SHORT COMMUNICATION

Early changes in plasma membrane *in vitro* by treatment with chemical carcinogen

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Abstract: To examine early changes in the cell membrane caused by treatment with 9.10-dimethyl-1.2-benzanthracene (DMBA), rat lingual epithelium *in vitro* was observed by means of electrophysiological, freeze-fracture and whole mount electron microscopic methods. Membrane potential showed a transient decrease at 6 h after the treatment but returned to almost the same level as that of the control group 1 day later. Membrane resistance decreased rapidly at 6 h after the treatment but increased at 12 h. Transient decrease of membrane resistance and membrane potential occurred prior to the decrease in coupling ratio at 6 h after treatment with DMBA. In freeze-fracture replicas, after 6 h of treatment with DMBA, the number of intramembranous particles (IMPs) was less than that of the control. This decrease was especially pronounced on the P-face. After 12 h treatment with DMBA, the number of IMPs increased by 20-40 % more than the control value. This increase was especially noticeable on the E-face, which was 70 % of the control value. At 3 days and thereafter, the number of IMPs decreased again and remained approximately the same as that of the control, though the E-face values remained larger than those of the control. Treatment with DMBA had a marked effect on the cytoskeletal organization. Treatment with DMBA caused morphologic changes as early as 6 h. These early changes might represent triggers for subsequent metabolic cellular abnormalities. The affected cells which were treated with DMBA appear to differ from normal cells through accelerated-abnormal cell membrane and cytoskeletal turnover.

Introduction

Cell division, proliferation, and mobility are controlled through the structure and function of the plasma membrane¹⁻⁵⁾. Moreover, it has also been suggested that the plasma membrane plays an important role in controlling cell proliferation; and it is assumed that the first event in carcinogenesis is a change in the protein structure of the cell membrane, and a secondary result is dysfunction of many plasma membranes⁵⁾. Recent studies

have clarified the altered characteristics of cell membranes following malignant transformation. One of the changes that takes place is a loss or decrease in cell-membrane surface receptors⁶⁻⁸⁾. Another is a change in cytoskeletal elements such as microfilaments and microtubules and their relations with the plasma membrane⁹⁻¹⁴⁾. As has been said, cell membranes are intimately related to the carcinogenesis mechanism. We have already reported that, in thin sections and freeze-fracture replicas of material treated *in vivo* with

DMBA, morphological alterations in plasma membrane occur during the process of carcinogenesis and in squamous cell carcinoma¹⁵⁾. Few reports¹⁶⁾, however, have dealt with initial changes in the cell membrane caused by the exposure of culture cells to chemical carcinogens.

In order to characterize early membrane alterations electrophysiologically and electron microscopically, rat lingual epithelium was cultivated in isolation, exposed to DMBA, and subsequently examined. The number of IMPs were measured quantitatively. In addition, membrane potential and membrane resistance were examined by electrophysiological methods. Morphological changes in intracellular microfilaments were observed by whole mount electron microscopy.

Materials and Methods

Cell Culture: Lingual epithelia were isolated from newborn rats (Sprague-Dawley strain) and dissociated into single, isolated cells by trypsinization. The cells were seeded in plastic dishes (Lux Co., 35 mm in diameter) at a density of 2×10^5 cells per dish. The culture medium consisted of Eagle's minimal essential medium (MEM) supplemented with 10 % fetal calf serum, 2 mM glutamine, 0.4 $\mu\text{g/ml}$ hydrocortisone, 50 unit/ml penicillin, and 50 $\mu\text{g/ml}$ streptomycin^{17,18)}. The medium was adjusted to pH 7.4 with 10 % NaHCO₃. Incubation temperature was 37 °C., and the gaseous environment was 95 % air and 5 % CO₂.

DMBA Treatment *In Vitro*: DMBA (SIGMA) was dissolved in acetone, diluted with MEM and added at a final concentration of 4.0 $\mu\text{g/ml}$ when the cells had attained a confluency of 60–70 %. Aliquots of acetone diluted with Eagle's MEM were added to the control cells. The final concentration of acetone in the medium was 0.1 % and this concentration of acetone had no substantial effect on cell coupling or morphology. Culture cells were treated with DMBA for periods of 6 h, 12 h, 1 day, 2 days, 3 days, and 10 days.

Electrophysiological Measurement: The microelectrodes used for the experiments were glass micropipettes filled with 3 M KCl having a d-c resistance of 20 to 30 M Ω by sta-

ndard technique. Two microelectrodes were inserted into two contiguous cells to measure membrane potential, membrane resistance, and electrical cell coupling under an inverted microscope. One electrode delivered the rectangular hyperpolarizing current pulses of 50 ms duration and the resulting potential changes (E 1) were simultaneously recorded in one of the cells using the balanced circuit of a preamplifier. The other was used to record the resulting potential changes (E 2) in an adjacent cell. The ratio of the potential changes (E 2/E 1), coupling ratio, was used to express the degree of cell-to-cell communication between the two cells and was calculated on the steady state voltage changes produced by the injected current¹⁹⁾. The current was supplied by a square pulse generator and potential changes were monitored on the screen of a cathod-ray oscilloscope. During electrical measurement, the temperature was kept at 35–37 °C by circulating warm water on the stage of the microscope.

Phase-Contrast Microscopy: Living cultures were viewed and photographed with a Nikon Diaphoto-TMD inverted-phase contrast microscope.

Electron Microscopy: For thin sectioning, the cultures were washed 3 times with a cacodylate buffer solution and fixed *in situ* with Karnovsky fixative (2 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for about 30 min at 4 °C. After repeated washing with the cold cacodylate buffer, the samples were postfixed in aqueous osmium tetroxide for 30 min, washed with distilled water and stained *en bloc* with 2 % aqueous uranyl acetate at 4 °C. They were then dehydrated in graded alcohol solutions and embedded in Epon 812. The polymerized Epon was removed from the dish and small areas containing the cells were cut from the embedded cultures to mount on a block. Thin sections were stained with uranyl acetate and lead citrate.

For freeze-fracture replicas, the cells grown on plastic sheets (Lux Co., 25 mm in diameter) were fixed with Karnovsky fixative for 30 min at 4 °C and immersed in 10, 20, 30, and 40 % glycerol in the same buffer for 30 min. The plastic sheets were cut in 5 $\times$ 5

Table 1 Quantitative Changes in Intramembranous Particles and Partition Coefficient (Kp) in Control and DMBA Treated groups

Status		Total membranous particle/ μm^2	P-face	E-face	Kp
Control group	1 day	1257	902 $\pm$ 169	355 $\pm$ 81	2.57
	3 day	1740	1368 $\pm$ 151	372 $\pm$ 96	3.68
	7 day	1761	1354 $\pm$ 64	408 $\pm$ 84	3.32
DMBA treated group	6 hour	1291 ²⁾	943 $\pm$ 472 ²⁾	348 $\pm$ 189 ⁴⁾	2.71
	12 hour	2163 ²⁾	1263 $\pm$ 387 ³⁾	900 $\pm$ 174 ¹⁾	1.4
	1 day	2642 ¹⁾	1756 $\pm$ 269 ¹⁾	886 $\pm$ 231 ¹⁾	1.98
	2 day	2345 ¹⁾	1440 $\pm$ 260 ⁴⁾	905 $\pm$ 127 ¹⁾	1.59
	3 day	1876 ⁴⁾	1102 $\pm$ 380 ⁴⁾	774 $\pm$ 194 ¹⁾	1.42
	10 day	2048 ⁴⁾	1299 $\pm$ 280 ⁴⁾	748 $\pm$ 113 ¹⁾	1.74

¹⁾ Differences from controls are statistically significant $P < 0.001$

²⁾ Differences from controls are statistically significant $P < 0.02$

³⁾ Differences from controls are statistically significant $P < 0.05$

⁴⁾ Differences from controls are not significant $P > 0.1$

mm and mounted on discs. They were then rapidly frozen in liquid Freon 22, cooled with liquid nitrogen and fractured at -100°C in a MILLITORR FE-600 freeze-etching unit. Platinum-carbon replicas were then cleaned by successive washings with sodium-hydrochloride and distilled water. These thin sections and replicas were examined with a Hitachi H-300 electron microscope.

Morphometric Analysis: IMPs were measured quantitatively by examination of 16-24 replicas from each of the control and DMBA treated groups. Particles of 9-12 nm were counted on randomly chosen photographs of freeze-fracture replicas at a magnification of $\times 50,000$ over an area of about $900 \mu\text{m}^2$ using IBAS-2 image analyzer (Interaktive Bildanalyse System II, Kontron). The partition coefficient (Kp), which is the ratio of particle counts for the P- and E-faces, represented the distribution of particles between the two faces²⁰⁾. A student's t-test was used to compare results from the two groups for significance.

Cytoskeletal Preparation: Cytoskeletons were prepared from cells essentially by the methods of Capco *et al.*²¹⁾ and Fey *et al.*²²⁾. Epithelial cells were grown on gold grids covered with formvar and coated with carbon. The grids were removed from the cul-

ture medium and rinsed in 0.1 M phosphate-buffered saline (pH 7.4) at 4°C . They were then placed in an extraction medium for 3 min and rinsed briefly in a digestion medium without enzymes. They were then placed in a digestion medium containing enzymes and incubated for 20 min at room temperature. These grids were placed in an extraction medium for 5 min at room temperature and then fixed by immersion in 2 % glutaraldehyde made of digestion medium without enzymes for 20 min at 4°C . The cells were dehydrated in alcohol and dried through the CO_2 critical point. They were examined with Hitachi H-800 transmission electron microscope at an accelerating voltage of 200 KeV at 6 h, 12 h, 1 day, 2 days, 3 days and 10 days. Stereomicrographs were generated at tilt angles of $+6$ - 10° .

Preparation of extraction medium, digestion medium and enzymes: The extraction medium contained 10 mM PIPES, 250 mM $(\text{NH}_4)_2\text{SO}_4$, 300 mM sucrose, 3 mM MgCl_2 , 1.2 mM PMSF, and 0.5 % Triton X-100, adjusted to pH 6.8 by 10 % NaHCO_3 . The digestion medium contained 10 mM PIPES, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl_2 , 1.2 mM PMSF, and 0.5 % Triton X-100, adjusted to pH 6.8 by 10 % NaHCO_3 . The enzymes used were bovine pancreatic DNAase I (SI-

Fig. 1 Phase contrast micrograph after 3 days of control culture. $\times 650$

Fig. 2 Phase contrast micrograph at 3 days after treatment with DMBA, The cells are slightly irregular in size and have enlarged intercellular spaces. $\times 650$

Fig. 3 Ten days after treatment with DMBA, cells are polygonal in shape and different in size. $\times 650$

Fig. 4 Electron micrograph at 1 day after treatment with DMBA. Mitochondria are scattered over the cytoplasm. One or two Golgi complexes are located near the nucleus. Bundles of tonofilaments have formed within the cytoplasm. $\times 8,000$

Fig. 5 Electron micrograph 3 days after treatment with DMBA. Free ribosomes have formed polysomes,

GMA), RNAase A (SIGMA), and micrococcal nuclease (SIGMA) DNAase and RNAase (preincubation at 100 °C for 3 min) were used at a concentration of 400 mg/ml of digestion medium. Micrococcal nuclease was used at a concentration of 12.5 unit/ml of digestion medium.

Results

1. Phase-contrast and electron microscopic observation.

After 3 days of control culture, cells of uniform size clumped together in small clusters of 3-5 cells. After 5-7 days in culture, these clusters had expanded to form mosaic-like sheets of cells. In some areas, multiple cell layers formed (Fig. 1). In 10-20 days after culture, the sheetlike structure enlarged. Many had formed multiple wholes with central keratinization.

One to three days after treatment with DMBA, the cells varied slightly in size and had enlarged intercellular spaces (Fig. 2). Ten days after treatment, the cells were polygonal in shape and different in size (Fig. 3). Electron microscopic observation showed mitochondria scattered in the cytoplasm at 1 day after treatment of DMBA. One or two Golgi complexes were located near the nucleus. Bundles of tonofilaments were formed within the cytoplasm (Fig. 4). Subsequently during culture, organelles developed well, and connections between desmosomes and tonofilaments became pronounced. Three days after treatment with DMBA, free ribosomes formed polysomes, and tonofilaments aggregated (Fig. 5). Ten days after treatment, mitochondria appeared at high density, and tonofilaments were short and aggregated. Some vacuoles had appeared in the cytoplasm (Fig. 6).

2. Electrophysiological findings

As the epithelial cells in the culture medium were very fragile, their membrane potential sometimes decreased quickly after insertion of the electrode. Therefore, the data were accepted when the membrane potential maintained its largest value for 2-3 min after penetration.

The average membrane potential was 9.8 ± 0.7 mV (mean $\pm$ S. E., $n=12$) after 1 day in a normal growth medium. Thereafter, it increased to -14.8 ± 0.8 mV ($n=20$) after 3 days of culture and maintained a steady level of about -15 mV within 3-7 days in the normal growth medium.

The average membrane resistance was 17.3 ± 2.8 M Ω ($n=9$) after 1 day and decreased gradually to about 5 M Ω during incubation in normal growth medium. This decrease of membrane resistance was due to the apparent increase in surface area of the cell membrane resulting from the formation of intercellular junctions with electrical low resistance between adjacent cells. The membrane resistance per unit area (specific membrane resistance) might not change.

The coupling ratio was 0.36 ± 0.05 ($n=8$) after 1 day of culture. It increased to 0.49 ± 0.04 ($n=13$) within 3 days and subsequently did not change or slightly decreased during incubation in the normal growth medium. Fig. 7 a-d shows an experiment in which junctional uncoupling ensues from treatment of DMBA. Decrease of coupling ratio, due to a fall in junctional membrane permeability, is indicated by a fall in E2. Fig. 7 e illustrates the change of membrane potential, membrane resistance and coupling ratio measured at various times after incubation in the culture medium with DMBA. After replacing the culture medium with DMBA, the membrane resistance fell transiently from 12.9 to 5.3 M Ω during 6 h but by 12 h markedly increased again to 17.0 M Ω which was higher than the control level. The transient fall of membrane resistance was also reflected in diminution of the membrane potential. During the first 6 h after treatment of DMBA, the membrane potential decreased transiently to -10.2 mV and returned to almost the same level as that of the control group. The coupling ratio was scarcely or slightly affected during the first 6 h after treatment with DMBA. The junctional coupling began to diminish after a transient decrease of membrane resistance and potential and, as a result, the coupling

and tonofilaments have aggregated. $\times 15,400$

Fig. 6 Ten days after treatment with DMBA, tonofilaments have shorted and aggregated. Some vacuoles have appeared in the cytoplasm. $\times 12,600$

Fig. 7 Junctional coupling in the control group after 3 days in normal growth medium (7a), and 12 h (7b), 1 day (7c) and 3 days (7d) after treatment with DMBA. Each oscilloscope record indicates E1 (upper trace), E2 (middle trace) and current with hyperpolarizing pulse. (7e) shows time course of junctional uncoupling by DMBA. Arrows indicate application of DMBA. —○—: Control, —●—: DMBA-treated group

ratio fell toward 0.18 at 12 h after treatment with DMBA. The transient reduction of junctional coupling suggested that, first of all, the function of the cell membrane as an ion barrier is disturbed temporarily by DMBA and then the inflow of extracellular ions and/or DMBA occurs. That these diminutions are not a result of cell damage is evident from their reversibility; as shown in Fig. 7e, the membrane resistance and potential were completely reversed after 12 h in the culture medium with DMBA. The delay in onset of junctional uncoupling in the culture medium with DMBA suggests that uncoupling may be the result of a critical change in in-

tercellular ionic environment and/or effect of DMBA on the junction.

3. Plasma membrane alteration

In replicas, the size of the IMPs were approximately 8–10 nm on both the P- and E-faces of the control group. The number of IMPs per μm^2 of membrane area was 1740 which was consistent with 1368/ μm^2 on the P-face and 372/ μm^2 on the E-face at 3 days after culture (Fig. 8). This gave a distribution index of 3.68, as can be seen in Table 1. The values obtained after 7 days of culture were similar to those after 3 days, which were in turn similar to values for basal cells in the squamous epithelium *in vivo*¹⁵.

Fig. 8 Freeze-fracture replicas of the control after 3 days of culture. The size of IMPs was approximately 8-10 nm in both P- and E-faces. $\times 131,000$

Fig. 9 Freeze-fracture replicas at 6 h after treatment with DMBA. The number of IMPs decreased on the P-face. $\times 150,000$

Fig. 10 and 11 The number of IMPs on the P-face has increased by 16-30 % of the control value at 12 h (Fig. 10) and 1 day (Fig. 11), after treatment with DMBA. The number of IMPs on the E-face has increased by 70 % of the control value. Fig. 10 $\times 100,000$, Fig. 11 $\times 125,000$

Fig. 12 and 13 The density of IMPs at 3 days (Fig. 12) and 10 days (Fig. 13) after treatment with DMBA is the same as the control. The number of IMPs on the E-face increased significantly over that of the control group. Fig. 12 $\times 120,000$, Fig. 13 $\times 125,000$

No cell membrane change occurring in response to treatment with DMBA was noted in the thin sections. In replicas, IMP size was 8–10 nm. No difference was noted between these values and the values of the control group. IMPs density was different, however, as is seen in Table 1. At 6 h after treatment with DMBA, the number of IMPs was $1291/\mu\text{m}^2$, which represented a decrease compared with the control value at 3 days after culture. The decrease was especially pronounced in the P-face (Fig. 9). The number of IMPs increased by 20–43 % of the control value after 12 h (Fig. 10), 1 day (Fig. 11), and 2 days after treatment with DMBA. IMPs on the E-face increased by 70 % of the

control value, which was a statistically significant difference from the control. After 3 days (Fig. 12) and thereafter (Fig. 13), the total number of IMPs was approximately the same as the control. The number of IMPs on the E-face, however, had increased significantly over those of the control group.

4. Epithelial cytoskeleton morphology

As is demonstrated in Fig. 14, a web of filaments extended throughout most of the cytoplasmic space. These cytoskeletons consisted of dimensional networks composed of 2–4 nm filaments, 5–8 nm filaments, and 13–21 nm filaments. The 2–4 nm and 5–8 nm filaments were apparently cross-linked to each other. In addition, these filaments were fre-

Fig. 14 Stereo electron micrographs of skeletal framework of cultured cells. A web of filament extends throughout most of the cytoplasmic space. These cytoskeletons consist of three dimensional networks composed of 2 filaments (small arrow), 5–8 nm filaments (double arrows), and 13–21 nm filaments (large arrows). Filaments of the 2–4 nm and 5–8 nm size associate in a dense network running parallel and beneath the plasma membrane (arrowheads). $\times 62,500$

Fig. 15 Stereo pairs of part of a cell at 3 days after treatment with DMBA. Cells appear partially devoid of 2–4 nm and 5–8 nm filaments (asterisk). The tonofilaments are composed of 13–21 nm filaments in a zigzag pattern and dense network aggregations or Compact foci of filamentous material (arrows). $\times 32,000$

Fig. 16 Numerous filaments are present on the lower side of the photograph, but the upper side is devoid of filaments (asterisk) in cytoplasm at 6 h after treatment with DMBA. $\times 35,700$

Fig. 17 Adjacent cells (upper and lower sides of photograph) appear particularly of filaments (asterisk) at 3 days after treatment with DMBA. The filament network beneath the plasma membrane decreased (arrowheads). $\times 36,000$

quently observed to form end-to-side associations with other filaments as well as with microtubules and tonofilaments. The 5-8 nm filaments appearing as Y-shaped branches were connected to each other and they are thought to be actin²²⁻²⁴⁾. The 32-43 nm filaments were assembled from tonofilaments or intermediate filaments of 13-21 nm filaments²²⁻²⁴⁾ and were connected to desmosomes. The 2-4 nm and 5-8 nm filaments appeared as a dense network beneath the plasma membrane (Fig. 14).

Treatment with DMBA resulted in a marked effect on the cytoskeletal network organization. Some cells were partially devoid of the 2-4 nm and 5-8 nm filaments (Figs. 15, 16 and 17). Dense network aggregates and even compact foci of filamentous material were formed (Fig. 15). Tonofilaments composed of 13-21 nm filaments were arranged in a zigzag pattern and were detached from the desmosomes. The density of filament networks beneath the plasma membrane decreased (Figs. 16 and 17).

Discussion

In electrophysiological studies, we found that a transient fall in membrane potential and resistance was noted 6 h after treatment with DMBA and prior to a fall in the coupling ratio. Also, we have reported these same results earlier¹⁶⁾. This may reflect the way a carcinogen such as DMBA acts on the cell membrane to disrupt its function as an ion barrier, and thus, concomitant passage of the carcinogen itself into the cell. In other words, changes of ion permeability are an expression of changes in the composition of the membrane. At the same time, this was morphologically expressed as a decrease in the number of IMPs. Twelve hours after treatment with DMBA, electrophysiological studies revealed a restoration of resistance and membrane potential to nearly normal values. Morphologically, the number of IMPs initially increased but, after 3 days of culture, decreased again to near control values, representing the autorepair process of the membrane. This transient decrease in membrane potential and resistance followed by recovery coincides with changes in membrane potential

and resistance in cases of cell fusion induced by the HVJ virus²⁵⁾. This fusion reaction temporarily destroys the ability of the membrane to function as an ion barrier. As the fusion reaction proceeds, however, the membrane's electrical property is restored, suggesting that plasma membrane electrical property is restored along with recombination and rearrangement of the membrane structure. After treatment with DMBA, the increase in the number of IMPs on the E-face persisted, as a result of a similar recombination and rearrangement. Subsequent phenomena are probably synergistic actions. For instance, it has been reported that, when chick embryo cells are transformed by the Rous sarcoma virus, reconstruction of actin filament occurs early, and the filaments aggregate in some location⁹⁾. This represents a loss of interrelationships between cancer cell surface protein and actin filaments. With exposure of the cells to substances such as colchicine which is toxic to microtubules, a lateral shift in intramembranous proteins and glycoproteins occurs¹²⁾. Microtubules have been reported to grow indistinct as a result of malignant cell transformation as the number of actin bundles decrease²⁶⁻²⁹⁾. In their review of the association between microfilaments and membranes, Weihing³⁰⁾ and Weatherbee³¹⁾ have pointed out dense filament networks in areas of active phagocytosis³²⁾ or endocytosis³³⁾, attachments of bundles of microfilaments to membrane-associated adhesion plaque of cultured cells³⁴⁾, and connections between membranes with microfilaments running parallel to them³⁵⁾. Using double-label immunofluorescence microscopy, Ash and Singer³⁶⁾ and Ash *et al.*³⁷⁾ have pointed out that the interpenetration and destruction of fibronectin and actin filaments occur because actin filaments are connected either directly or indirectly to protein in the membrane or through the membrane, to external proteins³⁸⁻⁴¹⁾. These findings suggest that the cytoplasm immediately beneath the cell membrane either indirectly or directly controls movement of IMPs. The increase in fluidity of the plasma membrane is the result of a disorder in the relationship of intracellular filaments⁴²⁾. The findings of transient changes in mem-

brane potential, membrane resistance and IMPs density in the early stage of carcinogenesis resulted in a disruption of plasma membrane among normal cells. At the same time, our studies indicate that an early degeneration of filaments is represented by aggregation and compact foci of filamentous materials. A focal decrease in density occurred in filament networks beneath the plasma membrane. It is important to note how an early inhibition of plasma membrane and an alteration of cytoskeletons by a carcinogen will transform a normal cell into a cancerous one. In addition, the attachment of the carcinogen to the cell membrane and/or its entrance into the cell as a cytotoxin must be kept in mind. However, the definite mode of connection between filaments, membrane, and the mechanism whereby filaments control the movement of membrane protein remains to be clarified. Further investigation should focus on the relationship between plasma membrane and cytoskeletons following exposure to a carcinogen.

The behavior of the cell membrane described in this study reveals in morphological and physiological ways abnormal cell metabolism. Permeability increased the passage of substances through the cell membrane which suggests a considerable breakdown in the membrane metabolism along with a loss of selective permeability.

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Reference

- 1) Burger, N. W., Bombik, B. M., Breckenridge, B. and Sheppard, J. R.: Growth control and cycle alteration of cyclic AMP in the cell cycle. *Nature* **239**: 161-163, 1977.
- 2) Grud, J. W. and Evans, W. M.: Relative rate of degradation of mouse liver surface membrane proteins. *Eur. J. Biochem.* **36**: 273-298, 1973.
- 3) Lenard, J. and Singer, S. J.: Alteration of conformation of protein in red blood cell membrane in solution by fixations used in electron microscopy. *J. Cell Biol* **37**: 117-121, 1968.
- 4) Singer, S. and Nicolson, G. L.: The fluid mosaic model of the structure of cell membrane. *Science*. **175**: 720-731, 1972.
- 5) Walach, D. F. H.: Cellular membrane and tumor behavior, a new hypothesis. *Proc. Natl. Acad. Sci. USA* **61**: 868-874, 1968.
- 6) Beltlane, M., Hermier, B., Dubert, J. M., and Paraf, P.: Differences in the cell surface proteins of drug resistance and sensitive cell lines. *Biochemie*. **58**: 731-736, 1976.
- 7) Marchesi, V. T., Tillack, T. W., Jackson, R. L., Segret, J. P. and Scott, R. E.: Chemical characterization and surface orientation of the major glycoprotein of the human erythrocyte membrane. *Proc. Natl. Acad. Sci. USA* **69**: 1445-1449, 1972.
- 8) Thoupoulos, P. and Roth, T.: Insulin receptor in normal and transformed fibroblast; Relationship to growth and transformation. *Cell*. **8**: 417-423, 1976.
- 9) Bosheck, C. B., Jockusch, B. M., Friis, R. R., Back, R. and Grundman, E. and Bauer, H.: Early changes in distribution and organization of microfilament protein during cell transformation. *Cell*. **24**: 175-184, 1981.
- 10) Carter, S. B.: Effect of cytocharasin on mammalian cells. *Nature*. **213**: 261-264, 1976.
- 11) Davis, A. T., Estensen, R. and Quir, P. G.: Cytocharasin B: Inhibition of human polynuclear leukocyte phagocytosis. *Proc. Soc. Exp. Med.* **137**: 161-164, 1971.
- 12) Olmsted, J. B. and Borisey, G. G.: Microtubules. *Ann Rev Biochem.* **42**: 507-540, 1973.
- 13) Weinstein, R. S. and McNutt, N. S.: Ultrastructure of red cell membrane. *Sem. Hematol.* **7**: 259-274, 1970.
- 14) Wood, D. A. and Smith, C. J.: Ultrastructure and development of epithelial pseudopodia in chemically induced premalignant lesions of the hamster cheek pouch. *Exp. Mol. Pathol.* **12**: 160-174, 1970.
- 15) Tachikawa, T., Yamamura, T. and Yoshiki, S.: Changes occurring in plasma membrane and intercellular junctions during the process of carcinogenesis and in squamous cell carcinoma. *Virchows Arch. B Cell Pathol.* **47**: 1-15, 1984.
- 16) Tachikawa, T., Kohno, Y., Matsui, Y. and Yoshiki, S.: *In Vitro* early changes in intercellular junctions by treatment with a chemical carcinogen. *Carcinogenesis* **7**: 885-892, 1986.
- 17) Reinwald, J. G. and Green, H.: Serial cultivation of strands of human epidermal keratinocytes: The formation of keratiniz-

- ing colonies from singel cells. *Cell*. **6** : 331-344, 1975.
- 18) Tachikawa, T. and Yoshiki, S.: Cultivation of rat lingual epithelium: Fine structure of cultured epithelial cells. *Tiss Culture in Dentist*, **20** : 29-30, 1983.
 - 19) Loewenstein, W. R., Socolar, S. J., Higashino, S., Kanno, Y. and Davidson, N.: Intercellular communication: Renal, urinary bladder, sensory, and salivary gland cells. *Science*. **149** : 295-298, 1965.
 - 20) Satir, P. and Satir, B.: Partition coefficient of membrane particles in the fusion rosette. *Exp. Cell Res.* **89** : 404-407, 1974.
 - 21) Capco, D. G., Wan, K. M. and Penman, S.: The nuclear matrix: Three-dimensional architecture and protein composition. *Cell*. **29** : 847-858, 1982.
 - 22) Fey, E., Wan, K. M. and Penman, S.: Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: Three-dimensional organization and protein composition. *J. Cell Biol.* **98** : 1973-1984, 1984.
 - 23) Schliwa, M.: Actin of cytoskeletal networks. *J. Cell Biol.* **92** : 79-91, 1982.
 - 24) Schliwa, M., Nakamura, T., Poter, R. K. and Euteneuer, U.: A tumor promoter induces rapid and coordinate reorganization of actin and vinculin in cultured cells. *J. Cell Biol.* **99** : 1045-1059, 1984.
 - 25) Okada, Y., Koseki, I., Kim, J., Hashimoto, T., Kanno, Y. and Matui, Y.: Modification of cell membrane with viral envelopes during fusion of cells with HJV (sendai virus). I. Interaction between cell membrane and virus in the early stage. *Exp. Cell Res.* **93** : 368-378, 1975.
 - 26) Edelman, G. M. and Yahara, I.: Temperature-sensitive changes in surface modulating assemblies of fibroblasts transformed by mutants of Rous sarcoma virus. *Proc. Natl Acad. Sci. USA*. **73** : 2047-2051, 1976.
 - 27) McNutt, N. S., Culp, L. A. and Black, P. H.: Contact-inhibited revertant cell lines isolated from SV-40 transformed cells, II. Ultrastructure study. *J. Cell Biol.* **50** : 691-707, 1971.
 - 28) McNutt, N. S., Culp, L. A. and Black, P. H.: Contact-inhibited revertant cell lines isolation from SV-40 transformed cells. IV. Microfilament distribution and cell shape in untransformed, transformed, and revertant Balb/3T3 cells. *J. Cell Biol.* **56** : 412-428, 1973.
 - 29) Perdue, J. F.: The distribution ultrastructure, and chemistry of microfilament in cultures chick embryo fibroblast. *J. Cell Biol.* **58** : 265-283, 1973.
 - 30) Weihing, R. R.: The cytoskeleton and plasma membrane. *Meth Achiev. Exp Pathol.* **8** : 42-109, 1979.
 - 31) Weatherbee, J. A. P.: Membrane and cell movement: Interactions of membrane with the proteins of the cytoskeleton. *Int. Rev. Cytol. suppl.* **12** : 113-176, 1981.
 - 32) Berlin, R. D., Caron, J. M. and Oliver, J. M. In: K. Roberts, J. S. Hyams. (eds), *Microtubules*, pp. 443-485. Academic press, N. Y, 1979.
 - 33) Albertini, D. F. and Anderson, E.: Microtubule and microfilament rearrangements during capping, of concanavalin A receptors on cultured ovarian granulosa cells. *J. Cell Biol.* **73** : 111-127, 1977.
 - 34) Abercrombie, M., Heaysman, J. E. M. and Pegrum, S. M.: The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella. *Exp. Cell Res.* **67** : 359-367, 1971.
 - 35) Mooseker, M. S. and Tilney, L. G.: Organization of an actin filament-membrane complex, filament polarity and membrane attachment in the microvilli of intestinal epithelial cells. *J. Cell Biol.* **67** : 725-743, 1975.
 - 36) Ash, J. F. and Singer, S. J.: Concanavalin A-induced transmembrane linkage of concanavalin A surface receptors to intracellular myosin-containing filaments. *Proc. Natl. Acad. Sci. USA*. **73** : 4575-4579, 1976.
 - 37) Ash, J. F., Louvard, D. and Singer, S. J.: Antibody-induced linkage of plasma membrane proteins to intracellular actomyosin containing filaments in cultured human fibroblasts. *Proc. Natl. Acad. Sci. USA*. **74** : 5584-5588, 1977.
 - 38) Heggeness, M. H., Ash, J. F. and Singer, S. J.: Transmembrane linkage of fibronectin to intercellular actin-containing filaments in cultured human fibroblasts. *Ann. N. Y. Acad. Sci.* **312** : 414-417, 1978.
 - 39) Hynes, R. O.: Role of surface alteration in cell transformation: The importance of protease and surface proteins. *Cell*. **1** : 147-156, 1974.
 - 40) Hynes, R. O. and Destree, A. T.: Relationships between fibronectin (LETS protein) and actin. *Cell*. **15** : 875-886, 1978.
 - 41) Singer, I. I.: The fibronexus: A transmembrane association of fibronectin-containing fibers and bundles of 5 nm microfilaments

- in hamster and human fibroblast. *Cell*. **16**: 675-685, 1979.
- 42) Nicolson, G. L.: The interaction lectins with animal cell surfaces. *Int. Rev. Cytol.* **39**: 89-195, 1974.