Short Pulse Cytochalasin B Treatment of Mouse Fibroblasts

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The drug cytochalasin B (CB), when applied to tissue cultures, was revealed by early studies to have inhibitory effects on various cellular activities (Carter 1967, Copeland 1974, Gail and Boone 1971, Wessells *et al.* 1971, Zigmond and Hirsch 1972). The possibility that cytochalasins might have direct activity on fibrils of F-actin (Spudich and Lin 1972) was experimentally repudiated by other workers (Forer and Behnke 1972). Upon viewing cytochalasin D (CD) drug effect on tissue culture cells with scanning electron microscopy, knobby protrusions, or so-called zeiotic blebs, were revealed to be a general cell response. The formation of zeiotic blebs, were studied intensively and described as resulting from a herniation of the cell endoplasm through the cell surface at sites of microfilament displacement (Godman *et al.* 1975, Miranda *et al.* 1974a, 1974b). Such displacement of microfilaments occurred in the cortex during cell contractions induced by the cytochalasins. This paper reports on the very early effects of CB on fibroblastic tissue culture cells as viewed by scanning electron microscopy and proposes a hypothetical model for the expansion of cell membranes into zeiotic blebs.

Materials and methods

Cells

Monolayer cultures of L-929 cells (mouse fibroblasts) were maintained at 37°C in Medium 199 (Grand Island Biological Co., Grand Island, NY.) supplemented with 10% fetal calf serum, 100 µg/ml streptomycin and 100 U/ml penicillin G. Subcultures were obtained after washing with phosphate buffered saline (PBS) and by trypsinization. Cells for microscopic examination were grown in the same medium as above on 9×22 mm glass coverslips in Leighton tubes. Only those cells which were grown in the tubes for 48 h were used for experiments.

Cytochalasin B treatment

Cytochalasin B (Aldrich Chem. Co., Milwaukee, WI) stock solutions were prepared in dimethylsulfoxide to 1 mg/ml and then added to the culture media giving a 10 µg/ml concentration. Treatment of the cells involved pipetting the standard growth medium from selected cultures and adding the CB-supplemented medium for selected times. Cells were treated for 20 sec and for 1, 5, 10, 30, and

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60 min. Coverslips were removed after CB treatment and prepared for scanning electron microscopy.

Scanning electron microscopy

Coverslips with attached cells were rinsed twice with prewarmed PBS and fixed in 2% glutaraldehyde buffered with 0.15 M cacodylate buffer (pH=7.3) at 21°C for 1 h. They were then subsequently washed with the same buffer, osmicated (1% O_3O_4 in 0.15 M cacodylate buffer for 1 h), and dehydrated through an ethanol series. Specimens were dried by critical point method in a Tousimis Samdri PVT unit using CO_2 as the transition fluid. The coverslips were then mounted on aluminum stubs and coated with approximately 200 Å gold in a cold sputtering device. Specimens were viewed with an Hitachi HHS-2R scanning electron microscope operating at 20 kV.

Results

Viewed with the SEM, typical log phase L-cell populations were primarily composed of two morphological types: 1) small rounded cells exhibiting numerous microvilli (Fig. 1) and; 2) more typical fibroblastic cells which were spindle or stellate in shape with fewer microvilli per unit surface area (Fig. 2). The blebbed morphology as described by Porter *et al.* (1973) in Chinese hamster ovary (CHO) cells appeared infrequently in log phase cultures, but increased when cells reached higher densities.

Zeiotic blebs appeared after 20 sec of cytochalasin B (CB) treatment in a log phase population. Initially, the blebs were distributed evenly over the cell surface and were more obvious in rounded cells than in spindle shaped and stellate cells. However, the disappearance of microvilli combined with cell surface folding was more pronounced in the latter (Figs. 3 and 4). The zeiotic blebs first appeared as hemispherical bodies (Fig. 4) and then enlarged to form clavated or bulbous ends on erect stalks (Fig. 5). The aggregation of zeiotic blebs near the tips of pseudopodia in spindle shaped and stellate cells occurred with 5 min after CB treatment, along with the virtual disappearance of microvilli in the central cell area (Fig. 6). The subsequent events in spindle and stellate cell development were similar to CD treatment on different cell lines as described by Godman *et al.* (1975). Within 10 min after CB treatment the zeiotic blebs aggregated toward specific sites of cell surfaces to form clusters which persisted as long as the drug was present.

Figs. 1-6. 1, control L-cell—no CB treatment. Cell is rounded with numerous microvilli. $\times 4800$. 2, control. Part of a spindle shaped cell with scattered microvilli. $\times 3000$. 3, CB treatment for 20 sec. Zeiotic blebs well established in rounded cells but are not so apparent in spindle shaped cells. $\times 800$. 4, CB treatment for 20 sec. Portion of spindle shaped cell with surface folds, rudiments of microvilli, and a semispherical zeiotic bleb, $\times 8000$. 5, CB treatment for 20 sec. Rounded cell with numerous zeiotic blebs amid many persisting microvilli. $\times 3000$. 6, CB treatment for 5 min. Stellate cell with zeiotic blebs aggregated at marginal sites and virtually lacking microvilli. $\times 2400$.

Discussion

Everhart and Rubin (1974), working with CB effects (5 $\mu\text{g/ml}$ for 5 min or longer) on synchronized CHO cell populations showed that the disappearance of blebs and microvilli, and the cell body contraction were typical surface morphology changes. Godman *et al.* (1975) suggested that the effect of CD (0.2 $\mu\text{g/ml}$ in combination with ^3H -thymidine for 10 min) was greatest in the G_1 phase of the cell cycle, but declined when the cells moved into the S phase. However, the difference became less distinct when higher drug concentrations were utilized. In one part of this experiment, we applied a high level (10 $\mu\text{g/ml}$) of CB for a very short pulse (20 sec) in which the formation of zeiotic blebs appeared throughout the population. The blebs were more obvious in rounded cells. In spindle shaped and stellate cells, there was a disappearance of microvilli and cell surface folding. In a CB dose level that suddenly caused an increased intracellular pressure and occurrence of gaps in the cortex, the level of an intracellular factor which could stabilize the surface membrane would not play a significant role. Johnson *et al.* (1972) postulated such a factor as being cyclic AMP. We suggest the possibility that in individual cells the amount of pre-existing membrane per unit area might be a key factor in determining the extensiveness of zeiotic bleb formation. More villated morphology in rounded L-cells and some other cell lines (Knutton *et al.* 1975) apparently have a higher ratio of membrane per unit area than flattened cells because of the large numbers of microvilli (Figs. 1 and 2). Upon an increase in intracellular pressure, the availability of membrane material is essential for zeiotic bleb formation. Microvilli appear to be an available source which can supply membrane material to cover the zeiotic protrusions. Thus, rounded cells have a better possibility to form the zeiotic blebs than spindle shaped ones from the same cell line. The common surface folding of spindle shaped cells might be caused by unevenly distributed intracellular pressure. The route for redistribution of blebs observed in this study is similar to that previously shown for the same cell line in the capping of Concanavalin A binding sites (Weller 1974). This further suggests a common pattern for surface membrane movement.

Summary

Mouse L-cells in log phase were treated with cytochalasin B for time periods ranging from 20 sec to 1 h and subsequently examined by means of scanning electron microscopy. Zeiotic blebs appeared within the shortest time period in all cells but was accompanied by a general loss of microvilli in spindle and stellate shaped cells. It is suggested that initial responses in zeiotic bleb formation may be dictated by the amount of pre-existing membrane per unit area of the cell surface, i. e., the extensiveness of microvilli.

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