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MODULATION OF THE DISTRIBUTION OF PLASMA MEMBRANE INTRAMEMBRANOUS PARTICLES IN CONTACT-INHIBITED AND TRANSFORMED CELLS

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SUMMARY

The intrinsic organization of the plasma membrane differs in normal and transformed cells. With the technique of freeze fracture and electron microscopy contact inhibited 3T3 cells have been shown to contain aggregated plasma membrane intramembranous particles, while transformed cells demonstrate a uniform particle distribution. The distribution of intramembranous particles in transformed cells can be affected by colchicine or vinblastine which induces a dose- and time-dependent particle aggregation. These observations suggest that microtubules and other membrane-associated colchicine-sensitive proteins probably influence the distribution of intrinsic membrane proteins and intramembranous particles in nucleated mammalian cells. An aggregated particle distribution has been observed in 3T3 cells or colchicine-treated transformed cells frozen in media, phosphate-buffered saline or following brief exposure to glycerol, sucrose or dimethyl sulfoxide containing solutions, independent of whether specimens were rapidly frozen from 37 °C, room temperature or 4 °C incubations. Cells briefly stabilized in 1 % formaldehyde yields similar patterns of particle distribution as cells rapidly frozen in media or in cryoprotectants. Glutaraldehyde fixation of cells, however, appears to alter the fracturing process in these cells, as visualized by an altered fracture face appearance, decreased numbers of particles, and no particle aggregates. Differences in membrane organization between normal and transformed cells have therefore been demonstrated using a series of preparative methods and colchicine and vinblastine have been shown to modulate intramembranous particle distribution in transformed 3T3 cells.

Cells transformed by oncogenic viruses demonstrate a loss of contact inhibition of cell proliferation [1], show decreased serum requirements for growth [2], increased rates of transport of sugars and nucleosides [3] and altered levels of intracellular cyclic nucleotides [4–6] compared to contact-inhibited cells. These characteristics together with the observations that transformed cells are more readily agglutinated by concanavalin A [7] and may have a decreased activity of adenylate cyclase [8],

a membrane-associated enzyme, suggest that viral transformation is associated with a number of functional alterations of the cell membrane. Ultrastructural confirmation of these observations has recently been presented. We demonstrated differences in the organization of the plasma membranes of transformed cells and contact-inhibited cells [9]. Plasma membrane intramembranous particles which have been shown to be associated with specific intrinsic membrane proteins in other cell systems, are aggregated in contact-inhibited cells, whereas they are randomly distributed in transformed cells [9].

The aggregation of intramembranous particles in contact-inhibited 3T3 cells has been shown to result from cell-to-cell contact [9] and particle disaggregation has been observed transiently during the mitotic phase of the cell cycle in synchronized populations of contact-inhibited cells [10].

Our initial observations have been confirmed by other investigators [11], however, it has been suggested that differential intramembranous particle aggregation may be induced by the cryoprotective agents, such as glycerol, which are used routinely to prepare cells for freeze fracture. This paper reports the results of studies to determine the effect of differences in cell preparation on the freeze fracture morphology of normal and transformed cells and the effect of vinblastine sulfate, colchicine and reduced temperature on the distribution of plasma membrane intramembranous particles.

Contact-inhibited 3T3 (clone A31) and SV40-transformed 3T3 cells (clone A31, subclone 6) (a gift of Dr George Todaro) were grown in Dulbecco's minimal essential media supplemented with 10 % calf serum, penicillin (100 I.U./ml) and streptomycin (10 mg/ml). Cultures were incubated in a humidified atmosphere containing 10 % CO_2. In all experiments, cell viability was assured by trypan blue dye exclusion before and after treatment with vinblastine, and cell cultures were shown to be free of mycoplasma contamination by transmission and scanning electron microscopy and by culture.

The saturation density of 3T3 and SV3T3 cells used in these studies was $4 \cdot 10^4$ cells $\cdot$ cm^{-2} and $4 \cdot 10^5$ cells $\cdot$ cm^{-2}, respectively. Individual cultures used to examine the effects of vinca alkaloids and various cell preparation schemes were allowed to grow to approx. 80 % confluence. The dose- and time-dependent effects of vinblastine sulfate, colchicine and reduced temperature were examined in cultures to which media alone as a control or media containing 10 % calf serum and drug concentrations of 10^{-7}–10^{-5} M and 10^{-9}–10^{-6}M, respectively, were added. The effect of reduced temperature (4 and 21 °C) was assayed in cultures of both normal and transformed cells in Dulbecco's minimal essential media, in 10 % glycerol or sucrose solutions at 4, 21 and 37 °C.

Control and experimental specimens were prepared for freeze fracture by the following methods: (1) in tissue culture media alone; (2) by stepwise glycerination of cells in situ in 10 % then 20 % glycerol phosphate-buffered saline for 15 min each; (3) by incubation of cells in 15 % glycerol or sucrose phosphate-buffered saline solutions at room temperature for between 5 min and 4 h; and (4) following incubation of cells in 15 % glycerol or sucrose solutions for 30 min at 4, 21 and 37 °C. Other specimens were fixed in: 1 % formaldehyde (or freshly prepared 1 % paraformaldehyde) or in 2.5 % glutaraldehyde phosphate-buffered saline for 10 min at room temperature or 37 °C prepared from either a stock 50 % biological grade glutaraldehyde (Electron Microscopy

Sciences) or from purified 70% glutaraldehyde (Ladd Co.). Thereafter, specimens were incubated with either 15% glycerol phosphate-buffered saline or media. Following these various procedures, cells were gently scraped from flasks with a rubber policeman and centrifuged at 60 $\times$ *g* for 5 min. Cell concentrates were then placed on gold freeze fracture planchets and rapidly frozen in Freon 22 cooled by liquid nitrogen. Specimens were then fractured at -100 °C, 10^{-6} Torr in a Balzars BAE 300 freeze-etch microtome. Platinum-carbon replicas were prepared, washed and examined in a Philips EM 300 electron microscope at 80 kV.

The percentage of fracture faces showing aggregated intramembranous particles was determined by counting approximately equal numbers of inner and outer fracture faces on at least 50 individual cells. Membranes were recorded to have aggregated particles when a significant portion of individual fracture faces contained clusters of $>$ 10 particles surrounded by particles-free surfaces [9, 10].

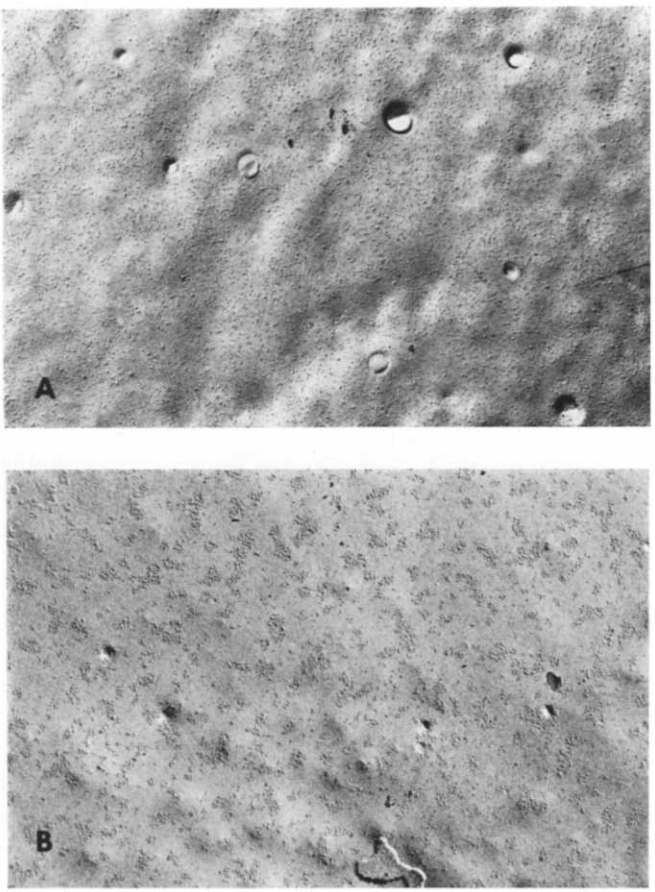

Fig. 1. Freeze fracture replica of a Balb/c SV_{40} transformed 3T3 cell (A) demonstrating a random distribution of intramembranous particles. Following treatment of SV3T3 cells with 10^{-6} M colchicine for 4 h, large aggregates of intramembranous particles are visualized on the plasma membrane fracture faces (B), (magnification 30 000 $\times$).

Control transformed cells demonstrate a random distribution of plasma membrane intramembranous particles (Fig. 1A) whereas vinblastine- or colchicine-treated transformed cells demonstrate aggregates of particles (Fig. 1B). Such particle aggregation was dose and time dependent (Fig. 2). Following addition of vinblastine there was an initial lag phase of 60–90 min, during which intramembranous particles remained randomly distributed. Between 2 and 3 h, aggregation of intramembranous particles developed and reached a maximum of 80% for 10^{-5} M and 60% for 10^{-7} M at 3–6 h, while controls demonstrated aggregates on less than 10% of fracture faces. Colchicine produced a similar effect with particle aggregation apparent on 90% of the fracture faces for 10^{-6} M and 60% for 10^{-9} M colchicine at 3–6 h.

We further observed that contact-inhibited cells and vinblastine-treated transformed cells have aggregated intramembranous particles and untreated transformed cells have random particles when cells were prepared by rapid freezing in: (1) tissue culture media alone, (2) phosphate-buffered saline alone, and (3) 10 or 20% sucrose or glycerol phosphate-buffered saline solutions for as short as 5 min or as long as 4 h (Table I). In all cases specimens of contact-inhibited cells or colchicine-treated transformed cells frozen in media or fixed in formaldehyde showed less pronounced particle aggregation than with other preparative methods. The aggregation was, however,

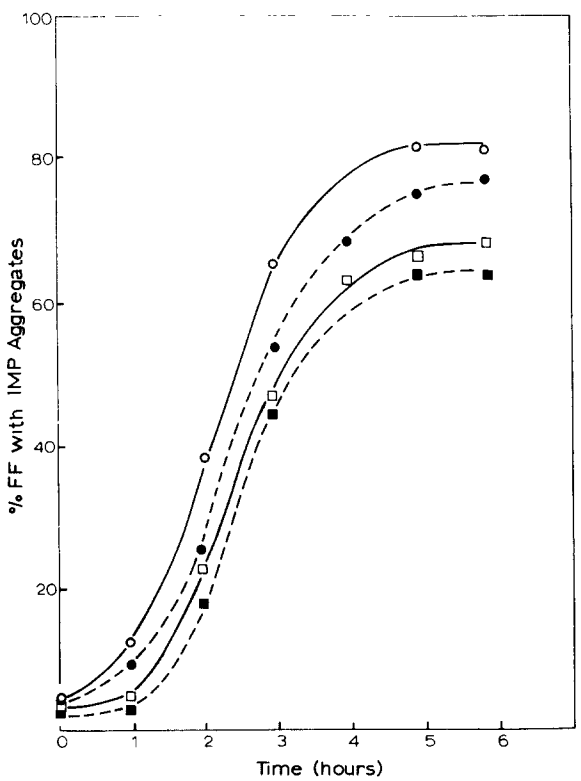

Fig. 2. The percentage of SV3T3 fracture faces demonstrating intramembranous particle aggregates as a function of time following vinblastine, 10^{-5} M (○-○), 10^{-7} M (□-□) or colchicine, 10^{-6} M (●- - -●), 10^{-9} M (■- - -■).

TABLE I

DISTRIBUTION OF PLASMA MEMBRANE INTRAMEMBRANOUS PARTICLES IN 3T3, SV3T3 AND SV3T3 TREATED WITH 10^{-6} M COLCHICINE FOR 4 h WITH CELLS RAPIDLY FROZEN FOLLOWING VARIOUS PREPARATION METHODS

Distribution was denoted as aggregated or random by criteria listed in the text. Following these criteria no consistent changes were observed in other cell membranes including nuclear and endoplasmic reticulum. To evaluate the adequacy of formaldehyde stabilization SV3T3 cells were incubated with 1 % formaldehyde for 10 min at 21 or 37 °C then treated with 10^{-6} M colchicine for 24 h. Colchicine treatment even under these prolonged conditions was unable to redistribute particles after brief formaldehyde stabilization. Cells fixed with 2.5 % glutaraldehyde for 10 min at 21 or 37 °C demonstrated an altered fracture face (AFF) as evidenced by: the appearance of plaques and pits on fracture faces, a decreased density of particles, and no particle aggregates in 3T3 cells or SV3T3 cells pre-treated with colchicine. Similar altered fracture faces were seen when cells were pre-incubated with glycerol then fixed with glutaraldehyde and re-incubated with glycerol. Cell viability was determined by trypan blue dye exclusion for both normal and transformed cells and following colchicine treatment; all samples reported had cell viabilities of greater than 90 %. N.A., not applicable; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide.

Conditions	3T3	SV3T3	4 h 10^{-6} M colchicine SV3T3
Media	Aggregated	Random	Aggregated
PBS	Aggregated	Random	Aggregated
10 % glycerol-PBS (5 min, 30 min, 2 or 4 h)	Aggregated	Random	Aggregated
20 % glycerol-PBS (5 min, 30 min, 2 or 4 h)	Aggregated	Random	Aggregated
10 % sucrose-PBS (5 or 30 min)	Aggregated	Random	Aggregated
20 % sucrose-PBS (5 or 30 min)	Aggregated	Random	Aggregated
10 % DMSO-PBS (30 min)	Aggregated	Random	Aggregated
1 % formaldehyde-PBS, 10 min; then frozen directly or in 15 % glycerol	Aggregated	Random	Aggregated
1 % formaldehyde, 10 min; then 10^{-6} M colchicine, 24 h	N.A.	Random	N.A.
2.5 % glutaraldehyde, 10 min; then frozen directly or in 15 % glycerol-PBS, 15 min	AFF	AFF	AFF
10 % glycerol-PBS, 15 min; followed by 2.5 % glutaraldehyde, 10 min; then incubation in 15 % glycerol, 15 min	AFF	AFF	AFF

quite distinct from the random distribution observed in viable control transformed cells. These results are not affected by variations in the temperature at which cells were incubated prior to freezing and were seen at 37, 21 and 4 °C (Table II). Fixation of cells in 2.5 % glutaraldehyde either before or after incubation with glycerol solutions

TABLE II

THE EFFECT OF TEMPERATURE ON INTRAMEMBRANOUS PARTICLE DISTRIBUTION OF 3T3 AND SV3T3 CELLS

Cells were incubated at the various temperatures for 30 min or 1 h with similar results and particle distribution evaluated as listed in text. PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide.

Conditions	3T3	SV3T3
Media (4, 21, 37 °C)	Aggregated	Random
10 % glycerol-PBS (4, 21, 37 °C)	Aggregated	Random
10 % sucrose-PBS (4, 21, 37 °C)	Aggregated	Random
10 % DMSO-PBS (21 °C)	Aggregated	Random

for 10 min or more leads to an apparent alteration of membrane cleavage that results in a decreased density of intramembranous particles, the appearance of numerous plaques and pits on the fracture faces and no particle aggregates are visualized in contact-inhibited or vinblastine-treated transformed cells. In addition, fixation in 0.1–1.0 % glutaraldehyde for 1 min as previously reported [9] has now also been found to cause altered fracture face morphology in this system. We have, however, observed that fixation of cells in 1 % formaldehyde or paraformaldehyde for 10 min at room temperature or 37 °C followed by direct freezing in media, phosphate-buffered saline or after incubation in 10 and 20 % glycerol phosphate-buffered saline routinely demonstrates small aggregates of particles in vinblastine-treated SV3T3 or contact-inhibited 3T3 cells, and randomly distributed particles in untreated SV3T3 cells. Furthermore, if SV3T3 cells are fixed in 1 % formaldehyde for 10 min, followed by incubation in 10^{-6} M colchicine for up to 24 h, the plasma membrane intramembranous particle distribution remains random, indicating that colchicine treatment after fixation is unable to redistribute particles.

The results of this study confirm our previous observations that there are significant differences in the distribution of intramembranous particles in contact-inhibited and transformed cells, which has been supported by others in this and other systems [11], though in some cases not [12, 13]. In addition, the results illustrate that that our observations are not secondary to cryoprotectants and that glutaraldehyde fixation of cells appears to alter the fracturing process. It should be noted that glutaraldehyde has been shown to seriously perturb the organization of membrane proteins in other systems [14, 15].

In addition, the results demonstrate that vinblastine and colchicine have a major effect on the organization of the plasma membrane of transformed cells. It has been shown that these agents disrupt microtubules [16], block mitotic spindle formation [17] and alter the polymerization of tubulin in vitro [18]. In addition, these agents have been shown to affect certain membrane transport activities [19, 20], lectin agglutination [21], ligand-induced receptor redistribution of immunoglobin and concanavalin A receptors in lymphocytes [22] and to induce shape changes in erythrocytes

[23] by mechanisms which apparently may not be associated with microtubule disruption.

The effect of vinblastine sulfate and colchicine on the freeze fracture morphology of SV3T3 cells may result in part from the disruption of cytoplasmic microtubules. However, our results in addition to previous studies demonstrating colchicine-induced alterations in other membrane phenomena [19–21] suggest a possible primary action on other membrane-associated moieties. This hypothesis is supported by the observation that reduced temperature which has been shown to disrupt cytoplasmic microtubules [24, 25] and to induce changes in cell shape [26, 27] does not significantly affect particle distribution in the 3T3/SV3T3 system under the conditions used in these experiments. Furthermore, elegant studies have shown a reduced temperature-induced clustering of particles in the alveolar membrane of *Tetrahymena pyriformis* [28] and that colchicine inhibits this phenomenon by a probable membrane effect rather than secondary to a disruption of microtubules [29].

The mechanisms accounting for our observations are undoubtedly quite complex and no unequivocal data have been presented in any nucleated mammalian system to provide a mechanism for the control of plasma membrane organization. A number of attractive possibilities arise which may even occur concomitantly. For example, the segregation of intramembranous particles could be based on thermodynamic considerations and be due to or promote phase separations occurring within the lipid matrix. Another attractive possibility, although there is no data to support it in nucleated cells, is that particles interact with other membrane proteins or membrane-associated protein networks similar to spectrin in the erythrocyte [30]. If this were the case, the integrity of this system and possible interactions between it and membrane particles, microfilaments or microtubules could be a mechanism for the control of the distribution of particles in nucleated mamalian cell plasma membrane.

These morphological observations suggest that real differences are present in the plasma membranes of normal and transformed cells. Results demonstrating that changes in the distribution of particles correlate with modulation in the metabolic activity of the cell in some systems [9, 10] suggest a yet undefined physiological significance. Subsequent studies are required to establish the biochemical and functional characteristics of intramembranous particles in native plasma membrane and the mechanisms that modulate their organization during various stages of cell growth and differentiation.

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