of immune effector cells.

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Mitogenic and Antimitogenic Transforming Growth Factors Secreted by Adenovirus 2- and Simian Virus 40-transformed Hamster Cells: Possible Roles in Promoting Tumorigenesis

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