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The Functional Role of Maturation Promoting Factor in the Two-cell Embryos

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A possible involvement of maturation promoting factor (MPF) in the two-cell block phenomenon was studied by fusion experiments. Germinal vesicle (GV) oocyte was fused with a blastomore from late or blocked 2-cell mouse embryos, and germinal vesicle breakdown (GVBD) of fused GV oocytes in the presence of dbcAMP (100 μg/ml) was scored as an index of MPF activity. GVBD was induced approximately 30% by fusion of a blastomere derived from late 2-cell embryos, but not from blocked 2-cell embryos. The rate of GVBD was changed when GV oocyte was fused with a blastomere from late 2-cell embryos which were treated with α-amanitin, puromycin or colcemid before and after fusion: Treatment of late 2-cell embryos with puromycin (50 μg/ml) but not with α-amanitin (100 μg/ml) clearly inhibited GVBD, indicating that *de novo* protein synthesis may be required for the appearance of MPF activity in late 2-cell embryos. Treatment of late 2-cell embryos with colcemid (0.1 μg/ml) doubled GVBD, presumably due to the maintenance of metaphase or mitotic phase. SDS-PAGE and two-dimensional electrophoresis revealed that there was no difference in protein synthetic pattern in late and blocked 2-cell embryos, but three phosphoproteins with 27, 35 and 46 kDa, presumably M-phase components were phosphorylated in late 2-cell embryos but not in blocked 2-cell embryos. It seems then that MPF activity is closely related to phosphorylation of M-phase components in late 2-cell embryos.

KEY WORDS: Maturation promoting factor, Cell fusion, 2-cell block, GVBD, Protein synthesis, Phosphorylation

In the most of Swiss albino or inbreed strain mice, embryonic development is rather restricted to 2-cell stage when embryos recovered from reproductive track prior to the early 2-cell stage and cultured under the conventional culture condition. This phenomenon is known as the '*in vitro* 2-cell block' and the blocked 2-cell embryos appear to be arrested in G2 phase of the second cleavage (Biggers *et al.*, 1971; Goddard and Pratt, 1983; Pratt, 1987). Although the cause of '*in vitro* 2-cell block' is not known, it has been known that the 2-cell block can be overcome by adding a trace amount of EDTA to culture medium (Abramczuk *et al.*, 1977; Whitten and Biggers, 1968), or replacing the blocked 2-cell embryos to the oviduct (Whittingham, 1968).

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Muggleton-Harris *et al.* (1982) previously showed that microinjection of 'non-blocking' cytoplasm to 'blocked 2-cell embryo' overcame the '*in vitro* 2-cell block' and achieved further development. The microinjection of cytoplasm from *in vivo* 2-cell embryo into *in vitro* 1-cell embryo also obviated the 2-cell block (Suzuki *et al.*, 1988). Kim *et al.* (1986) suggest that maturation promoting factor (MPF) activity appears to be present in 'late 2-cell embryo', but absent in 'blocked 2-cell embryo'. Pratt and Muggleton-Harris (1988) recently showed that a cytoplasmic factor from non-blocking cytoplasm is able to rescue the '*in vitro* 2-cell block' and the activity is high during the transition between G_2 and M phase and declines thereafter.

MPF is known to be the universal trigger for entry into M-phase (Masui and Shibuya, 1987). It is known that MPF exists in mouse 4-cell embryos (Balakier, 1978) and mammalian cultured cells (Sunkara *et al.*, 1979; Nelkin *et al.*, 1980). It is then possible to presume that the 2-cell block phenomenon in mouse embryos may be due to absence (or inactivation) of a cytoplasmic factor, presumably MPF. In order to elucidate the functional role of MPF in the 2-cell block phenomenon, a series of fusion experiments in which GV oocyte was fused with a blastomere obtained from late or blocked 2-cell embryos, was performed, and comparisons of protein synthesis and phosphorylation patterns between late and blocked 2-cell embryos were made.

Materials and Methods

Culture of oocytes and 2-cell embryos

Fully grown oocytes with GV were obtained from 3-week-old ICR strain mice (Seoul National University Animal Breeding Center). The zona pellucida of GV oocytes was removed by 0.5% pronase (Calbiochem) solution with 0.1% polyvinylpyrrolidone (PVP, Sigma) for 3 min. GV oocytes were thoroughly washed with Standard Egg Culture Medium plus 0.4% bovine serum albumin (SECM + BSA) (Biggers *et al.*, 1971). The oocytes were placed in SECM + BSA media containing 100 μg/ml of dbcAMP (Sigma) to prevent the spontaneous meiotic maturation (Cho *et al.*, 1974) under mineral oil in a humidified atmosphere of 5% CO_2 in air at 37° C until use.

Two-month-old ICR female mice were superovulated by injecting of 5 I.U. pregnant mare's serum gonadotropin (PMSG, Intervet) and followed by 5 I.U. human chorionic gonadotropin (hCG, Intervet) 46-48 hr later. The females were then mated with males and checked a vaginal plug in the morning of next day. One-cell or early 2-cell embryos were obtained from the oviduct 30 hr post hCG and cultured in SECM + BSA at 37° C for 18 hr to prepare blocked 2-cell embryos. Late 2-cell embryos were recovered by flushing the oviduct 48 hr post hCG. Cumulus cells were removed by incubation for 5 min with 300 USP/ml hyaluronidase (Sigma) in Dulbecco's phosphate buffered saline (PBS, pH 7.4).

Treatment of late 2-cell embryos with metabolic inhibitors

To determine the functional role of MPF in late 2-cell embryos, late 2-cell embryos were treated with several metabolic inhibitors: 1) α-amanitin (100 μg/ml, Behringer Mannheim Biochem.), an inhibitor of transcription of embryonic genes, 2) puromycin (50 μg/ml, Sigma) to inhibit embryonic protein synthesis, and 3) colcemid (0.1 μg/ml, Sigma) to arrest in M-phase by disrupting the formation of spindle fiber. The three metabolic inhibitors were treated for 4-6 hr prior to or after fusion.

Fusion of GV oocyte with a blastomere derived from 2-cell embryos

The zona pellucida of late or blocked 2-cell embryos was removed by treatment with 0.5% pronase for 4 min. Blastomeres of 2-cell embryos were separated by gentle pipetting after incubation with Ca^{++}-free SECM for 20 min. To recover from the damage, separated blastomeres were placed in SECM + BSA for 30 min. The fusion of oocyte with a blastomere was performed by method described previously (Gulyas *et al.*, 1984). Briefly, a blastomere was attached with GV oocyte using phytohemagglutinin (PHA, 400 μg/ml, Sigma). After washing with SECM thoroughly, pairs of oocyte-blastomere were transferred to media

containing 45% (w/v) polyethylene glycol (PEG, M.W. 1000, Sigma) in SECM for 60-90 sec. Pairs of oocyte-blastomere were then washed several times with SECM + BSA media containing 100 μg/ml dbcAMP. To prevent possible changes of pH and the spontaneous maturation of GV oocytes during operation, 20 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES, pH 7.4) and 100 μg/ml of dbcAMP were added to media used.

Observation of fused eggs

After inducing the fusion, pairs of oocyte-blastomere were transferred to a drop of SECM + BSA media containing 100 μg/ml dbcAMP, and cultured at 37 °C in 5% CO_2 for 4 hr. The fusion and GVBD were observed under stereomicroscope (x100), and the fusion number and the rate of GVBD of fused eggs were scored. For a better observation of GVBD, the fusants were fixed for 2 hr in fixative consisting of 3 volume of ethanol and 1 volume of glacial acetic acid, stained with 0.5% lacmoid (Sigma), and then observed under phase-contrast microscope.

^{35}S-methionine and ^{32}P-orthophosphate labeling of 2-cell embryos

To compare protein synthesis patterns, late and blocked 2-cell embryos were labeled in SECM + BSA media containing 100 μCi/ml of ^{35}S-methionine (specific activity; 1,100 Ci/mmole, New England Nuclear). Blocked 2-cell embryos obtained 48 hr and 60 hr post hCG were labeled for 4 hr and late 2-cell embryos derived 48 hr post hCG were labeled for 4 hr. For $^{32}PO_4$ labeling, late and block 2-cell embryos obtained 48 hr post hCG were cultured in phosphate-free SECM + BSA containing 500 μCi/ml of lyophilized ^{32}P-orthophosphate (carrier-free, Amersham) for 4 hr in the presence of colcemid (0.1 μg/ml). Colcemid was added to prevent mitotic cleavage during labeling. After labeling, embryos were thoroughly washed with PBS, placed in 20 μl of SDS-sample buffer for SDS-PAGE or lysis buffer for 2-D gel electrophoresis, and stored at −70° C until use.

SDS-polyacrylamide and Two-dimensional gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the methods of Laemmli (1970) using a linear 8-15% gradient separating gel and a 4% stacking gel. Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (1975). The isoelectric focusing was carried out with pH gradient (4.5 to 7.0) and the second dimension on a linear gradient (8-15%) polyacrylamide containing 0.1% SDS. One hundred embryos were usually used for 2-D gel electrophoresis. After electrophoresis, gels were fixed by immersing in 10% acetic acid, and processed for fluorography as described by Bonner and Laskey (1974). The gels were dried in vacuum at 80° C and exposed to X-ray film (Kodak, X-Omat AR) under intensifying screen at −70° C for 1-4 weeks.

Results

Effect of metabolic inhibitors on MPF activity in late 2-cell embryos

To confirm the presence of MPF activity in mouse 2-cell embryos, GV oocytes were fused with either blocked or late 2-cell embryos, and the rate of GVBD of fusants in the presence of 100 μg/ml dbcAMP was examined (Fig. 1). GVBD was approximately 30% in GV oocytes fused with a blastomere from late 2-cell embryos, but null in GV oocytes fused with blastomere from blocked 2-cell embryos (Table 1). These data indicate that late 2-cell embryos have a capacity of MPF activation and are able to overcome the meiotic inhibiting effect of dbcAMP on GVBD as shown previously (Cho *et al.*, 1974).

To examine the effect of metabolic inhibitors on the activation of MPF in late 2-cell embryos, late 2-cell embryos were treated with α-amanitin, puromycin and/or colcemid for 6 hr prior to fusion and then fused with GV oocytes. As shown in Table 2, the blastomere pretreated with α-amanitin induced GVBD of fused GV oocytes, which was similar to that observed in the control group, whereas the blastomere pretreated with puromycin failed to undergo GVBD of fused GV oocytes. GV oocytes fused with blastomeres pretreated with colcemid doubled the rate of

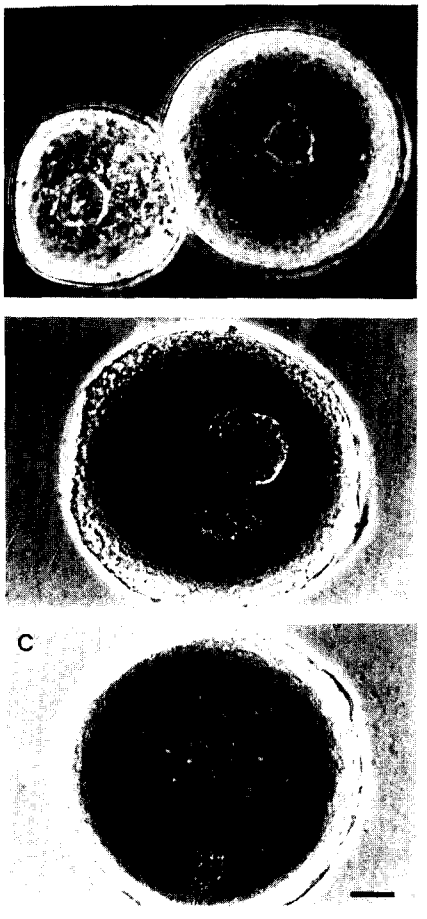

Fig. 1. Photomicrographs showing the fusion between GV oocyte and blastomere from 2-cell embryo. A: The pair of GV oocyte and blastomere agglutinated by phytohemagglutinin. B: The GV oocyte fused with blastomere by treatment with polyethylene glycol. C: The fused eggs showing GVBD of GV oocytes. Bar is 15 μm.

GVBD (60 %) in comparison with the control value. Similar results were also evident when GV oocytes were fused with blastomere derived from late 2-cell embryos which were treated with some combination of the drugs (Table 3). To further confirm the above results and to test the possibility of appearance of MPF activity after fusion, a blastomere of late 2-cell embryos was fused with GV oocyte, and then incubated in the presence of α-amanitin, puromycin and/or colcemid, respectively (Table 4). In all cases, GVBD was quite similar to that shown in Table 2.

Comparison of protein synthesis and phosphorylation patterns between late and blocked 2-cell embryos

The above results indicate that protein synthesis may be required for the appearance of MPF activity in late 2-cell embryos. The protein synthesis pattern was thus analyzed using late and blocked 2-cell embryos. Two-dimensional gel electrophoresis showed that the overall pattern of protein synthesis was very similar between the late and blocked 2-cell embryos. Several proteins (designated by arrowheads in Fig. 2) were, however, synthesized in late 2-cell embryos, but not in blocked 2-cell embryos. Blocked 2-cell embryos persisted to synthesize other proteins (designated by arrows in Fig. 2), which were found in stage prior to M-phase during the second cleavage of mouse embryos (Kang *et al.*, 1989). Moreover, when protein synthesis pattern of blocked 2-cell embryos was examined during degeneration period, the overall activity appeared to be decreased, although the pattern of protein synthesis was nearly unchanged (Fig. 2C). It is

Table 1. GVBD of GV oocyte fused with a blastomeres from blocked or late 2-cell embryos in the presence of dbcAMP

Type of embryos	No. of pairs	No. of fusion	No. of GVBD (%)	No. of degeneration*
Blocked 2-cell embryos	46	22	0(0.0)	4
Late 2-cell embryos	45	21	6(28.6)	6

The pairs of oocyte-blastomere were incubated in SECM + BSA media containing 100 μg/ml dbcAMP for 4 hr and then examined GVBD of fused GV oocytes. All of unfused GV oocytes were arrested at GV stage. Unfused blastomeres from late 2-cell embryos, but not from blocked 2-cell embryos were cleaved. Experiments were the sum of four replicates. *, Number of degenerated oocytes or blastomeres.

Table 2. GVBD of GV oocyte fused with a blastomere from late 2-cell embryos pretreated with metabloic inhibitors

Pretreatment	No. of pairs	No. of fusion	No. of GVBD (%)	No. of degeneration*
control	53	32	9(28.1)	1
α-amanitin (100 μg/ml)	40	26	7(26.9)	6
puromycin (50 μg/ml)	55	33	0(0.0)	0
colcemid (0.1 μg/ml)	39	22	13(59.1)	1

Late 2-cell embryos obtained 48 hr post hCG were pretreated with either α-amanitin, puromycin or colcemid for 6 hr and then the blastomeres were fused with GV oocytes. Observation was made following 4 hr-culture in the presence of dbcAMP (100 μg/ml). All of unfused GV oocytes were maintained at GV stage. Unfused blastomere pretreated with α-amanitin were cleaved, but the unfused blastomere pretreated with puromycin or colcemid failed to cleave. Data from four experiments were pooled. *, Number of degenerated oocytes or blastomeres.

Table 3. Effects of combination of metabolic inhibitors on GVBD of GV oocyte when fused with a blastomere from late 2-cell embryos

Pretreatment	No. of pairs	No. of fusion	No. of GVBD (%)	No. of degeneration*
Control	58	30	9(30.0)	2
Colcemid + α-amanitin	61	29	13(44.8)	5
Colcemid + puromycin	71	35	0(0.0)	2
α-amanitin + puromycin	56	31	0(0.0)	3

Late 2-cell embryos were pretreated with combination of α-amanitin (100 μg/ml), puromycin (50 μg/ml) and/or colcemid (0.1 μg/ml) for 6 hr prior to fusion. A blastomere of 2-cell embryos was fused with GV oocytes, and fused GV oocytes were cultured in medium containing dbcAMP (100 μg/ml) for 4 hr. Data were the sum of five replicates. *, Number of degenerated oocytes or blastomeres.

Table 4. Effect of metabolic inhibitors on GVBD of GV oocyte after fusion with a blastomere of late 2-cell embryos

Treatment	No. of pairs	No. of fusion	No. of GVBD (%)	No. of degeneration*
Control	48	23	7(30.4)	4
α-amanitin	50	23	7(30.4)	3
puromycin	51	24	0(0.0)	3
colcemid	50	21	11(52.4)	3

Late 2-cell embryos obtained 48 hr later post hCG were fused with GV oocytes. The pairs of oocyte-blastomere were incubated with medium containing α-amanitin (100 μg/ml), puromycin (50 μg/ml) or colcemid (0.1 μg/ml) in the presence of dbcAMP (100 μg/ml) for 4 hr. Experiments were repeated by five times. *, Number of degenerated oocytes or blastomeres.

evident that specific proteins found in late 2-cell embryos were not synthesized in blocked 2-cell embryos.

It appears to be possible that there exists a possible difference in the pattern of protein phosphorylation between late and block 2-cell embryos, since protein phosphorylation and MPF activity are known to be closely correlated and increased during transition from G_2 stage to M-phase (Miake-Lye and Kirschner, 1985; Karsenti

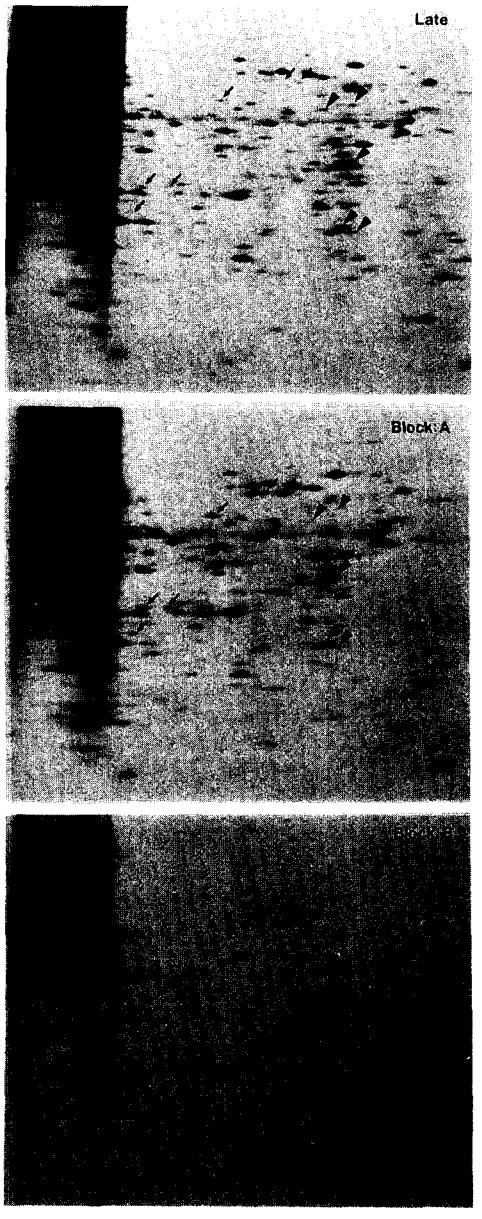

Fig. 2. Protein synthesis patterns of late and blocked 2-cell embryos. Late 2-cell embryos collected 48 hr post hCG were labeled with ^{35}S-methionine for 4 hr. Blocked 2-cell embryos obtained 48 hr (Block A) and 60 hr (Block B) post hCG were labeled with ^{35}S-methionine for 4 hr. Labeled 2-cell embryos were thoroughly washed with BSA-free medium and subjected to two-dimensional gel electrophoresis. Proteins (arrow-heads) were mainly synthesized in late 2-cell embryos. Proteins (arrows) were synthesized only in blocked 2-cell embryos.

et al., 1987). To explore the above possibility, late and blocked 2-cell embryos were labeled with $^{32}PO_4$ for 4 hr in the presence of 0.1 μg/ml of colcemid to prevent further development to 4-cell stage, and then subjected to analysis by SDS-PAGE and two-dimensional gel electrophoresis. One dimensional SDS-PAGE showed that three proteins with 27, 35 and 46 kDa were mainly phosphorylated in late 2-cell embryos, but not in blocked 2-cell embryos (Figs. 3 and 4). And the overall pattern of protein phosphorylation increased in late 2-cell embryos, presumably due to transition to M-phase.

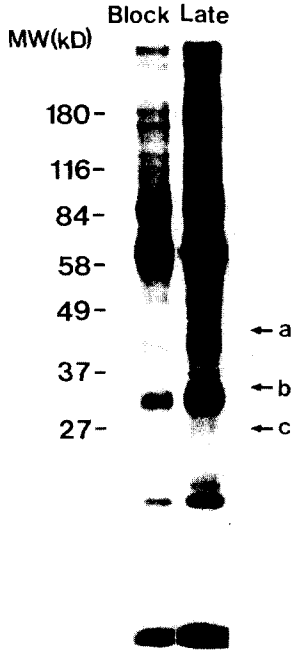

Fig. 3. Comparison of protein phosphorylation patterns in late and blocked 2-cell embryos. Late and blocked 2-cell embryos were incubated in medium containing $^{32}PO_4$ in the presence of colcemid (0.1 μg/ml) for 4 hr. Arrows, a, b, and c indicate 46, 35, and 27 kda proteins, respectively.

Discussion

The present experiment clearly demonstrates that the late 2-cell embryos but not blocked 2-cell

embryos are able to active MPF leading to GVBD. MPF activity in late 2-cell embryos may be originated from *de novo* protein synthesis from stored mRNA, and increased to high level at metaphase stage. A cytoplasmic factor, presumably MPF is known to overcome the 2-cell block phenomenon by microinjection of non-blocking cytoplasm (Muggleton-Harris *et al.*, 1982). The MPF activity is identified in mouse 4-cell embryos as well as somatic cells (Balakier, 1978; Sunkara *et al.*, 1979). Taken together, the present study suggests that active MPF in late 2-cell mouse embryos may be very similar to or the same as a cytoplasmic factor to obviate the *in vitro* 2-cell block.

MPF activity is known to fluctuate in a cyclic manner during cleavage in mouse 2-cell embryos (Pratt and Muggleton-Harris, 1988) and the elevated level of MPF appears to be well correlated with each metaphase in the normal cell cycle (Doree *et al.*, 1983; Gehart *et al.*, 1984). The critical level to enter M-phase during each cell cycle and then to be inactivated following each division. Protein synthesis is also required for appearance of MPF activity in M-phase during each cell cycle (Gehart *et al.*, 1984; Picard *et al.*, 1985) and the second meiosis of mouse oocyte (Clarke and Masui, 1985; Hashimoto and Kishimoto, 1988). In the present study, two-D gel analysis showed a very similar protein synthesis pattern in the late and blocked 2-cell embryos. These results may reflect that blocked 2-cell embryos are just arrested in G_2 stage of cell cycle, but not degenerated as indicated previously (Goddard and Pratt, 1983). Blocked 2-cell embryos may persist the continuous protein synthesis (see Fig. 2, arrows), which is synthesized in the stage just prior to G_2 during the second cleavage. Late 2-cell embryos, however, synthesized new proteins (Fig. 2, arrowheads), which are not found in blocked 2-cell embryos. It appears that these polypeptides are involved in

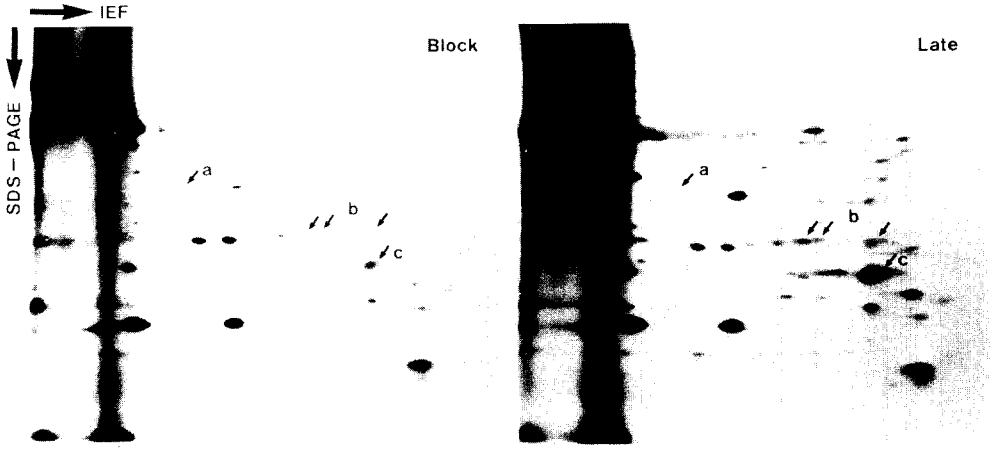

Fig. 4. Two-dimensional gel electrophoretic analysis of protein phosphorylation in late and blocked 2-cell embryos. Two-cell embryos were prepared as Fig. 3. Arrows, a, b, and c were respectively corresponded to the polypeptides indicated in Fig. 3.

treatment of late 2-cell embryos with colcemid seems to increase MPF activity, presumably due to the maintenance of metaphase. In the early mitotic division of mouse embryos and the most growing cells, inhibition of protein synthesis resulted in arrest of interphase (Prescott, 1976) which termed as 'restriction point' (Pardee, 1974). It appears that protein(s) which must reach a the induction of GVBD when GV oocytes were fused with blastomere from late 2-cell embryos. Therefore, inhibition of protein synthesis with puromycin probably blocked the synthesis of presumed MPF in late 2-cell embryos. The altered synthesis of these proteins in blocked 2-cell embryos may influence further development to 4-cell stage. It is still unclear whether these proteins

are related to triggering mitosis.

On the other hand, substantial evidence indicates that protein phosphorylation may play an important role in the transition from interphase to M-phase (Miake-Lye and Kirschner, 1985; Kang *et al.*, 1990, 1991). Analysis of protein phosphorylation revealed that the overall phosphorylation of late 2-cell embryos was increased when compared with that of blocked 2-cell embryos. Furthermore, several specific proteins with 27, 35 and 46 kDa, presumably M-phase components (Howlett, 1986) are phosphorylated in late 2-cell embryos, but not in blocked 2-cell embryos. It has been reported that during the early cleavage of fertilized mouse embryos, M-phase components are phosphorylated in a cell cycle-dependent manner and may be related to MPF activity or a mitosis trigger factor (Howlett, 1986). And M-phase components are also phosphorylated during meiotic maturation as shown recently (Kang *et al.*, 1991). Taken together, the present data suggest that M-phase components may be involved in G_2-M phase transition, and phosphorylation of these proteins may be closely related to MPF activity in the regulation of oocyte maturation and early cleavage in mouse.

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생쥐 2-세포기 배아에서 성숙유도물질의 기능적 역할

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생쥐 배아의 체외 배양시 나타나는 '2-cell block' 현상에서 성숙유도물질(MPF)의 관련성을 세포융합 방법을 이용하여 조사하였다. 후기 또는 blocked 2-세포기 배아에서 얻은 할구를 생쥐의 GV 난자와 세포융합한 후, dbcAMP(100 μg/ml)가 들어있는 배양액에서 배양하면서 GV 난자의 핵막붕괴(GVBD)를 관찰하여 MPF 활성도를 측정하였다. GV 난자를 후기 2-세포기 배아의 할구와 융합시켰을 때 약 30% 정도의 GV 난자는 GVBD를 보였으나, bloked 2-세포기와 융합시켰을 때는 GVBD가 전혀 일어나지 않았다. 후기 2-세포기 배아를 융합하기 전 또는 후에 α-amanitin, puromycin 또는 colcemid를 처리하였을 때 GVBD 율이 변화되었는데, puromycin(50 μg/ml) 처리시 GVBD가 일어나지 않았으며, α-amanitin(100 μg/ml)의 경우에는 GVBD 율이 변하지 않은 것으로 보아 새로운 단백질 합성이 MPF 활성에 필요한 것으로 보인다. 또한 colcemid(0.1 μg/ml) 처리시 GVBD 율이 2배 높아졌는데, 이는 분열기의 상태가 지속되기 때문이라 사료된다. SDS-PAGE나 2차원 전기영동 수행시, 후기 2-세포기와 blocked 2-세포기 간에 단백질 합성 양상은 차이가 없었으나, M-시기 인자라 추측되는 27, 35, 46 KDa 인산화 단백질은 후기 2-세포기 배아에서만 관찰되었다. 따라서 후기 2-세포기 배아에서 MPF 활성화는 M-시기 인자의 인산화와 밀접한 관련이 있는 것으로 사료된다.