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Effect of Dibutyryl Cyclic AMP and Serum Deprivation on the Membranes of Transformed Mouse Fibroblasts. A Freeze Fracture Study (38304)

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Mammalian cells transformed by oncogenic viruses cause tumors in syngeneic animals (1) and show loss of contact inhibition in tissue culture (2). The loss of growth control associated with malignant transformation is thought to result at least in part from changes in cell membrane structure and function. This may be associated with the presence of abnormal membrane glycolipids (3, 4) and glycoproteins (5–7) as well as with changes in the activity of certain membrane enzymes (8, 9).

We have also recently demonstrated that the membranes of contact inhibited cells differ from virally transformed cells in their ultrastructural freeze fracture morphology (10). Transformed 3T3 cells demonstrated randomly distributed intramembranous particles (IMP), which are thought to represent intrinsic membrane proteins (11, 12) whereas contact inhibited 3T3 fibroblasts show cell contact associated aggregation of intramembranous particles.

In view of recent studies which have demonstrated that treatment of transformed cells with exogenous cyclic 3′-5′ adenosine monophosphate (cAMP) or serum deprivation can induce "reverse transformation" in cellular morphology, concanavalin A agglutinability and growth characteristics, (13–16) we have employed freeze fracture and electron microscopy to determine whether these treatments can restore the membrane ultrastructure of transformed cells to that observed in contact inhibited cells.

BALB/c 3T3 fibroblasts (clone A31) (3T3), Simian Virus 40 transformed BALB/c 3T3 fibroblasts (clone A31, subclone 6) (SV3T3), SV40 transformed BALB/c 3T3 fibroblasts (SVT2) and Polyoma transformed BALB/c 3T3 fibroblasts (PY3T3) (gifts of Dr. Todaro and Dr. Aaronson) were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% calf serum, penicillin (100 IU/ml) and streptomycin (10 mg/ml). Cells were incubated at 37° in a humidified atmosphere containing 10% CO_2. Contact inhibited cell lines were passed routinely at a cell density of 1×10^4 cells/cm^2 to avoid spontaneous transformation whereas transformed cell lines were allowed to grow to a higher density before passage.

In experiments to examine the effect of exogenous dibutyryl cAMP ((But)$_2$cAMP) (Boehringer Laboratories), theophylline (Sigma) or serum deprivation (Colorado Serum Co.) on cell growth and membrane morphology, cells were grown in standard media for 24 hr after passage. Medium containing either (But)$_2$cAMP with or without theophylline, or 0.1% calf serum was then added and cell growth was monitored by cell counts in a hemocytometer. Cell viability was routinely tested by Trypan blue dye exclusion. Specimens for freeze fracture studies were stepwise glycerinated *in situ* with 10% glycerol-phosphate buffered saline (PBS) for 10 min followed by 20% glycerol-PBS for 20 min at room temperature (10). Thereafter, cells were removed by scraping with a rubber policeman and then centrifuged at 60*g* for 10 min at room temperature. Cell pellets were then placed on planchets and rapidly frozen in Freon 22 cooled by liquid nitrogen. Representative samples were also fixed *in situ* in either 1.0% gluteraldehyde-PBS for 1 min or 0.1% gluteraldehyde-PBS for 5 min at room temperature followed by treatment with 10% glycerol-PBS for 10 min and 20% glycerol-PBS for 20

min. The results described in this paper for control and experimental samples were comparable for both preparation schemes. Freeze fracture was performed in a Balzars BAE 300 freeze etch microtome at $-100°$, 10^{-6} Torr vacuum. Platinum-carbon replicas were cast, washed and examined in a Phillips EM 300 electron microscope (10, 11).

The percent of fracture faces showing aggregation of intramembranous particles was calculated by examination of approximately equal numbers of inner and outer fracture faces of at least 40 individual cells. Cells were designated to show IMP aggregation when the majority of individual fracture faces showed clusters of IMP containing greater than 10 particles surrounded by significant surface areas devoid of such particles (10).

The growth of SV3T3 or PY3T3 fibroblasts in (But)$_2$cAMP, theophylline (Fig. 1), or 0.1% calf serum (Fig. 2) resulted in variable inhibition of cell growth. These results verify previous reports that PY3T3 fibroblasts are more responsive to (But)$_2$cAMP growth inhibition than SV3T3 fibroblasts (13). However, Fig. 2 illustrates that

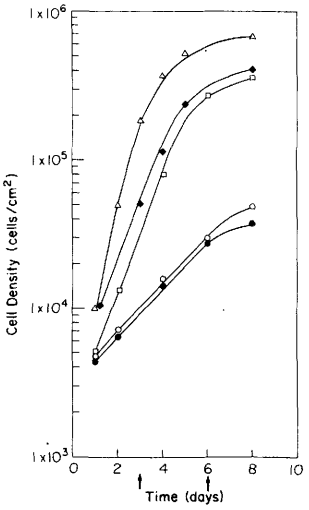

FIG. 1. The effect of (But)$_2$cAMP and theophylline on growth of virally-transformed 3T3 fibroblasts. Following passage cells were planted in 10% calf serum without additives for 24 hr to facilitate cell attachment, after which medium containing (But)$_2$cAMP plus or minus theophylline was added. Media were subsequently changed at 3-day intervals (↑). (Δ—Δ) SV3T3; (◆—◆) SV3T3 plus 1.0 m*M* (But)$_2$cAMP and 1.0 m*M* theophylline; (□ — □) PY3T3; (○ — ○) PY3T3 plus 1.0 m*M* (But)$_2$cAMP and 1.0 m*M* theophylline; and (● — ●) contact inhibited 3T3 controls.

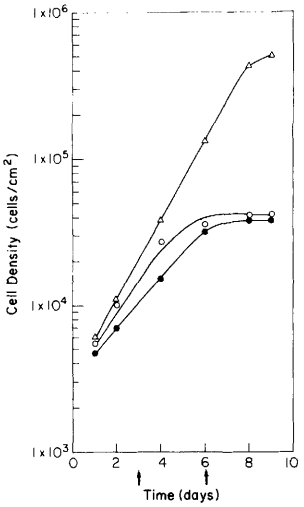

FIG. 2. The effect of serum deprivation on growth of SV40 transformed 3T3 fibroblasts. Following passage cells were planted in 10% calf serum for 24 hr to facilitate cell attachment, after which media containing 0.1% calf serum were added. Media were subsequently changed at 3-day intervals (↑). (Δ—Δ) SV3T3 control grown in medium containing 10% calf serum; (○ — ○) SV3T3 grown in 0.1% calf serum; and (● — ●) contact inhibited 3T3 controls grown in medium containing 10% calf serum.

SV3T3 are sensitive to growth inhibition by serum deprivation (17).

Table I correlates the growth and morphological characteristics of normal and transformed cells with their plasma membrane freeze fracture ultrastructure. Contact inhibited 3T3 cells grew to a saturation density of 4×10^4 cells/cm^2, showed an epithelial morphology and demonstrated IMP aggregation on 85% of the fracture faces (Fig. 3). SV3T3 and PY3T3 cells grew to a saturation density of between 4 and 7×10^5 cells/cm^2, had a fibroblastic morphology and showed no significant IMP aggregation (Fig. 4). Treatment of SV3T3 cells with various concentrations of (But)$_2$cAMP with or without theophylline did not significantly inhibit cell growth and did not induce morphological reverse transformation or IMP aggregation. In contrast, treatment of PY3T3 cells with various concentrations of (But)$_2$cAMP plus theophylline or growth of SV3T3 cells in 0.1% serum inhibited cell growth to a saturation density of between 5 and 7×10^4 cells/cm^2. This inhibition of growth was associated with morphological reverse transformation but not with significant IMP aggregation.

TABLE I. Effect of $(But)_2cAMP$ and Serum on Transformed Mouse Fibroblasts.

Cell Type	Additives	Maximum percent of FF with aggregated IMP (±5%)	Saturation density (cells/cm²)[a]	Morphological reverse transformation	Number of experiments[b]
Contact inhibited controls					
3T3(A31)	—	85	4×10^4	NA	6
Transformed Controls					
SVT2	—	7.5	7×10^5	NA	2
SV3T3 (A31, subclone 6)	—	5	7×10^5	NA	2
PY3T3 (A31)	—	7.5	4×10^5	NA	2
Experimental					
SVT2 or SV3T3 (A31, subclone 6)	0.5 m*M* $(But)_2cAMP$	7.5	5.5×10^5	—	2
	0.1 m*M* $(But)_2cAMP$+ 0.1 m*M* theophylline	7.5	5.0×10^5	—	2
	1.0 m*M* $(But)_2cAMP$+ 1.0 m*M* theophylline	10	3.5×10^5	—	2
PY3T3 (A31)	0.5 m*M* $(But)_2cAMP$	10	7×10^4	+	1
	1.0 m*M* $(But)_2cAMP$+ 1.0 m*M* theophylline	10	5×10^4	+	2
SV3T3 (A31, subclone 6)	0.1% Calf serum	7.5	5×10^4	+	3

[a] All cells grown in 10% calf serum unless otherwise designated and were > 95% viable as determined by trypan blue dye exclusion. Viability of cell populations in these studies is essential since IMP aggregation can be observed in transformed cell populations at high cell densities where the percentage of nonviable cells is increased (30).

[b] Samples for freeze-fracture studies were routinely examined on Days 2, 4 and 8 after subculture or the addition of additives.

NA—not applicable.

The results of this study and studies on membrane transport (18) clearly show that elevation of cellular cAMP levels by $(But)_2cAMP$ (19) or serum deprivation (20, 21) does not restore normal membrane structure and function to transformed cells even though they do show reverse transformation of cell morphology, cell agglutination by concanavalin A and growth characteristics. The cell synchrony studies of Smets (22) also show a failure of $(But)_2cAMP$ to restore normal cell cycle kinetics to transformed cells.

Control of cell growth in normal contact inhibited cells seems to be regulated by a complex sequence of events probably initiated by cell-to-cell contact. Cell contact induces the aggregation of IMP (10) and preliminary studies suggest that cell contact also induces an ordering of cellular membrane lipids (Scott, Furcht & Barnett, unpublished observations). Subsequent to the onset of cell-to-cell contact it has been reported that the rate of 2-deoxyglucose transport decreases (23) and the level of cellular cAMP increases (24, 25). Furthermore, Goldberg *et al.* (26) have also shown a decrease in cellular cGMP levels prior to cessation of cellular proliferation. In contrast untreated transformed cells maintain fluid cellular membrane lipids (27), randomly distributed IMP (10), low cAMP levels (24) and high cGMP levels at all cell densities (26).

Based on these observations we have presented a hypothesis to explain how the activity of membrane enzymes, which are critical to the regulation of cell growth, may be modulated by changes in the organization of membrane pro-

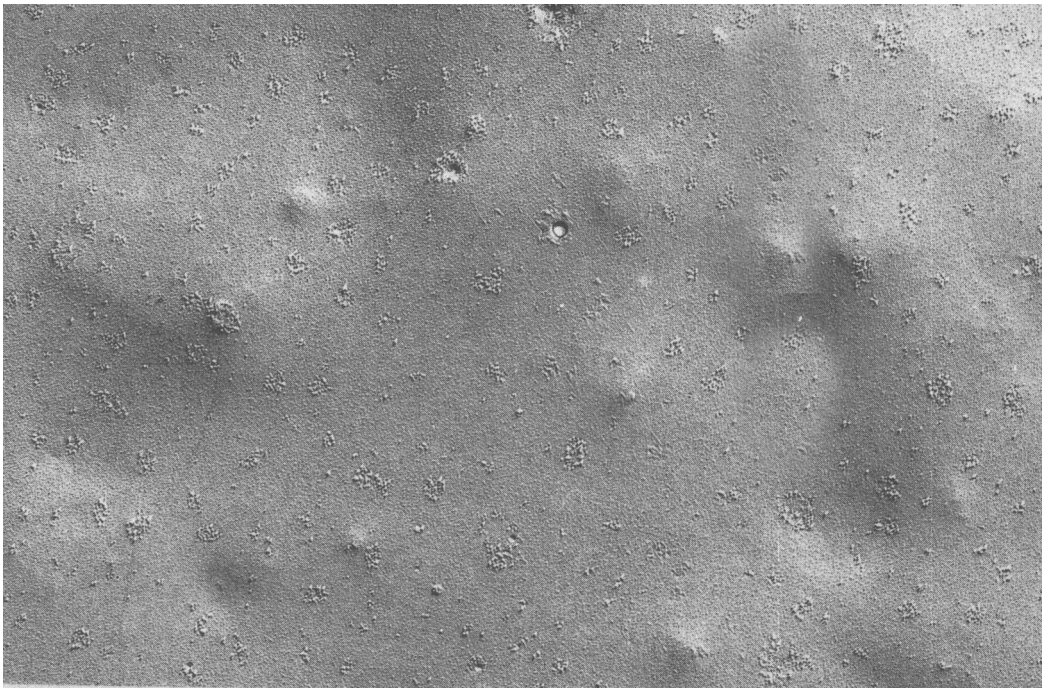

FIG. 3. Freeze fracture replica of the inner fracture face of a contact inhibited 3T3 fibroblast showing aggregated IMP (35,000×).

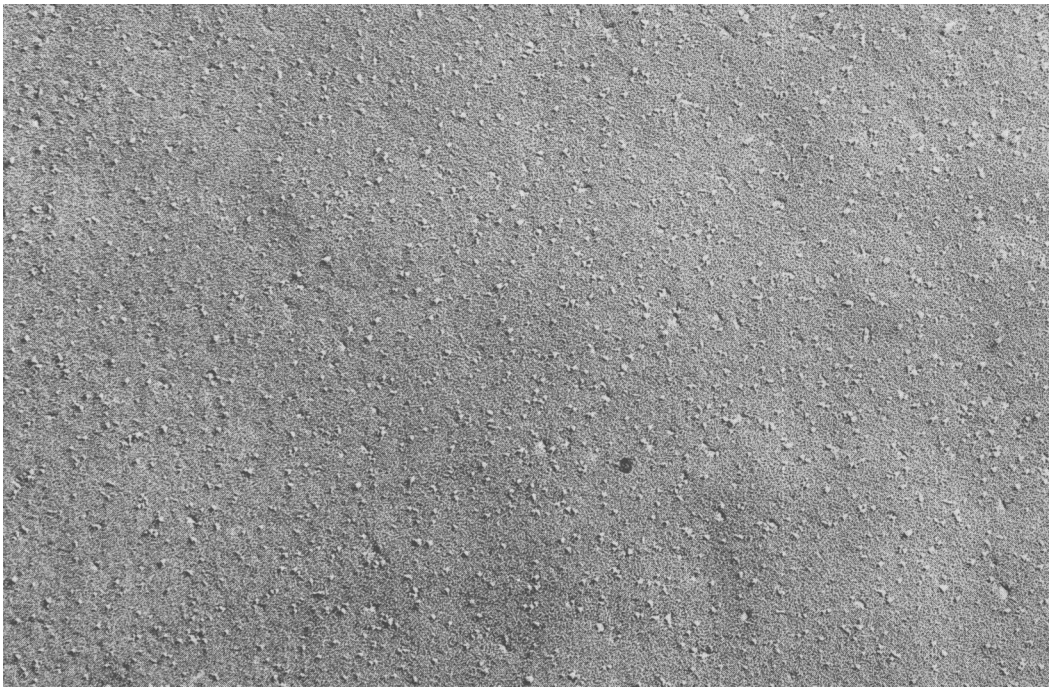

FIG. 4. Freeze fracture replica of the inner fracture face of an SV3T3 fibroblast showing randomly distributed IMP. An identical morphological pattern is observed in transformed fibroblasts treated with $(But)_2$cAMP or following serum deprivation (35,000×).

teins and lipids (27). This hypothesis predicts that cell-to-cell contact between normal cells should induce a change in membrane organization which eventually results in cessation of cell growth. It seems probable that the normal contact induced sequence of events that lead to the control of cell proliferation can be partially bypassed under certain conditions. Our studies suggest that (But)$_2$cAMP and serum deprivation may function in this manner. The observations that treatment with (But)$_2$cAMP or serum deprivation can only temporarily modulate cellular cAMP levels and cell growth rates (28) (Scott & Furcht, unpublished observations) in transformed cells suggest that such treatments do indeed only bypass a normal membrane associated growth control mechanism. We interpret the failure of serum deprivation and (But)$_2$cAMP treatment to restore the normal contact inhibited architecture to the plasma membranes of transformed cells to indicate that the organization and distribution of the membrane proteins and lipids are critical for the development and maintenance of the normal contact inhibited state.

Summary. Dibutyryl cyclic AMP inhibits growth of certain transformed mouse fibroblasts as does serum starvation. These treatments, however, fail to affect membrane structure as visualized by freeze fracture and electron microscopy. These observations suggest that such treatments do not restore the membranes of transformed cells to normal and that the organization and distribution of certain membrane proteins may be critical for the development and maintenance of the normal contact inhibited state.

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