Reorganization of cytoplasmic structures during cell fusion

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Summary

In order to provide a better understanding of the dynamic process of cell fusion, we studied the reorganization of cytoplasmic structures in electrofused CV-1 cells. Using fluorescence microscopy and double staining methods, we examined correlations between the structural patterns of the major cytoskeletal proteins (microtubules, actin and vimentin intermediate filaments) and the distribution of various organelles (endoplasmic reticulum, mitochondria and nuclei) at different stages of cell fusion. Our results suggest that microtubules appear to play a primary role in the process of cytoplasmic reorganization. At the early stage of cell fusion, microtubules were observed to infiltrate rapidly into the newly formed cytoplasmic bridges and establish a connection

between the cytoskeletal networks of fusing cells. The reorganization of microtubules was found to be correlated with the redistribution of endoplasmic reticulum (ER), vimentin intermediate filaments, mitochondria, and the aggregation of nuclei. The F-actin system, on the other hand, appeared to be independent of the reorganization of the other cytoplasmic structures. The principal function of F-actin during cell fusion is probably to widen the cytoplasmic bridges by lamellipodial extension.

Key words: microtubule, vimentin, actin, endoplasmic reticulum, mitochondrion, nucleus, CV-1 cells, electrofusion, polykaryon, syncytium, immunofluorescence.

Introduction

Cell division and cell fusion are two related processes that play an important role in the development of biological organisms. Cell fusion also has important uses in the study of somatic cell genetics including gene mapping, and the production of hybridomas and monoclonal antibodies (for review, see Ringertz and Savage, 1976; Evered and Whelan, 1984; Sowers, 1987). But unlike cell division, the mechanism of cell fusion is largely unknown. When two cells fuse, the fusion process consists of at least three major steps: (1) fusion of membranes to establish the cytoplasmic bridge; (2) expansion of the cytoplasmic bridge to allow the cytoplasm of the fusing cells to merge; and (3) reorganization of the cytoplasmic structures to form a newly integrated system. Membrane fusion, as the first step of cell fusion, has been actively studied in many laboratories (for reviews, see Poste and Nicolson, 1978; Blumenthal, 1987; Ohki *et al.* 1988). The mechanism of cytoplasmic fusion, on the other hand, has not been investigated in detail. One of the reasons for this may be due to the technical difficulties of studying cytoplasmic fusion. First, the assay method is far more complicated in the study of cytoplasmic fusion than in membrane fusion. Secondly, after the initiation of fusion, the cells must be maintained in their normal physiological state for a long period for observation. Such a requirement excludes the use of some of the common fusogens that may have toxic effects. And thirdly, in order to examine the dynamic changes of the cytoplasmic structures, cell fusion must be induced by a treatment that requires very little time and the fusion process must be relatively well synchronized.

Most of the previous studies of cytoplasmic fusion relied on either chemical fusogens (e.g. PEG, Ca^{2+} , lysolecithin) or the action of viruses. These treatments cannot satisfy our requirements because they may either have toxic side-effects or require a long period of incubation. Furthermore, these chemical or biological methods usually caused uncontrollable fusion of too many cells to produce giant polykaryons. In such cases, it was difficult to examine the reorganization of cellular structures between two fusing partners.

With the development of the electric field-induced fusion method (Zimmermann, 1982; Teissie *et al.* 1982; Bates *et al.* 1987), most of these technical difficulties can be overcome. Specifically, we recently developed a method to directly fuse cells attached to the substratum by applying a very short (sub-millisecond) pulse of radio-frequency (RF) electric field (Chang, 1989a,b; Zheng and Chang, 1990, 1991). Membrane fusion occurs almost immediately following the application of the electrical pulse. The fusion yield was usually quite high (about 70%) and the fusion process was highly synchronous. Furthermore, fusion can be limited to a small number of cells by controlling cell density and electrical parameters. Thus, electrofusion of attached cultured cells provides an ideal model system for studying the dynamic process of cytoplasmic fusion.

In the study reported here, we used immunostaining methods and fluorescence microscopy to examine the dynamic changes of the various cytoskeletal structures (microtubules, F-actin and vimentin intermediate filaments) and the distribution of major organelles in the electrofused CV-1 cells. It has been suggested previously that cytoskeletal structures are involved in the reorganiz-

ation of the cytoplasmic structures during cell fusion (Zheng and Chang, 1990, 1991; Holmes and Choppin, 1968; Wang *et al.* 1979). We wanted to investigate their functional roles by examining the correlations between the cytoskeletal structures and the distribution of the major organelles at the different stages of cytoplasmic fusion.

Materials and methods

Cells

CV-1 cells (from green monkey kidney) were obtained from the American Type Tissue Collection (Rockville, MD). The cells were cultured in Dubecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 i.u. ml⁻¹ each of penicillin and streptomycin). All reagents for tissue culture were obtained from GIBCO (Gaithersburg, MD).

Electrofusion

CV-1 cells were plated on 18 mm coverslips one day ahead with a final cell density of approximately 100 cells mm⁻². Before fusion, cells were washed in fusion medium (2 mM Hepes, 280 mM mannitol, 1 mM MgCl₂, pH 7.2) twice. Then, cells were induced to fuse by exposure to a single pulse of radio-frequency electric field (pulse width 0.2 ms, pulse amplitude 1 kV cm⁻¹, frequency 100 kHz) following the procedure of Zheng and Chang (1990). After the electrical treatment, cells were recultured in normal culture medium at 37°C. After various periods of reculturing, the cells were fixed and processed for fluorescence labeling.

Drug treatment

In one of the experiments, microtubules were depolymerized by treatment with Nocodazole (methyl (5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl)-carbamate). The drug was obtained from Sigma Chemical Co. (St Louis, MO) and stocked at 1 mg ml⁻¹ in dimethylsulfoxide (DMSO). CV-1 cells grown on coverslips were first treated with 10 µg ml⁻¹ nocodazole for 1 h at 37°C before electrical treatment. After then, the cells were recultured in medium containing 10 µg ml⁻¹ nocodazole.

Immunofluorescence staining of microtubules (MTs) and intermediate filaments (IFs)

CV-1 cells were fixed in dry methanol (at -20°C) for 2 min following the procedure of Osborn and Weber (1982). After an immediate wash with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.2), the cells were incubated with individual (or a mixture of) primary antibodies (anti-tubulin or anti-vimentin mAbs in PBS containing 10% normal goat serum) at 37°C for 45 min. After washing with PBS, 0.05% Tween 20-PBS and double distilled water, the cells were incubated with secondary antibodies at 37°C for another 45 min. Then, after processing by similar washing steps, the cells were mounted in a mounting medium containing Mowiol 4-88 (Osborn and Weber, 1982).

For primary antibodies: rat anti-tubulin monoclonal antibody was obtained from Bioproducts for Science (Indianapolis, Indiana). The working dilution was 1:100. Mouse anti-vimentin monoclonal antibody was purchased from Amersham (Arlington Heights, Illinois). The working dilution was 1:20. For secondary antibodies: fluorescein (DFAT)-conjugated goat anti-mouse IgG antiserum was obtained from Jackson Immunoresearch Lab, while Texas Red-conjugated goat-anti-rat IgG antiserum was obtained from Chappel (West Chester, PA). The working dilution was 1:100.

Fluorescence staining of F-actin

CV-1 cells were fixed in 0.25% glutaraldehyde for 10 min, and permeabilized in 0.1% Triton X-100 in PBS for 5 min. After quenching of the fluorescence background of aldehyde groups with 0.5 mg ml⁻¹ NaBH₄ following the procedure of Osborn and

Weber (1982), the cells were incubated with rhodamine-conjugated phalloidin (Sigma Chemical, St Louis, 1:100 dilution from 3.3 mM stock solution in methanol) for 10 min at room temperature. After washing with PBS twice, the cells were mounted in Mowiol 4-88 medium and viewed immediately.

Fluorescence labelling of endoplasmic reticulum (ER) and mitochondria

The ER and mitochondria in glutaraldehyde-fixed cells were labeled with 2.5 µg ml⁻¹ DiOC₆(3) (3,3'-dihexyloxacarbocyanine iodide; from Sigma Chemical, St Louis, MO) following the procedure of Terasaki *et al.* (1984, 1986).

Double fluorescence staining of cytoskeletal and organelles

After the glutaraldehyde-fixed cells were labeled with DiOC₆(3), they were mounted on a glass slide in cacodylate buffer (0.1 M cacodylate, 0.1 M sucrose, pH 7.4). The structures of ER and mitochondria were examined using a fluorescence microscope and photographs were taken. Then, the cells were permeabilized by treatment with dry methanol (at -20°C) for 2 min (for MTs and vimentin IFs labeling) or with 0.1% Triton X-100 for 5 min (for F-actin labeling), and the cytoskeletal proteins were labeled following the described procedures. After mounting in Mowiol medium, the cells that had their ER and mitochondrial structures recorded previously were identified again under the microscope. Then, their cytoskeletal structures were examined and photographed. In such experiments, the coverslips were marked lightly with a diamond pen to facilitate the identification of cells.

Fluorescent images were obtained using a Zeiss Axiophot microscope (Carl Zeiss, Germany) equipped with 63× and 100× (NA 1.3 oil) objectives.

Results

Sample micrographs of control (i.e. non-fused) and fused CV-1 cells (with phase optics) are shown in Fig. 1. The CV-1 cells are relatively flat, with most of its cytoplasm concentrated near the perinuclear region. After the electrical treatment, membranes between some of the neighboring cells began to fuse within minutes. In about 10 min, numerous fusing cells connected by cytoplasmic bridges could be observed. These cytoplasmic bridges continued to expand in the next hour, while organelles (including nuclei) of the fusing cells gradually merged. Normally, it took several hours for cells to complete their fusion process at 37°C. At the end of this process, all nuclei were aggregated at the center of the polykaryon (Fig. 1b).

Cytoskeletal reorganization

Three major classes of cytoskeletal proteins: microtubules (MTs), vimentin intermediate filaments (vimentin IFs) and actin microfilaments, were observed in CV-1 cells. Each of these cytoskeletal structures was found to undergo specific reorganization during fusion process.

Microtubules. Microtubules in control CV-1 cells appeared in a pattern typical of most fibroblast cells; namely, microtubules were distributed radially from a brightly stained microtubule organizing center (MTOC) near the nucleus towards the cell periphery (Fig. 2a). When cell fusion was initiated by applying an electric pulse, narrow cytoplasmic bridges were formed between neighboring cells within a few minutes. These cytoplasmic bridges were initially free of microtubules. But within 10–15 min, microtubules began to infiltrate gradually into these cytoplasmic bridges (Fig. 2b). As the cytoplasmic bridges became wider with time, more microtubules (in the form of parallel bundles or arrays) were observed inside the

Fig. 1. Electric-field-induced fusion of CV-1 cells (shown with phase-contrast optics). (a) Control CV-1 cells; (b) a polykaryon formed by fusing 4 cells at 3 h after the electrical treatment. Bar, 35 μm .

bridges (Fig. 2c). These parallel microtubule arrays appeared as a continuous structure that connected the MTOCs of the neighboring fusing cells (Fig. 2c and d).

Later, as the cytoplasmic bridges became wider to allow the cytoplasm of the fusing cells to merge, nuclei of the fusing partners gradually moved towards each other. Parallel microtubule arrays were always observed linking these nuclei. As the nuclei moved closer together, the microtubules between them became very dense and shortened (Fig. 2e). It usually took several hours for the fusion process to be completed. At that time the nuclei were found to aggregate in the center of the polykaryon and the microtubule distribution returned to a pattern similar to that of control cells (Fig. 2f). The microtubule network appeared to radiate from an integrated MTOC that was attached to the aggregated nuclei.

Vimentin IFs. The distribution of vimentin IFs in control CV-1 cells was similar to that of microtubules, except that vimentin intermediate filaments were more heavily concentrated in the perinuclear region (Fig. 3a). When neighboring cells were induced to fuse by applying an electric pulse, the vimentin IFs were also found to infiltrate gradually into the cytoplasmic bridge (Fig. 3b). They then established linkages between the individual networks of the fusing cells (Fig. 3c), and eventually

merged to form the single vimentin IF network of the polykaryon (Fig. 3d). This process was very similar to that for microtubules.

However, there was a significant difference between the dynamics of the reorganization of microtubules and that of the vimentin intermediate filaments. Such a difference can be clearly seen in the study using a double immunostaining method (Fig. 4). Probably because the vimentin IFs in the control cells normally did not extend into the cell periphery as far as the microtubule network did (Fig. 4a and b), they were found to infiltrate the cytoplasmic bridges at a much later time than microtubules. For example, at 30 min after electrical treatment, many microtubule bundles had appeared at the cytoplasmic bridges, but very little vimentin IFs were found there (Fig. 4c and d). At 90 min after the initiation of fusion, the microtubule networks of the two fusing cells were almost connected to each other, while the vimentin IFs networks were still largely separate and were only linked with a few connecting IF bundles (Fig. 4e and f).

Like that of microtubules, the distribution of vimentin was closely related to the positioning of the nuclei. In some fusing cells, the nuclei had completed their aggregation at 2 h after the initiation of fusion. Under this situation, vimentin IFs were found to be integrated to form a combined network surrounding the nuclear cluster, a pattern similar to that of microtubules in the polykaryon (Fig. 4g and h).

Actin filaments. The distribution pattern of actin filaments (or F-actin) was completely different from those of microtubules and vimentin intermediate filaments. In control cells, F-actin appeared mainly in the form of thick bundles (stress fibers) distributed throughout the cell body in a non-radial pattern (Fig. 5a). In addition, F-actin in a diffuse form or in fine filamentous structures could normally be observed.

After cell fusion was initiated, some of the actin stress fibers gradually disappeared. With time, some actin filaments were found to be aligned along the edges of the cytoplasmic bridges (see arrow in Fig. 5b). Large sheets of cytoplasm containing diffuse or filamentous F-actin were frequently observed to extend outwards from both sides of the cytoplasmic bridges. At the edges of these sheets, lamellipodia (ruffles) that were rich in actin (see arrow-head in Fig. 5) could also be found.

At about 60 min after the initiation of fusion, the mass of the cytoplasm of the fusing cells had merged. However, the actin structures of the individual fusing partners basically remained unmixed (Fig. 5c). If the fusion involved only two cells, the actin filaments would be reorganized into an integrated network by about 2 h. But, if the polykaryon was formed by fusing a larger number of cells, the reorganization of actin would take much longer. For example, in some of the polykaryons, even at 3 h after the initiation of fusion, when the aggregation of nuclei (marked by asterisks in Fig. 5) was already complete, the actin structures still appear as separated patches with different orientation (see Fig. 5d).

Reorganization of major organelles

The organelles were observed by fluorescence staining using the lipophilic dye DiOC₆(3). With such a dye the cell membrane was very dimly stained and was barely visible in the fluorescent micrographs. The ER structures could be clearly observed, while mitochondria and some vesicular structures were very brightly stained (see Fig. 6).

Endoplasmic reticulum. With the DiOC₆(3) staining,

Fig. 2. Distribution patterns of microtubule networks in CV-1 cells during fusion. (a) Control cells; (b) CV-1 cells at $t=15$ min after the initiation of fusion. (c) $t=30$ min; (d) $t=45$ min; (e) $t=90$ min; (f) $t=2$ h. Cytoplasmic bridges filled with microtubules are marked by arrowheads. Bars: $17\ \mu\text{m}$ (d); $25\ \mu\text{m}$ for all other panels.

endoplasmic reticulum in CV-1 cells appeared as a continuous lace-like reticular structure distributed throughout most parts of the cell (Fig. 6a). ER networks could be observed most clearly in the flat areas near the cell periphery where mitochondria or other brightly stained vesicular structures were absent. Near the cell edges, even single ER tubules could be seen.

Minutes after neighbouring cells were fused, ER structures were found to infiltrate into the newly formed cytoplasmic bridges and they gradually formed a continuous structure connecting the ER networks of the fusing

cells (Fig. 6b and c). As the cytoplasmic bridges widened with time, more ER structures were observed there (Fig. 6d). Later, as the cytoplasm of the fusing cells merged, ER became a large inter-connecting network (Fig. 6e and f), similar to that observed in the microtubule system. A few hours after the initiation of fusion, when the nuclear aggregation was complete, ER were found to be reorganized into a single integrated system and appeared in a pattern similar to that of the control cells (Fig. 6g).

Mitochondria. In control CV-1 cells, the distribution of mitochondria differed from that of ER, in that mitochon-

Fig. 3. Distribution patterns of vimentin IFs in CV-1 cells at various times (t) after the initiation of fusion. (a) Control; (b) $t=30$ min; (c) $t=60$ min; and (d) $t=2$ h. Cytoplasmic bridges between fusing cells are indicated by arrowheads. Bar, 25 μ m.

dria were located mainly in the perinuclear region (Fig. 6a). The filamentous mitochondria normally had their longitudinal axes oriented in a radial direction.

When the cells were induced to fuse, mitochondria also infiltrated into the cytoplasmic bridges (Fig. 6). However, such infiltration was slower than that of ER. In most fusing cells, mitochondria began to appear to be distributed continuously through the cytoplasmic bridge at about 30 min after initiation of fusion (Fig. 6d). As the cytoplasmic bridges became wider, more mitochondria were seen inside the bridges (Fig. 6e and f). Once the fusion was complete, mitochondria accumulated around the aggregated nuclei in a pattern similar to that of control cells (Fig. 6g).

Correlation between the redistribution of organelles and the reorganization of the cytoskeletal structures

Correlation between reorganization of ER and cytoskeleton. The structural correlation between ER and microtubules was examined using the double fluorescence-labeling method. Sample micrographs showing the comparison between the structure of ER and that of microtubules can be seen in Fig. 7. The distribution of ER (Fig. 7a) appeared to be closely related to the distribution of microtubules (Fig. 7b). In fact, the distribution pattern of ER completely overlapped that of microtubules. Wherever ER structures were observed, one could find microtubule bundles in the same location. This observation

suggests that the distribution of ER must follow that of microtubules.

The distribution of ER structures, on the other hand, does not seem to be correlated with the structural patterns of vimentin IFs or actin filaments. Fig. 8 shows the paired images of ER and MTs (a and b), ER and vimentin IFs (c and d), and ER and F-actin (e and f) in fusing cells. It is clear that the ER distribution was closely correlated only with the structures of microtubules. One can see that even an isolated ER tubule corresponded to a single microtubule or bundle (see arrowheads in Fig. 8a and b). The distribution of ER overlapped with neither vimentin IFs nor with actin filaments.

Correlation between mitochondria and cytoskeleton. The correlation between the distributions of mitochondria and cytoskeleton can also be examined from results shown in Fig. 7 and 8. The mitochondria appeared to be distributed following the migration of ER, indicating that the movement of mitochondria was probably along the MT bundles (see Fig. 7). Interestingly, it appeared that the distribution of mitochondria might also be roughly correlated with that of vimentin IFs (Fig. 8c and d).

We have made a quantitative examination of the time course taken for the various cytoplasmic components to infiltrate into the cytoplasmic bridges. The results are shown in Fig. 9. MTs were by far the earliest to appear at the cytoplasmic bridges. Both the vimentin intermediate filaments and mitochondria entered the cytoplasmic

Fig. 4. Double staining of Mts and vimentin IFs in CV-1 cells at different time (*t*) after the initiation of fusion. (a,c,e,g) MTs; (b,d,f,h) vimentin IFs. (a,b) Control; (c,d) *t*=30 min; (e,f) *t*=90 min; (g,h) *t*=120 min. Cytoplasmic bridges are marked by arrowheads. Bar, 25 μ m.

bridges at almost the same rate, which was much slower than that of microtubules.

We found no correlation between the distribution of mitochondria and the structure of actin filaments (Fig. 8e and f). The reorganizations of these two structures were apparently not related.

Effect of a MT-depolymerizing drug

To investigate further the functional role of microtubules in the reorganization of the various cytoplasmic structures, we applied nocodazole to CV 1 cells to depolymerize their microtubules and studied the fusion process of these drug-treated cells. We found that: (1) the membrane fusion appeared to be unaffected by the nocodazole treatment. Neighboring cells could still be fused by applying an electric pulse. (2) Following membrane fusion, cytoplasmic bridges could also expand with time so that the cytoplasm of the fusing cells would eventually merge. (3) Reorganization of most of the cytoplasmic structures, however, was apparently inhibited by the drug treatment. Sample micrographs of these drug-treated cells at 60 min after the

initiation of fusion are shown in Fig. 10. In these nocodazole-treated cells, microtubules were completely depolymerized (Fig. 10a). The morphology of the vimentin IFs was also greatly altered; they appeared as bundles surrounding the individual nuclei, which remained unaggregated (Fig. 10b). ER and mitochondria had also collapsed around the individual nuclei. Their structures did not infiltrate into the cytoplasmic bridges (Fig. 10c).

Discussion

The results of this study clearly demonstrate that cell fusion is a highly complicated process. After the fusion of their membranes, the neighboring fusing cells must enlarge their cytoplasmic bridges, merge their cytoplasm, and reorganize their cytoplasmic components into the integrated system of the polykaryon. This is very different from the fusion of model systems used for membrane studies, such as liposomes or human red blood cells, in which the internal contents simply mix following membrane fusion.

This study suggests that reorganization of the cytoskeleton plays an important role in the process of cytoplasmic fusion, and that the reorganization of the cytoskeletal networks and the redistribution of cellular organelles are correlated. Teissie *et al.* (1989) reported that when electric-field-treated culture cells were incubated at 4°C (instead

Fig. 5. Changes in actin networks in CV-1 cells during fusion. (a) Control cells; (b) fusing cells at *t*=30 min after the initiation of fusion; (c) *t*=60 min; (d) *t*=3 h. Arrowheads point to actin ruffles, arrows point to F-actin bundles aligned along the cytoplasmic bridges. Bar, 30 μ m.

Fig. 6. Distribution patterns of ER and mitochondria in CV-1 cells during fusion. (a) Control; (b and c) $t=15$ min; (d) $t=30$ min; (e and f) $t=60$ min; and (g) $t=2$ h after the initiation of fusion. The positions of nuclei are indicated by asterisks. Bar, $25\ \mu\text{m}$.

of 37°C), cells did not fuse to form polykaryons even though the membrane fusion had taken place. Later when the cells were reincubated at 37°C for a few hours, a large number of polykaryons were observed. This result can be understood very easily on the basis of the findings of our study. When the cells were incubated at 4°C , the cellular activities (including cytoskeletal reorganization) were blocked. And thus, the process of cytoplasmic fusion could not proceed. When the cells were returned to 37°C , normal fusion could be resumed because the reorganization of the cytoskeletal structures was no longer inhibited.

The findings of this study suggest that each of the cytoskeletal proteins may play a different functional role during the fusion process. Basically, we think that microtubules probably play the most important role in cell fusion because the reorganization of MTs seems to be closely related to the redistribution of almost all the other cytoplasmic structures. The only exception is actin. F-actin seems to play an independent role. Reorganization of actin appeared to facilitate the widening of the cytoplasmic bridges and thus allow the cytoplasm of the fusing cells to merge. This interpretation is supported by at least

Fig. 7. Comparison between the distribution patterns of ER and mitochondria (a) and that of MTs (b) by a double staining method. The cells had been fused for 45 min. Bar, 10 μ m.

two observations: (1) there was no overlap between the structure of actin and the distribution patterns of other cytoplasmic components during fusion; and (2) the drug treatment experiment showed that while depolymerizing microtubules affected the reorganization of other cytoplasmic structures, it did not prevent the widening of the cytoplasmic bridges. Using time-lapse video microscopy, we have observed that the widening of cytoplasmic bridges was caused by spontaneous extension of lamellipodia, a

process clearly driven by the reorganization of F-actin (Smith 1988, Conrad *et al.* 1989).

We envisage that soon after membranes of neighboring cells were induced to fuse by electrical treatment, MTs began to infiltrate into the newly formed cytoplasmic bridges. These MT arrays appeared as bundles that connected the cytoskeletal networks of the fusing cells. These MT networks then gradually merged as the cytoplasmic bridges expanded. During the same time, the

Fig. 8. Correlation between the distribution patterns of ER and cytoskeleton in fusing cells as revealed by double staining. (a and b) ER and MTs, respectively, in fusing cells at 60 min after electrical treatment, arrowheads indicate that a single ER tubule was co-localized with a MT in the newly extended flat sheets of cytoplasm; (c and d), ER and vimentin IFs in fusing cells at 45 min after initiation of fusion. Arrowheads mark the boundary of the flat sheets of cytoplasm that were free of vimentin IFs. Arrows indicate the distributions of mitochondria and vimentin IFs in the cytoplasmic bridge; (e and f) ER and actin in fusing cells at 30 min after electric pulse. Arrowheads point to the actin-rich membrane ruffles, while the arrow in f indicates the actin-condensed edges of a cytoplasmic bridge. Asterisks indicate the positions of nuclei. Bar, 20 μ m.

other cytoplasmic structures were redistributed by migrating along the microtubules bundles in the cytoplasmic bridges.

The conclusion that reorganization of microtubules plays a leading role in the process of cytoplasmic fusion is supported by many of our observations. First, micro-

Fig. 9. Time course of infiltration of microtubules (MT), vimentin IFs (IF) and mitochondria (MC) into cytoplasmic bridges. Infiltration was defined as the appearance of one specific cytoplasmic component in the middle region of the cytoplasmic bridge. Data were summarized from observation of 20–40 cytoplasmic bridges in two experiments.

tubules were the first cytoplasmic structures that appeared inside the newly formed cytoplasmic bridges. Second, the reorganization of all major organelles (ER, vimentin IFs, mitochondria, and nuclei) was found to be closely correlated with the reorganization of MTs. Third, many known MT-depolymerizing drugs, including colchicine, colcemid and nocodazole, all had the ability to inhibit cytoplasmic reorganization (Zheng and Chang, 1990, 1991; Holmes and Choppin, 1968; Wang *et al.* 1979). When nocodazole-treated cells were fused, nuclear aggregation was inhibited; vimentin IFs, ER and mitochondria were found to have collapsed around the individual nuclei instead of becoming reorganized into the integrated system of the syncytium.

Our finding that the reorganization of ER is dependent on the reorganization of MTs is consistent with several recent studies. For example, Terasaki *et al.* (1986) reported that the structures of ER and microtubules are closely correlated during cell spreading. It was also suggested that ER may be formed by movement of a membrane system along MTs driven by MT-associated motor proteins such as kinesin (Vale and Hotani, 1988; Dabora and Sheetz, 1988; Lee *et al.* 1989).

The dependence of nuclear aggregation on the reorganization of MTs has been suggested previously (Holmes and Choppin, 1968; Wang and Choppin, 1979; Zheng and Chang, 1990), even though the precise mechanisms were not clear. During cytoplasmic fusion, many parallel microtubule bundles were found between the nuclei of the fusing cells, appearing to connect the nuclei in pairs and allow them to be pulled towards each other. The length of such MT bundles became shortened as the nuclei moved closer. Hence, it is highly probable that nuclear aggregation may be related to a MT-based motility system (Zheng and Chang, 1990).

Like ER, the redistribution of mitochondria during cell fusion apparently was related to the reorganization of microtubules. This finding is not surprising, since association of mitochondria with microtubules has been observed in many other systems (Smith *et al.* 1977; Wang and Goldman, 1978; Ball and Singer, 1982; Vale *et al.* 1985). In dissociated squid axoplasm, it was clearly demonstrated by video microscopy that mitochondria moved along microtubules (Vale *et al.* 1985; Schnapp *et al.* 1986).

The relationships between the redistribution of vimentin intermediate filaments, mitochondria and microtubules appear to be relatively complicated. Even in

Fig. 10. Distribution patterns of MTs (a), vimentin IFs (b) and ER (c) in nocodazole-treated fusing cells. The cells were fixed at 60 min after the initiation of fusion. Arrows point to the cytoplasmic bridges. Bar, 20 μ m.

control CV-1 cells, the distribution patterns of vimentin IFs or mitochondria were not the same as that of microtubules. This observations suggests that although the movement of mitochondria or the distribution of vimentin IFs may be guided by the microtubules, there are probably other factor(s) that may keep them concentrated near the nucleus. For example, several studies suggested that intermediate filaments can also affect the distribution of organelles (Wang and Goldman, 1978;

Mose-Larsen *et al.* 1982; Summerhayes *et al.* 1983; Goldman *et al.* 1986). Such a consideration might partially explain why both vimentin IFs and mitochondria infiltrated the cytoplasmic bridges at a later stage than the microtubules.

Even though this study suggests that actin bundles are not directly involved in organelle reorganization, we cannot rule out the possibility that isolated actin filaments, or actin structures associated with the plasma membrane, may play a certain role in the cell fusion process, since low concentrations of actin are very difficult to detect using the fluorescence microscopy method.

In conclusion, this study demonstrates that cytoplasmic fusion is a highly complicated process that involves a series of specific reorganizations of cytoskeletal networks and redistribution of major organelles. Our results also suggest different functional roles for the various cytoskeletal proteins. We found that microtubules appear to be the most important cytoskeletal structures and are largely responsible for the reorganization of the cytoplasmic components during cell fusion.

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