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—Original—

Electrofusion of Blastomeres of Hamster 2-Cell Embryos and Dynamic Changes of the Cytoskeletal Distribution

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Abstract. Cell fusion is an important process in current animal biotechnology. This study was designed to establish optimal parameters for the electrofusion of hamster 2-cell blastomeres and to examine the dynamic changes in the cytoskeletal distribution using fluorescence staining. Various electric fields (70–2000 V/mm) and durations (50–1000 μsec) of electric pulse were applied. Electric fields higher than 300 V/mm or of duration longer than 200 μsec caused disruption of the blastomere. Blastomere fusion occurred at 70–170 V/mm of 100 μsec duration, with high yields (91%). However, electrofused embryos did not develop. Fluorescence observations showed that the control 2-cell embryos possessed a dense network of microtubules around the nucleus and abundant microfilaments at the cell-to-cell contact region. In the fused embryos, two domains of cytoskeleton, consisting of both microfilaments and microtubules, concentrated around each nucleus were observed. In the embryos which failed to fuse, however, a thin layer of microtubules and very thick bundle of microfilaments appeared at the cell-to-cell contact region. The results suggest that blastomere fusion is accompanied with cytoskeletal reorganization and that dynamic changes of the cytoskeleton occur in different modes between fused and non-fused embryos.
Key words: Electrofusion, Embryo, Cytoskeleton, Hamster

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Recent studies have shown that cells can be fused by electric stimulation [1] and such electrofusion has been applied to nuclear transfer, in which either an embryonic cell or a somatic cell was fused with an enucleated oocyte in sheep [2–4], mice [5], cattle [6–9] and pigs [10]. Similarly, the blastomeres of 2-cell embryos have been electrofused in mice [11–13], rabbits [14] and cattle [15]. However, little is known about the optimal parameters required for the electrofusion of hamster 2-cell embryos, or about the behavior and tolerance of embryos exposed to an electric field. Also, dynamic changes in the cytoskeleton of embryonic cells during fusion have not yet been investigated, although the cytoskeleton plays an important role in fusion of somatic cells [16, 17]. Thus, the objectives of the present study were 1) to examine the efficiency of electrofusion applicable to hamster embryos, and 2) to evaluate the distributional characteristics of microtubules and microfilaments in hamster 2-cell embryos after electrostimulation.

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Materials and Methods

Collection of embryos

The experimental design was approved by the Ethical Committee for Experimentation with

Animals, Hirosaki University. Golden hamsters (*Mesocricetus auratus*), 10–12 weeks old, were kept under conditions of constant temperature (21–23 C) and a cycle of 14 h light (0500 h–1900 h) and 10 h dark. They were given laboratory chow (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and water *ad libitum*. The estrous cycle of each female was determined according to the method of Orsini [18]. In the evening on the 4th day of the estrous cycle, females were caged with males and checked for the presence of spermatozoa in the vaginal smear. Successfully mated female hamsters were sedated with ether and sacrificed by cervical dislocation. Two-cell embryos were flushed from the oviducts with Dulbecco's phosphate buffer solution (PBS; Gibco, Grand Island, NY, USA) supplemented with 0.1% polyvinylalcohol (PVA; Sigma, St. Louis, MO, USA) at 1300 h on Day 2, 32 h after the estimated time of sperm penetration [19]. The collected embryos were therefore considered to be at the middle of the 2-cell stage. Higher fusion rates of blastomeres were reported in the middle stage of mouse 2-cell embryos [13].

Electrode chamber

Two types of electrode chambers were used. One was similar to the type reported by Kubiak and Tarkowski [11]. Briefly, the electrode chamber was made of a glass Petri dish (60 mm in diameter) with a platinum block electrode (3 mm wide) glued to the bottom. The partner electrode was a needle type, made of a platinum wire (100 μm in diameter), attached to a simple micromanipulator. This is referred to as a block-and-needle electrode. One embryo was placed between the electrodes and the cleavage plane was oriented perpendicularly to the line between both electrodes. The distance between the electrodes was almost equal to the diameter of the zona pellucida (~100 μm).

The other type of electrode chamber was made of a glass Petri dish (60 mm in diameter) with two parallel platinum block electrodes spaced 0.5 mm apart (referred to as a block-and-block electrode). An alternating current (AC) field of 1 MHz and voltage of 5 V/mm was applied for 15 sec to induce alignment of the blastomeres, when this type was used.

Both types of electrode chambers were half-filled with low-conductive (14.3 kΩ) pulse solution of 0.3 M mannitol supplemented with 0.05 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.05% BSA. Before electrical stimulation, embryos were equilibrated for 5 min in the pulse solution. Electric stimulation was delivered with either LF101L- or CUY21-Electro Cell Fusion apparatus (Bex, Tokyo, Japan).

Experimental design

Trial 1. Effects of the pulse strength and duration on embryos: To examine the tolerance of hamster 2-cell embryos to the electric field, various electric fields (100–2000 V/mm) and durations (50–1000 μsec) of direct current (DC) pulses were applied using a block-and-needle type of electrode. After electric stimulation, the morphology of the embryos was observed under an inverted microscope at 5 min intervals for the first 20 min and at 10 min intervals for the next 40 min. The electrostimulated embryos were then processed for either aceto-lacmoid staining or fluorescence labeling to examine the integrity of the plasma membrane and to evaluate the distributional characteristics of the cytoskeleton, respectively.

Trial 2. Changes in cytoskeleton and development of fused embryos: On the basis of the results from Trial 1, the magnitude of DC pulses and the pulse number were examined to choose a suitable parameter for blastomere fusion of hamster 2-cell embryos. Fusion of the blastomeres was evaluated similarly to Trial 1 for 1 h post-stimulation and classified into 3 groups, completely fused, partially fused or failed to fuse, followed by processing for fluorescence or lacmoid staining.

Some of the electrofused embryos were cultured in 100-μl drops of modified HECM-3 [20] containing 0.1% PVA under mineral oil at 37.5 C in an humidified atmosphere of 10% CO_2, 10% O_2 and 80% N_2. *In vitro* development of the fused embryos was compared with that of diploid control embryos with or without mannitol treatment.

Fluorescence observations

Methods for preparing samples for fluorescence observations have been reported previously [21]. Briefly, embryos were fixed in a microtubule stabilizing buffer [22] at 37 C for 1 h, washed extensively and blocked overnight at 4 C in the wash medium (calcium-free PBS containing 2% BSA, 2% goat serum, 0.2% milk powder, 0.2% sodium azide and 0.1% Triton-X). The fixed samples were then exposed overnight (at 4 C) to anti-β tubulin primary antibodies (1:200; Sigma),

washed, and then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200; Sigma) at 37 C for 2 h. After rinsing, the samples were stained with rhodamine-phalloidin (1:1000; Molecular Probes, Eugene, OR, USA) for microfilaments for 1 h, and washed with the wash medium, then stained for DNA with Hoechst 33342 (10 μg/ml) in mounting medium containing PBS and glycerol (1:1). The oocytes were finally mounted on slides and examined to assess their nuclear morphology and the distribution of microtubules and microfilaments.

The samples were viewed under an Olympus microscope (BX-FLA, Olympus, Tokyo, Japan). A U-MNIBA filter set (Olympus) was used for FITC, a U-MWIB set (Olympus) was used for rhodamine, and a U-MWU set (Olympus) for Hoechst. A cooled CCD video system (ImagePoint, Photometrics Ltd., Tucson, AZ, USA) was used to obtain images on a computer and color adjustment was done with IPLab-Spectrum P software (Signal Analytics Corporation, Vienna, VA, USA).

Data analysis

Data on fusion rates were arc sin transformed and then assessed by analysis of variance with the help of the BMDP program (BMDP Statistical Software, Inc., Los Angeles, CA). Differences between the treatments were determined using Tukey's multiple range test.

Results

Trial 1. Effects of pulse strength and duration on embryos

Pulse Strength: The output voltages of 10-200 V were tested using a block-and-needle type of electrode when the pulse duration was kept constant at 50 μsec. Therefore, the electric field strength ranged from 100 to 2000 V/mm, because of the approximately 100 μm distance between the electrodes. The results are shown in Table 1. Non-stimulated controls and almost all embryos stimulated at 100–150 V/mm suffered no remarkable changes in the membrane integrity until 1 h post-stimulation. Half of the embryos stimulated at 300 V/mm showed deformation of the blastomeres and decreased size of the nucleus in either or both blastomeres. At electric fields greater than 600 V/mm, all embryos were deformed or lysed.

Pulse duration: Table 2 summarizes the effect of pulse duration on the integrity of the plasma membrane of the blastomeres at a constant electric field (100 V/mm). The majority of embryos treated at durations longer than 200 μsec were lysed during the 1-h incubation. The embryos stimulated for durations longer than 400 μsec often showed rupture or melting of the zona pellucida facing the anode, which was on the needle side of the block-and-needle type of electrode, with 66, 50 and 75% of incidence for 400-, 600- and 800-μsec groups, respectively. Fusion of the blastomeres occurred with double pulses of 100 μsec and 200 μsec (19 and 20%, respectively).

Morphological observations: Intact nuclei of the blastomeres became encircled with long microtubules radiating from the nuclear envelope towards the cytoplasm (Fig. 1a and b). Microfilaments were observed beneath the cell membrane and were more concentrated in the region of cell-to-cell contact (Fig. 1a and c). Rupture of the zona pellucida facing toward the anode following long electric pulses as mentioned above allowed us to distinguish which blastomere was on the anode side at the time of observation. The blastomere nearest the anode appeared to expand and the cytoskeleton decreased in density compared with the controls (Fig. 1d and e). On the other hand, the sister blastomere on the cathode side was affected in different modes, and the cytoskeletal elements diffused throughout the cytoplasm (Fig. 1e and f). Tubulin staining also showed some leakage of tubulin particles into the perivitelline space (Fig. 1d and e). The nucleus and nucleoli in the blastomere often decreased in size on the anode side as observed by lacmoid staining (photo not shown).

Trial 2. Changes in cytoskeleton and development of fused embryos

Optimum fusion condition: The strength of DC pulses (70, 150 and 170 V/mm) and the pulse number (1 or 2 times) were examined at a constant duration of 100 μsec. The results are summarized in Table 3. The maximum rate of fusion was observed when the embryos were exposed to a single DC pulse at 150 V/mm in both types of electrodes: 77% in the block-and-needle type and 91% in the block-and-block type. The minimum fusion rate was noticed when exposed to double

Table 1. Effect of pulse strength on the integrity of plasma membrane of hamster 2-cell embryos

DC pulse (V/mm)*	No. of embryos	Percentage of embryos showing the plasma membrane was:		
		Intact	Deformed	Lysed
Control	19	100		
100	14	100		
125	10	100		
150	20	70	30	
300	8	50	50	
600	8		100	
1000	7		15	85
1250	5			100
2000	15			100

*, Pulse duration was 50 μsec. Block-and-needle type of electrode was used.

Table 2. Effect of pulse duration on the integrity of plasma membrane of hamster 2-cell embryos

Pulse duration* (μ sec)	No. of embryos	Percentage of embryos showing the plasma membrane was:		
		Intact**	Deformed	Lysed
Control	16	100 (0)		
50 × 2	6	50 (50)		
100 × 2	21	29 (33)	33	5
100 × 4	18	27 (17)	39	17
200	5	40 (0)		60
200 × 2	12	8 (8)	25	59
200 × 4	6		50	50
400	6	17 (0)	33	50
400 × 2	8		38	62
600	6		33	67
600 × 2	8		13	87
800	4	25 (0)		75
800 × 2	8		50	50
1000 × 2	8			100

*, Pulse strength was 100 V/mm. Block-and-needle type of electrode was used.
**, Figures in parentheses show percentages of the embryos whose blastomere(s) displayed the nucleus reduced in size.

170 V/mm DC pulses in both types of electrodes. The electrofusion procedure was more efficient in the block-and-block type of electrode chamber, because many two-cell embryos were charged with AC to orient the cleavage plane without manipulation.

Course of fusion: Within 20 min after applying a single 150 V/mm DC pulse, about 80% of the embryos became more compact and the boundary between the blastomeres became less distinct. Namely, the blastomeres changed from a point-to-point contact of the membrane to a flattened membrane contact zone. At this time, the embryo became oval in shape, and then merged into one round cell (Fig. 2a).

Dynamic changes in cytoskeletal distribution: In the embryos fused completely, the microtubules in the peripheral cytoplasm were distributed more sparsely (Fig. 2a and b) than that in non-stimulated control embryos (Fig. 1a and b), and showed a delicate microtubule network. Microfilaments at the cell boundary disappeared either completely or almost completely and abundant microfilaments appeared around the nucleus, suggesting

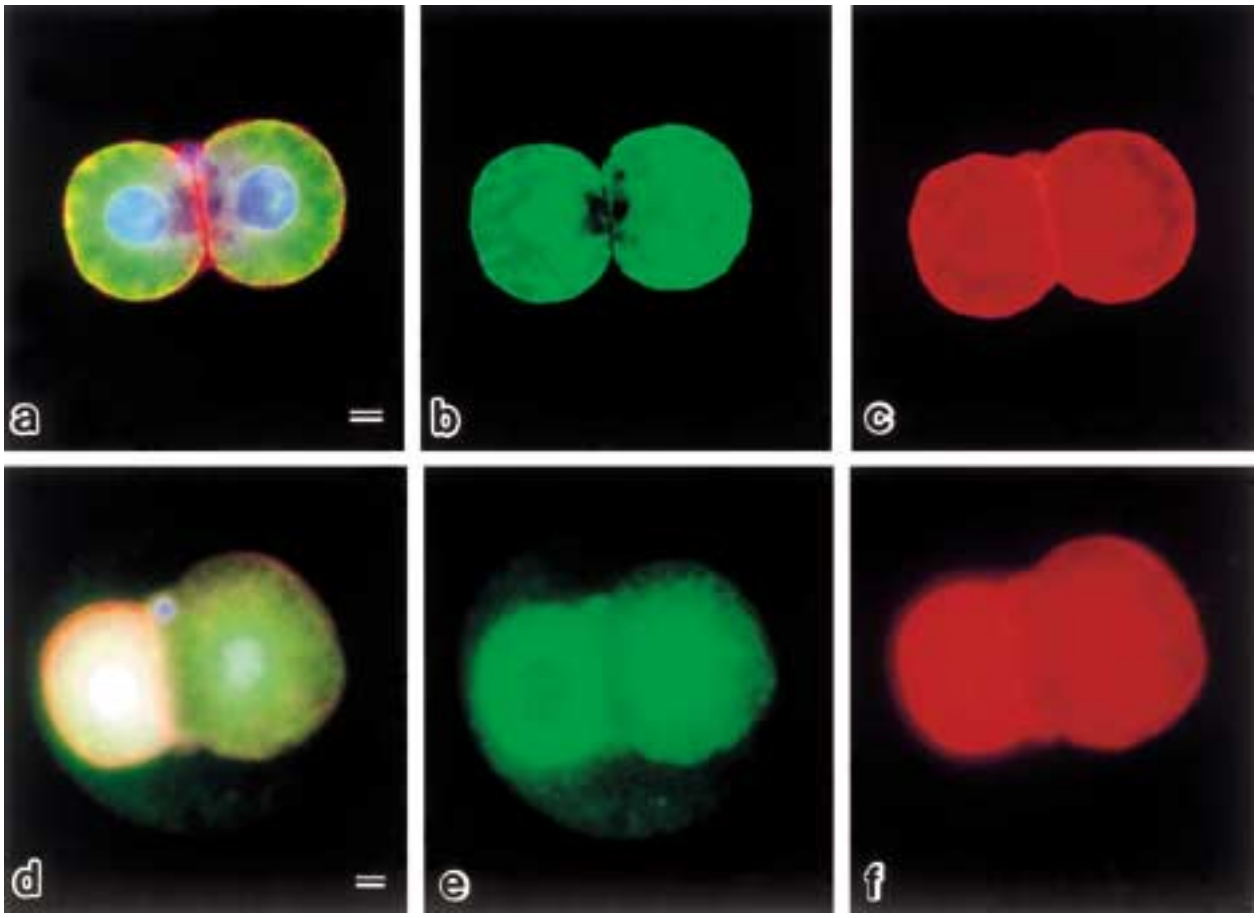

Fig. 1. Fluorescent micrographs of hamster 2-cell embryos before (a–c) and after (d–f) electrostimulation (double DC pulses at 100 V/mm of 600 μsec). Bar represents 10 μm. Microtubules are green; microfilaments are red and nuclei are blue; yellow shows the overlap of microtubules and microfilaments. The blastomere on the cathode (on the left side) decreased greatly in size compared to its counterpart. Notice the diffusion of microtubules (e) and microfilaments (f) throughout the cytoplasm on the cathode. Microtubule staining (e) shows some leakage of depolymerized elements into the perivitelline space.

Table 3. Fusion rates of hamster 2-cell blastomeres in different electric fields

Electrode type	DC pulse (V/mm)	Pulse number	No. of embryos	Percentage of embryos* showing:		
				Nonfused	Fusing	Fused
Block and needle	150	1	35	8 ± 6	15 ± 6	77 ± 5
	150	2	30	11 ± 5	33 ± 7	56 ± 9
	170	1	29	23 ± 9	24 ± 5	52 ± 11
	170	2	30	27 ± 11	26 ± 9	47 ± 7
Block and block	70	1	32	28 ± 7	14 ± 5	53 ± 9[b]
	70	2	32	16 ± 10	15 ± 6	62 ± 8
	150	1	96	2 ± 2	8 ± 4	91 ± 4[a]
	150	2	64	12 ± 9	17 ± 8	71 ± 10
	170	1	62	9 ± 8	18 ± 4	70 ± 9
	170	2	63	13 ± 7	40 ± 9	48 ± 10[c]

*, Means ± s.e.m.

[a-c], Fusion rates with different superscripts are significantly different ([a,b] $P<0.05$; [a,c] $P<0.01$).

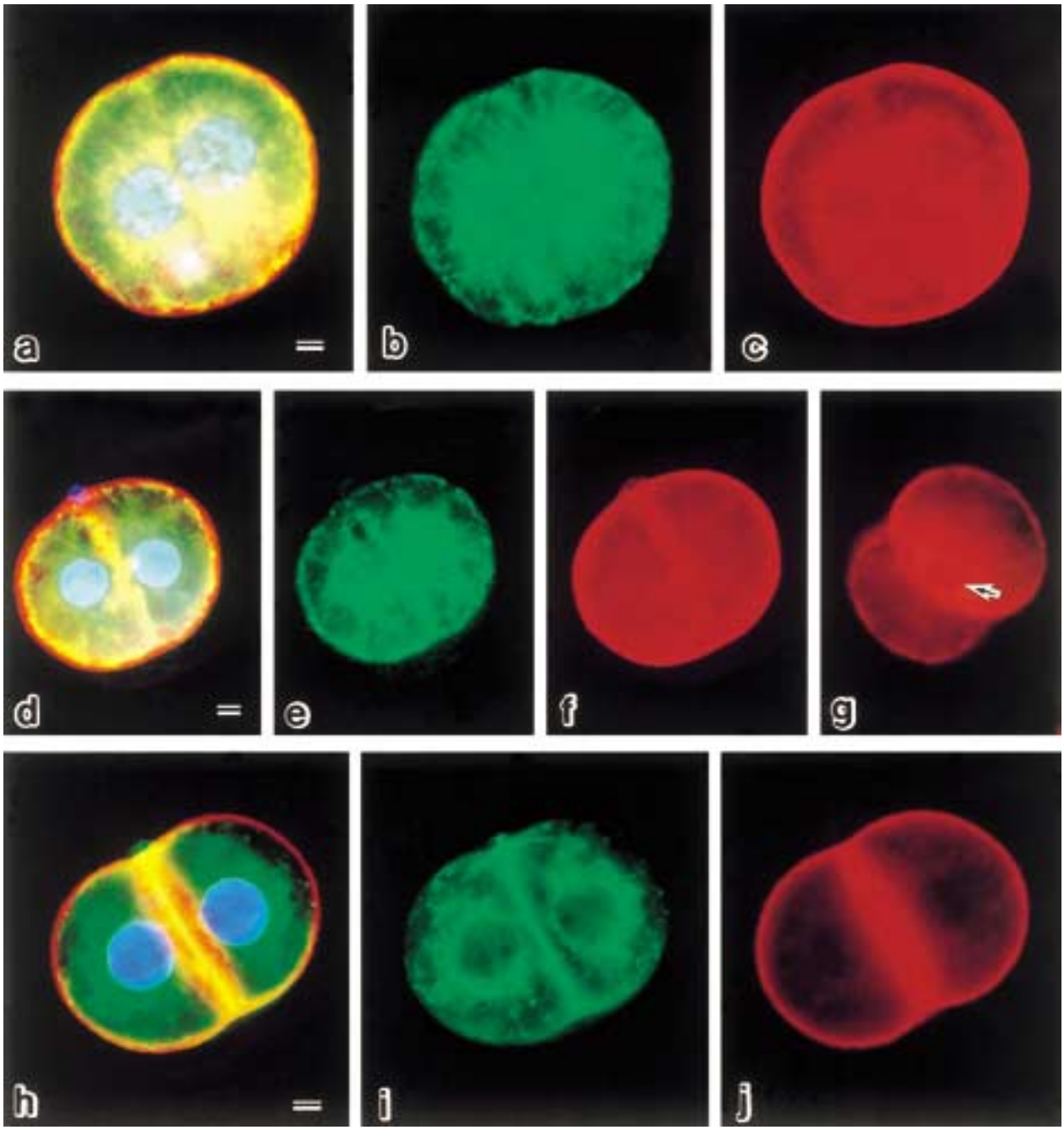

Fig. 2. Fluorescent micrographs of the embryos fused completely (a–c) or partially (d–g), and those which failed to fuse (h–j) after electrostimulation of 2-cell embryos. Bar represents 10 μm. Explanations of colors are the same as in Fig.1. A fused embryo stimulated by double pulses at 70 V/mm of 150 μsec (a–c). A partially fused embryo post-stimulation of a single 150 V/mm pulse of 150 μsec (d–f). An embryo treated by double 150 V/mm pulse of 150 μsec (g). Note a pore (a decreased portion in staining) on the microfilament zone between the sister blastomeres (arrow). An embryo failed to fuse after a single 70 V/mm pulse of 150 μsec (h–j). Note distinct zones of microtubules (i) and microfilaments (j) at the cell-to-cell contact region.

redistribution of actin structures (Fig. 2a and c). Nuclear fusion was never observed in these embryos (Fig. 2a).

The embryos judged as 'partially fused' showed an oval shape, and formed a relatively large zone of contact (Fig. 2d). Two microtubule domains

including each nucleus became closer (Fig. 2e) and the microfilament zone of the cell boundary was usually fractured (Fig. 2f). A pore was observed on the microfilament zone of the cell-to-cell contact region, when viewed from an oblique angle (Fig. 2g).

In the embryos which failed to fuse, some flattening of the blastomeres was achieved in the area of membrane contact, where a distinct cell membrane was still observed (Fig. 2h). In these embryos, a thin layer of microtubules was found, although this structure was never observed in the control embryos (Fig. 2i vs. 1b). In addition, a clearly discernible boundary of microfilaments, which became thicker than that in the controls, was noticed at the cell-to-cell contact region (Fig. 2j).

Development of electrofused embryos: The majority (73%, 22/30) of the freshly collected embryos developed to the morula and blastocyst stages, whereas 20 (67%) of 30 embryos treated with mannitol solution were blocked in their development at the 4- to 8-cell stages. None of the 37 embryos electrofused by a single DC pulse at 70-170 V/mm exhibited further cleavage in contrast to the non-stimulated controls.

Discussion

High fusion rates of 2-cell embryos were reported in rabbits at field strengths of 250 or 300 V/mm and 35 to 1000 μsec duration [14]. In mouse 2-cell embryos, the maximum rate of fusion was obtained by double pulses of 250 μsec [11] or 150 to 200 μsec [12] at 100 V/mm. The optimal fusion condition for hamster 2-cell blastomeres revealed in this study (150 V/mm, 100 μsec) was almost similar to that in mice [11, 12], but very different from that in rabbits [14]. Although blastomere fusion of the hamster 2-cell embryos was induced with high yields (91%) in this study, the electrofused embryos underwent no further development *in vitro*. In addition, development of non-stimulated embryos after equilibration with a 0.3 M mannitol fusion medium for 5 min was blocked by the 8-cell stage, whereas 73% of freshly collected embryos developed beyond the morula stage *in vitro*. These observations suggest that the fusion medium and/or longer manipulation *in vitro* may adversely affect the early development of the hamster embryos. This contrasts with the findings in other species. The blastomeres of 2-cell embryos have been induced to fuse using electric pulses at high rates and the tetraploid embryos developed to the morulae and blastocysts in mice [11–13], rabbits [14] and cattle [15].

Fluorescence observations demonstrate that cell fusion is accompanied by dynamic changes in the microtubules and microfilaments. A great decrease in the distribution density of microtubules after electrostimulation suggests a partial depolymerization. The alterations in distribution and behavior of microtubules and microfilaments might affect normal formation of the mitotic spindle and the contractile ring, respectively. This may be one of the explanations for the lack of progress in development of the electrofused hamster embryos. It is noteworthy that distribution of the cytoskeleton is characteristically different between the fused and non-fused embryos. In the latter, abundant microfilaments were concentrated at the cell boundary rather than near the nucleus. The microtubules also appeared at the cell boundary of such embryos unlike the controls. The embryos fused partially showed a fragmented or pored microfilament zone at the cell-to-cell contact region, thereby the intracellular components might be exchanged. However, it is not clear if the blastomeres would have fused completely into one tetoraploid cell accompanied by redistribution of the cytoskeleton, if the incubation time had been prolonged.

The alternative explanation for the loss of developmental ability in the hamster embryos is that ionic concentration gradients directly affect the viability by subjecting the ooplasm to deleterious effects of ionic stress. The electrofusion medium used in this study contained an extremely low ionic concentration of Mg^{2+} (0.1 mM) and Ca^{2+} (0.05 mM) compared with those in modified HECM-3 (0.46 mM and 1.9 mM, respectively [20]). Hamster 2-cell embryos can develop to the blastocyst stages in media with a relatively wide range of both calcium (0.5–2.0 mM) and magnesium ions (0.125–0.5 mM) [23]. In a preliminary study, however, we observed a great decrease in development of the hamster 2-cell embryos when cultured in modified HECM-3 with low concentrations of $MgCl_2$. Namely, the developmental rates beyond the morula stage were 0 and 7% in the HECM-3 containing 0 and 0.23 mM of $MgCl_2$, respectively, whereas 71% developed in the original HECM-3 containing 0.46 mM of $MgCl_2$

(Suzuki, unpublished observation). Therefore, it is possible that optimal development occurs in a much smaller range of ionic concentrations, and is dependent on the culture conditions employed: e.g. amino acids supplemented were 4 in [23] (2 amino acids in ours), and oxygen concentration at culture was 19% (10% in ours). Lane *et al.* [24] showed that increasing the magnesium concentration in the medium prevented an increase in intracellular calcium ion concentrations of cultured 2-cell hamster embryos, suggesting that the extracellular magnesium concentration is acting as a regulatory factor for calcium homeostasis in the embryos. The stress of transferring the embryo between media with wide ranges of calcium and magnesium ions (e.g. from the physiological condition to the extremely low concentration of extracellular ions and vice versa) might disrupt the intracellular calcium homeostasis, resulting in the loss in development observed in culture of electrofused embryos. Optimal culture condition for hamster embryos is a matter for further investigation.

In conclusion, the cell function of the embryos may be more drastically affected by electrostimulation and the electrofusion medium in the hamster than in the other species [11–14], even if the blastomeres fuse and the plasma membrane is maintained normally. Further research is warranted to develop a reliable method for blastomere fusion of hamster embryos by using a weaker electric field (e.g. repeated pulses of lower electric strength) or by using physiologically balanced media (e.g. PBS [12, 25]) to minimize the effects of the electrofusion medium.

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