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26. We thank M. Byrne and A. Fox for technical assistance, K. Stromberg for the pathology studies on the tumor tissues, and A. Frankel and E. Scolnick for the sarcoma-specific DNA probes. This work was supported in part by the Virus Cancer Program of the National Cancer Institute.

8 May 1978

Growth Inhibition of Transformed Cells with Succinylated Concanavalin A

Abstract. *Succinylated concanavalin A reversibly inhibits the growth of SV40 transformed mouse 3T3 cells and thus causes an accumulation of the cells in the G_1 phase of the cell cycle. In a soft substrate (methylcellulose) succinylated concanavalin A also restores in transformed cells the growth behavior typical of untransformed cells.*

Morphological, biochemical, and functional changes of the cell surface are associated with neoplastic transformation both in vivo and in vitro. Lectins, multivalent proteins with carbohydrate specificities, have been used to probe and perturb the surfaces of a wide variety of cell types (*1*). A preferential agglutinability with lectins was one of the early indications that surfaces of transformed cells differed from those of the untransformed parent cells (*2*, *3*). With the aim of altering and possibly readjusting the cell surface architecture, we have grown cells in the presence of succinylated concanavalin A (succinyl-Con A) a nontoxic, nonagglutinating derivative of the jack bean lectin concanavalin A (Con A).

It was reported (*4*) that succinyl-Con A reversibly inhibits the growth of untransformed mouse fibroblasts (3T3) through an interaction with cells in the mitotic and early G_1 phases of the cell cycle. During these phases, many untransformed cells assume several of the cell surface characteristics of their transformed derivatives (*5*, *6*). We report here that succinyl-Con A reversibly inhibits the growth of SV40 virus transformed 3T3 mouse cells and thus causes an accumulation of cells in the G_1 phase, thereby inducing a growth phenotype similar to that of the untransformed parent cell.

When grown on a solid substratum in Dulbecco's modified Eagle's medium containing either low (2 percent) or high (10 percent) calf serum concentrations, SV40 3T3 cells characteristically grow to high cell densities until nutrient depletion or medium acidity, or both, result in cessation of growth followed by cell death. In the presence of succinyl-Con A, however, SV40 3T3 cells stop growing at low cell densities and maintain their density for several days (Fig. 1A).

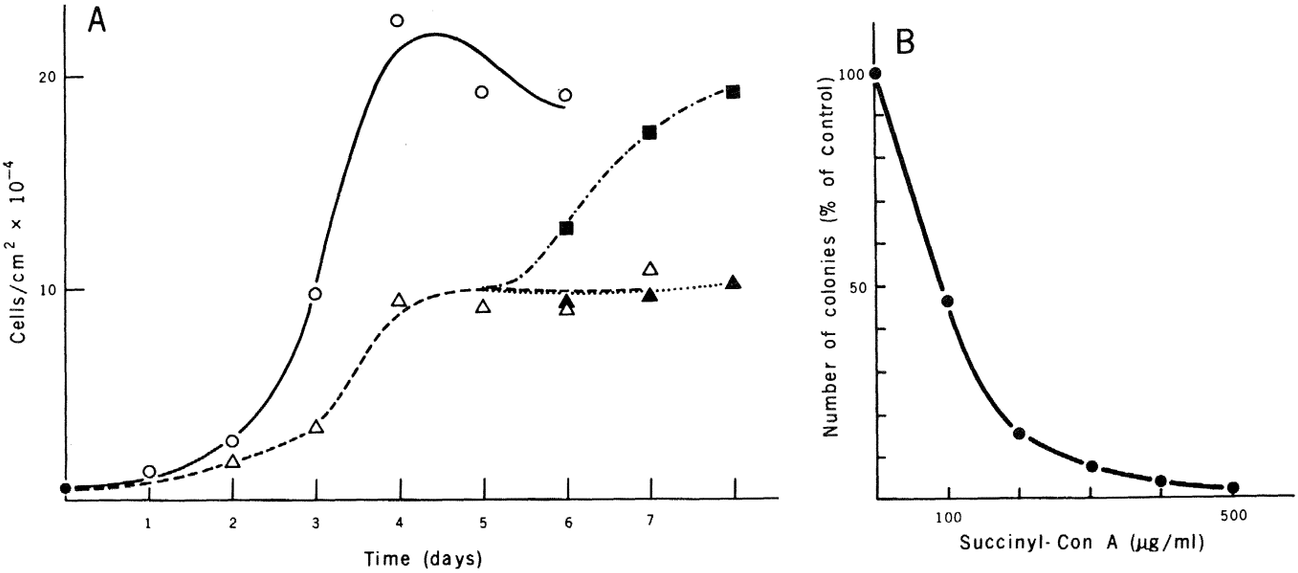

Fig. 1. (A) Inhibition of growth on plastic to high cell densities. The SV40 3T3 cells (6×10^4) were plated into 35-mm Falcon plastic dishes containing 2 ml of Dulbecco's modified Eagle's medium (DME) (4.5 g per liter of glucose) plus 10 percent calf serum (CS). After 24 hours the medium was changed to DME + 2 percent CS with (△) or without (○) 400 μg of succinyl-Con A per milliliter. To keep the inhibited cultures viable for up to 10 days the medium was changed to DME plus 0.1 percent CS with 400 μg of succinyl-Con A per milliliter on day 5 (▲). The cells were released from growth inhibition by changing the medium to DME plus 0.1 percent CS with 10 m*M* α-methyl-D-mannoside on day 5 (■). Cells were harvested with a buffered trypsin solution and counted in a Coulter counter. (B) Inhibition of anchorage-independent growth. The cells were subcultured into DME plus 10 percent CS, 1.2 percent methylcellulose (4000 count/sec, Dow Chemical), and succinyl-Con A as indicated over a 4-ml layer of DME plus 10 percent CS, succinyl-Con A, and 0.9 percent agar in a 6-cm Falcon plastic tissue culture dish; 1 and 2 weeks after subculturing, 4 ml of fresh DME containing 10 percent CS and 1.2 percent methylcellulose were added to each dish; 3 weeks after subculturing the colonies were counted; each point is the total of four dishes; 100 percent represents 2590 colonies.

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The cell density at which growth ceases is dependent upon the concentration of succinyl-Con A present in the medium—the higher the lectin concentration the lower the final density.

The stable, low density maintained by cells in the presence of succinyl-Con A is not due to an equilibrium between growing and dying cells because (i) cell populations that have reached a constant density also show a low rate of DNA synthesis, similar to that of density inhibited untransformed 3T3 cells; (ii) time-lapse studies of a quiescent succinyl-Con A–treated culture of SV40 3T3 cells demonstrated a reduction in cell division to the level of confluent 3T3 cells; (iii) SV40 3T3 cells plated at different initial densities, but treated with the same concentration of succinyl-Con A, grow to about the same final density. This then rules out a time-dependent toxic effect and suggests that cell density as well as succinyl-Con A concentration, and not time of exposure to succinyl-Con A, regulate cell growth. Furthermore, the growth-inhibitory effect of succinyl-Con A can not only be prevented but can also be reversed by the addition of α-methyl-D-mannoside, a Con A–specific hapten sugar (Fig. 1A).

Among the growth characteristics commonly associated with transformation, anchorage-independent growth—that is, growth in the absence of a solid surface (as, for example, in soft agar)—has been suggested to be well correlated with in vivo tumorigenicity of tissue culture cells (*7*). Figure 1B demonstrates that succinyl-Con A also inhibits anchorage-independent growth. The growth inhibitory effect of succinyl-Con A can be overcome to 50 percent by the addition of α-methyl-D-mannoside at a concentration corresponding to the dissociation constant of succinyl-Con A for this sugar hapten (2 m*M*).

Untransformed 3T3 fibroblasts accumulate in G_1 when arrested either by high cell density or nutrient starvation (*8*). The SV40 transformed cells, however, show only a slight accumulation in G_1 once they reach their peak density when deprived of nutrients. It was of interest, therefore, to examine the cell cycle distribution of SV40 3T3 cells whose proliferation had been inhibited by succinyl-Con A. A logarithmically growing SV40 3T3 cell culture has more cells in the S and G_2 phases combined than in the G_1 phase (Fig. 2A). Succinyl-Con A–inhibited SV40 3T3 cells accumulate in the G_1 phase (Fig. 2B), thus exhibiting a cell cycle pattern more similar to that of density-inhibited 3T3 cell monolayers (Fig. 2C).

Trowbridge and Hilborn (*9*) reported that succinyl-Con A did not cause inhibition to any appreciable extent of the growth of SV40 3T3 cells in the presence of 10 percent serum. For transformed fibroblasts such serum concentrations are in the supersaturated range for both growth rate and final cell density, and inhibitory effects with most agents can hardly be seen.

Therefore, we used 2 percent calf serum and consistently found that cells grown in the presence of succinyl-Con A were inhibited, whereas control cells still grew to high densities. This observation is in agreement with earlier work where a nonagglutinating Con A preparation obtained by trypsin digestion (*10*) was shown to inhibit the growth of polyoma-transformed 3T3 cells in conditioned medium (medium that had been used previously for growing cells and was therefore partially depleted of growth-stimulating factors). These data, as well as recent studies of the effect of succinyl-Con A on the growth of chick embryo fibroblasts and baby hamster kidney cells (*11*), indicate that the serum and the succinyl-Con A concentration must be determined for each individual cell line.

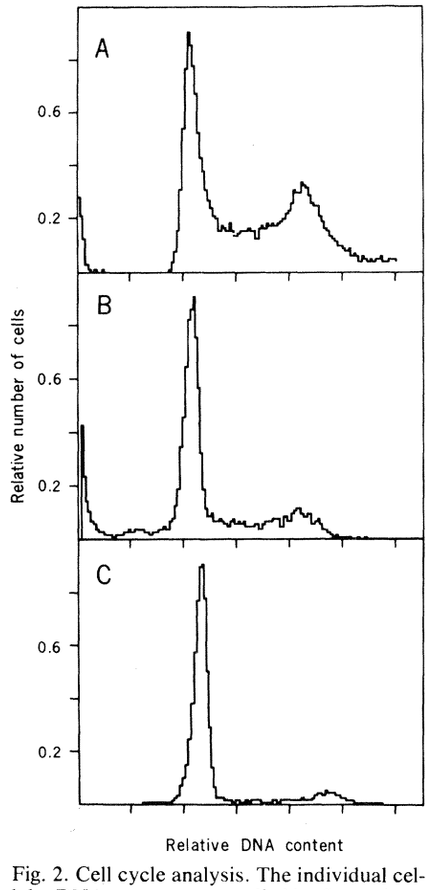

Fig. 2. Cell cycle analysis. The individual cellular DNA content was studied by flow-microfluorometric methods in an impulse cytophotometer. The SV40 3T3 cells (5×10^5) were plated into 10-cm Falcon dishes containing 15 ml of DME plus 10 percent CS. After 24 hours the medium was changed to DME plus 2 percent CS with (B) or without (A) 400 μg of succinyl-Con A per milliliter. Cells were harvested on day 5 with trypsin and EDTA, fixed in ethanol, and prepared for impulse cytophotometry with ethidium bromide (*17*). The stained nuclei were measured in a Phywe ICP 11 impulse cytophotometer. In (C), 3T3 cells were grown to monolayers and prepared and analyzed as for (A) and (B).

It is a general characteristic of transformed cells that they are resistant to growth inhibition. Although some transformed cell lines have been synchronized (*12–14*) the techniques normally used to inhibit and synchronize the growth of untransformed cells cause transformed cells to stop randomly (*15*) throughout the cell cycle and die thereafter. Succinyl-Con A stops and maintains transformed 3T3 cells in a nonproliferating state. This results in an accumulation of cells in the G_1 phase of the cell cycle as is generally observed for untransformed 3T3 cells at high densities.

Pardee (*16*) has proposed that a "restriction point" mechanism exists that regulates the reentry of resting untransformed cells into the cell cycle. However, SV40 virus-transformed 3T3 cells respond poorly to restricting conditions such as low serum concentration, nutrient deprivation, or high cell densities. Succinyl-Con A treatment of such cells seems to reestablish some aspects of "restriction point" growth control, similar to that observed in untransformed cells.

Studies with time-lapse cinemicroscopy have indicated that succinyl-Con A induces an increased intercellular adhesion between daughter cells completing mitosis or cells that come into contact while migrating about the substratum. This succinyl-Con A–induced increase in cell-cell adhesion may be functionally related to growth inhibition. Alternatively, succinyl-Con A may exert its inhibition by directly binding to serum growth factors. We ruled out the latter possibility for SV40 transformed 3T3 cells by using a series of different serum concentrations at a constant succinyl-Con A concentration. The results we obtained were similar to those discussed for 3T3 cells (*4*). Investigations of whether binding and uptake of serum growth factors by the cell surface is inhibited by succinyl-Con A might provide some additional insights into membrane-mediated growth control processes in general.

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18. Supported by the Swiss National Foundation, grant 3.720-76.

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21 February 1978; revised 10 May 1978

Cytidine 3′,5′-Monophosphate Phosphodiesterase: Decreased Activity in the Regenerating and Developing Liver

Abstract. *A decrease in the activity of the enzyme cytidine 3′,5′-monophosphate (cyclic CMP) phosphodiesterase was noted in the regenerating liver of young rats as early as 8 hours after partial hepatectomy, with a maximum decrease occurring 12 hours after the surgery. In comparison, in old rats which showed a slower liver growth, the maximum decrease in the activity of cyclic CMP phosphodiesterase was smaller and occurred at a much later time (2 days after surgery). A similar decrease in the enzyme activity was observed in the fetal liver of guinea pigs. These findings suggest that regulation of tissue concentration of cyclic CMP may be crucial for the regeneration and development of the liver.*

Experimental evidence, although often contradictory, has linked adenosine 3′,5′-monophosphate (cyclic AMP) and guanosine 3′,5′-monophosphate (cyclic GMP) to liver regeneration in rats subjected to partial hepatectomy (*1*, *2*). However, many of the pathophysiologic processes of tissues, including regeneration of the liver, cannot be satisfactorily explained solely on the basis of the presumed actions of these two purine cyclic nucleotides. It has been suspected that other cyclic nucleotides may serve to mediate biological processes that are different from or complementary to those mediated by cyclic AMP and cyclic GMP. Recently, Bloch and co-workers (*3*) identified cytidine 3′,5′-monophosphate (cyclic CMP), a pyrimidine cyclic nucleotide, from leukemia L-1210 cells, and demonstrated that addition of exogenous cyclic CMP, but not cyclic AMP, cyclic GMP, or uridine 3′,5′-monophosphate (cyclic UMP), stimulates the growth of the leukemic cells in culture. These workers also reported that L-1210 cells and regenerating liver have increased concentrations of cyclic CMP compared to their respective controls. Subsequently, Cech and Ignarro (*4*) reported the existence of cytidylate cyclase, the enzyme that catalyzes the formation of cyclic CMP from cytidine triphosphate (CTP), and showed that its activity is higher in regenerating liver and myeloid leukemic tumors than in normal tissue.

We have reported (*5*) the occurrence of cyclic CMP phosphodiesterase in all of many rat tissues examined and have noted that its activity is decreased in tissues undergoing rapid cell proliferation and growth (*5*). In the experiments described herein we compared the changes in the activity of cyclic CMP phosphodiesterase in regenerating and developing liver with changes in the activities of the phosphodiesterases (E.C. 3.1.4) of cyclic AMP and cyclic GMP.

In young male rats (weighing 120 to 150 g) we noted that the activity of cyclic CMP phosphodiesterase decreased as early as 8 hours after partial hepatectomy (Fig. 1) in which about 75 percent of the total liver mass was removed (*6*). The enzyme, shown to be the species with a high Michaelis constant (K_m) (in the millimolar range of substrate), was assayed with both 1 μM and 1 mM concentrations of cyclic CMP in the presence of 10 mM Fe^{2+} (*5*, *7*). The activity was lowest 12 hours after the operation, then gradually recovered thereafter but remained depressed during the entire experimental period; at day 5, when the liver had grown back to nearly its original weight, the enzyme activity was still about 20 percent lower than in the control rats that received sham operations. Increased cytidylate cyclase activity was reported earlier in regenerating liver by Cech and Ignarro (*4*). This, coupled with the depressed cyclic CMP phosphodiesterase activity found in the present study, may account for the higher cyclic CMP content in regenerating liver shown by Bloch (*3*).

In the same animals we also studied changes in the activities of the phosphodiesterases of cyclic AMP and cyclic GMP. Both enzymes were shown to be predominantly the low K_m species (in the micromolar range of substrate). For these assays we used a 1 μM concentration of the respective cyclic nucleotides as substrates in the presence of 20 mM Mg^{2+} (*8*, *9*). Although the changes in activities of these enzymes were less pronounced than the changes that occurred in cyclic CMP phosphodiesterase, their activities were similarly depressed during the earlier phase of the liver regeneration (Fig. 1). However, their activities returned to the control values during days 2 and 3 after partial hepatectomy, at which time the liver was still undergoing rapid growth. We also noted the patterns of changes in cyclic AMP and cyclic GMP phosphodiesterase activity using 1 mM concentrations of substrate. These data (not shown) were qualitatively similar to those obtained with the 1 μM substrate concentrations. Thus decreased hydrolytic destruction of cyclic AMP and cyclic GMP may also be crucial in the earlier phase of liver regeneration, a con-

Table 1. Comparison of the activities of phosphodiesterases for cyclic CMP, cyclic AMP, and cyclic GMP in the liver of the guinea pig fetus (20 days before birth) and adult (over 200 days old). The enzyme activities in the whole liver homogenates were assayed with 1 μM and 1 mM concentrations of the individual cyclic nucleotides as substrates as indicated. Activities are expressed as nanomoles of substrate hydrolyzed per minute per gram of tissue, and the data are presented as means ± standard errors of the means from five fetuses and three adults.

Developmental stage	Phosphodiesterase activity assayed with					
	Cyclic CMP		Cyclic AMP		Cyclic GMP	
	1 μM	1 mM	1 μM	1 mM	1 μM	1 mM
Fetus	0.14 ± 0.01*	90 ± 6*	9.1 ± 0.6†	235 ± 43	9.3 ± 0.9	504 ± 46†
Adult	0.25 ± 0.02	127 ± 6	12.1 ± 0.9	298 ± 43	9.9 ± 1.4	744 ± 90

* $P < .005$. † $P < .05$.

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