Abilities of cumulus and granulosa cells to enhance the developmental competence of bovine oocytes during *in vitro* maturation period are promoted by midkine; a possible implication of its apoptosis suppressing effects

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Abstract

We previously reported that when midkine (MK), a heparin-binding growth differentiation factor was used in *in vitro* maturation (IVM) culture of bovine cumulus-enclosed oocytes (CEOs), their developmental competence to the blastocyst stage after *in vitro* fertilization (IVF) was enhanced and the effect of MK might be mediated by its action upon mural granulosa cells and cumulus cells that closely surround the oocyte. In the present study, when denuded oocytes (DOs) were matured in IVM medium with or without MK (200 ng/ml) in the presence or absence of isolated cumulus cell masses and subjected to IVF, the enhancing effects of MK on the developmental competence of DOs to the blastocyst stage after IVF were exerted only in the presence of cumulus cells. In addition, we prepared the conditioned media of granulosa cells cultured with or without 200 ng MK/ml (CMMK+ or CMMK– respectively) and examined their effects on the IVM of DOs in terms of their developmental competence to the blastocyst stage after IVF. The supplementation of CMMK+ into IVM medium at 40% (v/v) significantly enhanced the blastocyst development compared with the no additive control and the CMMK– supplemented groups. Furthermore, the effects of MK during IVM of bovine CEOs on the cumulus cell apoptosis were investigated. CEOs were cultured up to 24 h in IVM medium without (control) or with 200 ng MK/ml. The genomic DNA was extracted from CEOs at 0, 6, 12, 18 and 24 h of IVM and subjected to ligation-mediated PCR (LM-PCR) to detect the apoptotic internucleosomal DNA fragmentation. DNA fragmentation was scarcely detected at the start of IVM, whereas it increased time-dependently as the IVM culture progressed. The degree of the fragmentation was significantly lower in the MK-treatment group compared with the control group at 18 and 24 h of IVM. The apoptosis-suppressing effect of MK on cumulus cells was further confirmed *in situ* by using TUNEL on CEOs. In conclusion, data from the present study further confirmed that MK enhances the developmental competence of bovine oocytes via cumulus and granulosa cells. It was also demonstrated that MK suppresses the apoptosis that occurs in cumulus cells during the period of IVM of bovine CEOs. The putative soluble factor(s) from cumulus cells was suggested from the experiment using CMMK+. MK may promote the production of such factors in part by its anti-apoptotic effects on cumulus cells.

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Introduction

In the course of growth and maturation of oocytes *in vivo*, follicular cells surrounding oocytes secrete various bioactive substances, some of which might affect the characteristics of the oocyte (Driancourt & Thuel 1998). Mammalian oocytes removed from the follicles can spontaneously undergo meiotic maturation *in vitro*,

resulting in the formation of morphologically normal secondary oocytes arrested at the metaphase stage (MII) of the second meiotic division (Edwards 1965). However, post-fertilization development to the blastocyst stage of oocytes that have reached the MII stage in *in vitro* maturation (IVM) culture is generally less successful than that of oocytes matured *in vivo* (Leibfried-Rutledge *et al.* 1987, van de Leemput *et al.*

1999, Rizos *et al.* 2002). This is often attributed to the lack of physiological factors in IVM that regulate the acquisition of the developmental competence of oocytes during the maturation period. Several growth factors contained in the follicular fluid have been listed as developmental competence-enhancing factors, and include epidermal growth factor (Harper & Brackett 1993, Kobayashi *et al.* 1994, Lonergan *et al.* 1996), transforming growth factor- α (Kobayashi *et al.* 1994), insulin-like growth factor-I (Harper & Brackett 1992), activin A (Stock *et al.* 1997, Silva & Knight 1998), inhibin A (Stock *et al.* 1997), vascular endothelial growth factor (Einspanier *et al.* 2002, Luo *et al.* 2002), brain-derived neurotrophic factor (Kawamura *et al.* 2005, Martins da Silva *et al.* 2005) and midkine (MK) (Ikeda *et al.* 2000a, 2000b).

MK is one of the heparin-binding growth differentiation factors that is known to be quite rich (125 ng/ml) in bovine follicular fluid. We previously found that the addition of recombinant MK into IVM medium of bovine cumulus-enclosed oocytes (CEOs), enhanced the developmental competence of oocytes to the blastocyst stage after *in vitro* fertilization (IVF) and suggested that the MK effects are not exerted by its direct action upon the oocytes, but mediated through the cumulus cells in CEOs. However, the concrete action of MK upon cumulus cells remains unknown. Recently, we reported that cumulus cells undergo apoptosis during the IVM period and suggested the negative correlation between the developmental competence of CEOs and the degree of apoptosis of the cumulus cells during IVM (Ikeda *et al.* 2003). On the other hand, MK has been reported to have anti-apoptotic effects on certain types of cells, including neuronal (Owada *et al.* 1999a and 1999b) and tumour (Qi *et al.* 2000, Ohuchida *et al.* 2004) cells.

In the present study, the requirement of cumulus and granulosa cells for the enhancing effects of MK on the developmental competence of bovine oocytes was examined using the following IVM culture systems: (1) IVM of denuded oocytes (DOs) co-cultured with isolated cumulus cells and (2) IVM of DOs with granulosa cell derived conditioned medium prepared in the presence or absence of MK. Furthermore, the effects of MK on apoptosis of cumulus cells in CEOs during the IVM period were also investigated on the basis of our hypothesis that MK enhances the viability of the cumulus cells through its anti-apoptotic property.

Materials and Methods

All chemicals, except where specified otherwise were purchased from Sigma.

Culture media

The washing medium for the collection and sampling of CEOs was modified PBS (mPBS) that contained

0.9 mmol CaCl_2/l , 0.49 mmol MgCl_2/l , 1.19 mmol NaHCO_3/l , 0.33 mmol sodium pyruvate/l, 1.5 mmol glucose/l and 0.5 mg polyvinylalcohol/ml (PVA). The basal medium for IVM (IVM medium) was a modified synthetic oviduct fluid (mSOF) described by Ikeda *et al.* (2000a). This medium consisted of SOF (Tervit *et al.* 1972), 2% (v/v) basal medium Eagle amino acids solution, 1% (v/v) minimum essential medium non-essential amino acids solution, 0.5 mg PVA/ml, 1–2 μg oestradiol-17 β /ml and 100 IU human chorionic gonadotrophin/ml (Sankyo Co., Tokyo, Japan).

The medium for IVF was modified Tyrode's balanced salt solution (BO) (Brackett & Oliphant 1975). The washing medium for frozen-thawed bull spermatozoa was BO supplemented with 10 mmol caffeine sodium benzoate/l and 20 μg heparin/ml (BO-1). The medium used for the co-incubation of spermatozoa and oocytes was a 50:50 (v/v) mixture of BO-1 and BO supplemented with 20 mg BSA/ml (BO-2). The medium for *in vitro* culture (IVC) of embryos after IVF (IVC medium) was the mSOF described above, supplemented with 1 or 5% (v/v) heat-inactivated foetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) without PVA and hormones.

Preparation of MK

Recombinant bovine MK was produced by means of a baculovirus expression system as described previously (Ikeda *et al.* 2000a).

Preparation of denuded oocytes (DOs)

Ovaries collected from Japanese beef cattle at a local abattoir were transported to the laboratory within 3 h in saline (0.9% (w/v) NaCl) at 35–38 °C. Follicular contents were aspirated from follicles with a diameter of 2–5 mm and diluted with mPBS. The CEOs consisting of oocytes with homogeneous and evenly granulated cytoplasm and an intact cumulus cell mass were selected from the follicular contents. To prepare DOs, groups of ~100 CEOs were transferred into microcentrifuge tubes with 100 μl mPBS and cumulus cells were removed from the CEOs by vortexing for about 5 min.

Experiment 1: Effects of MK in the presence or absence of isolated cumulus cells during IVM of DOs on their post-fertilization development

IVM medium drops containing a cumulus cell mass were prepared as follows: ten CEOs were introduced into 50 μl drops of IVM medium with or without 200 ng MK/ml under mineral oil. Oocytes were mechanically removed from the cumulus mass by pipetting and only the cumulus mass was left in the drop. Ten to twelve DOs prepared as described above were introduced into the

drop and matured with or without MK in combination with the presence or absence of cumulus mass (see Fig. 1) at 39 °C under 5% CO₂ in air for 24 h. To estimate the number of cells per drop, cumulus cells in ten CEOs were counted as follows. Ten CEOs were vortexed in 100 µl mPBS as described earlier, so that completely denuded oocytes and suspension of dispersed cumulus cells were prepared. The numbers of cumulus cells in suspension were calculated using haemocytometer. The cell number from ten CEOs was calculated as 8×10^4 . The DOs after IVM culture were subjected to the IVF and IVC as described elsewhere (Ikeda *et al.* 2000a, 2000b). Briefly, after thawing frozen bull semen, spermatozoa were washed with BO-1, resuspended at a concentration of $4\text{--}5 \times 10^6/\text{ml}$ in BO-2 and prepared as 100 µl suspension. Groups of 10–12 DOs after IVM were transferred into the sperm suspension and incubated for 6 h. Thereafter, oocytes were transferred into 50 µl drops of IVC medium containing 1% (v/v) FBS and cultured at 39 °C under 5% CO₂, 5% O₂ and 90% N₂. At 48 h post-insemination, the morphologically normal cleaved embryos were transferred to IVC medium containing 5% (v/v) FBS and cultured until day 8 (fertilization = day 0). The cleavage rates were assessed at 48 h post-insemination. The percentages of embryos reaching the blastocyst stage were recorded on day 8.

Experiment 2: Effects of conditioned media of granulosa cells cultured with or without MK

The granulosa cells obtained from small follicles at CEO collection were washed in mPBS twice by centrifugation at 200 *g* for 5 min. After the second centrifugation, the granulosa cells were resuspended in 10 ml IVM medium and then washed using centrifugation. After the supernatant was discarded, the granulosa cell pellet was resuspended in 5 ml IVM medium with or without 200 ng MK/ml and cultured for 24 h at 39 °C under 5% CO₂ in air in a 15 ml plastic tube. To estimate the number of cells per tube, GC pellet was dispersed by vortexing and cell number was calculated by haemocytometer. Cell density was adjusted to 4×10^6 cells/ml. After culturing, the granulosa cells were sedimented by centrifugation at 1500 *g* for 20 min. The supernatant was

filtered and stored at –80 °C until use. The medium conditioned by granulosa cells in the presence or absence of MK was designated as CMMK+ or CMMK– respectively. Ten to twelve DOs were introduced into the 50 µl drop of IVM medium containing 10, 20, 40 and 50% (v/v) of CMMK+ or CMMK– and 40% (v/v) CMMK– containing IVM medium supplemented with 200 ng MK/ml and cultured for 24 h at 39 °C under 5% CO₂ in air. No additive group served as control. Sixty to hundred and twenty-two oocytes in each group were examined in three replicates. The DOs after IVM culture were subjected to IVF and IVC and the developmental rates were recorded as in experiment 1.

Experiment 3: Effects of MK on apoptosis of cumulus cells in CEOs during IVM culture

Ten CEOs collected as described earlier were introduced into a 50 µl drop of IVM medium with or without 200 ng MK/ml under mineral oil. CEOs were cultured for 0, 6, 12, 18 and 24 h at 39 °C under 5% CO₂ in air. The detection of the nucleosomal DNA ladder of apoptotic cumulus cells was performed using an ApoAlert ligation-mediated PCR (LM-PCR) ladder assay kit (Clontech) as reported previously (Ikeda *et al.* 2003). The LM-PCR assay has been reported to be semiquantitative, allowing the comparison of the relative extent of apoptosis in different samples (Staley *et al.* 1997). The individual groups of ten CEOs were washed with mPBS and transferred into microcentrifugal tubes with 50 µl of mPBS. The tubes, each containing ten CEOs, were immersed in liquid nitrogen and stored at –80 °C until DNA extraction. After thawing, the CEOs were lysed by adding 50 µl of two times strengthened lysis buffer (20 mmol Tris–HCl/l, 200 mmol NaCl/l, 50 mmol EDTA/l, 1% (w/v) SDS, 0.2 mg proteinase K/ml, pH 8.0) and incubating at 50 °C for 18 h by gentle shaking. The DNA was extracted by the phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v, PCI) method and ethanol precipitated. The DNA pellets were dried and resuspended in 20 µl TE buffer (10 mmol Tris–HCl/l, 1 mmol EDTA/l, pH 8.0). This solution (20 µl) contained DNA corresponding to eight CEOs. To verify the equality of the amounts of the DNA extracted, PCRs upon the β-actin gene as an internal control were performed using the DNA solution. Two microlitres of DNA solution (corresponding to 0.8 CEO) were mixed in a 50 µl reaction mixture containing 200 µmol dNTPs/l, 2.5 U Ex-Taq DNA polymerase (Takara, Japan) and a pair of primers (0.25 µmol/l) specific for the β-actin gene and amplified for 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C. DNA corresponding to three CEOs was mixed with 1 nmol each of 24 bp (5'-AGCACTCTCGAGCCTCTCACCGCA-3') and 12 bp (5'-TGCGGTGAGAGG-3') unphosphorylated oligonucleotides in a 49 µl reaction volume with 1× Ligation mix (Clontech). The oligonucleotides were annealed by heating to 55 °C for 10 min and cooling

Figure 1 Bovine denuded oocytes (DOs, A) and DOs with isolated cumulus cell mass (B) at the beginning of IVM culture. Bar represents 150 µm.

gradually to 10 °C over 1 h followed by incubation at 10 °C for 10 min. Four hundred units (1 µl) of T4 DNA ligase (Clontech) were added and ligation was performed at 16 °C for 16 h. This procedure allowed the unphosphorylated adaptors to ligate to the 5'-phosphorylated blunt ends of the DNA fragments generated during the IVM culture. Adaptor-ligated DNA solution (50 µl) was stored at -20 °C until PCR was performed.

Adaptor-ligated DNA solution (8 µl) corresponding to half of a CEO was mixed with 5 µl 10× LM-PCR mix (Clontech) containing the 24 bp oligonucleotides (linker primer) and 36.5 µl water in a PCR tube. The tube was heated to 72 °C for 3 min followed by the addition of 2.5 U (0.5 µl) of Ex-Taq DNA polymerase and additional incubation at 72 °C for 5 min to fill in the 5' protruding ends of the ligated DNA. The 24 bp oligonucleotide could now serve as a primer and the DNA fragments with adaptors on both ends could be exponentially amplified. PCRs were performed for 24–26 cycles of 1 min at 94 °C and 3 min at 72 °C. All the PCR products (50 µl) were subjected to electrophoresis through 2% (w/v) agarose gels containing 0.8 µg ethidium bromide/ml. After electrophoresis for 60 min at 8 V/cm in Tris–borate/EDTA buffer, the gels were photographed on a UV transilluminator.

The band intensities of the PCR products were measured by densitometry using a model 4.0 Atto densitograph (Atto, Tokyo, Japan). The intensity of ladder-like bands derived from the apoptotic DNA fragments (<1 kb) was expressed relative to the intensity of the band for β-actin. The relative intensity for the onset of IVM (0 h) was subtracted from that for each time point and the difference was designated as the apoptotic index. Experiments were repeated at least twice for each time point in each group.

Effects of MK on cumulus cell apoptosis were further examined using TUNEL assay. After the 24 h IVM without (control) or with 200 ng MK/ml, CEOs were fixed in 4% (w/v) paraformaldehyde in PBS and permeabilised with PBS containing 0.5% (v/v) Triton-X 100. After washing with PBS containing 0.01% (w/v) PVA (PBS–PVA), the samples were incubated for 1 h at 37 °C in a TUNEL reaction mixture (*In situ* Cell Death Detection Kit, Roche). The negative control did not contain TdT, while the positive control was subjected to TUNEL after treatment with 100 U DNase I/ml for 1 h at 37 °C. After the TUNEL reaction, all the nuclei were stained with 0.001% (w/v) Hoechst 33342 diluted in PBS–PVA for 5 min. The CEOs were mounted onto a slide with a droplet of VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA) and flattened with a coverslip. Slides were examined under a Carl Zeiss Axiophot2 microscope (Oberkochen, Germany) equipped with No. 2 filter (excitation 365 nm, emission 420 nm for Hoechst stain) and No. 10 filter (excitation 450–490 nm, emission 515–565 nm for TUNEL assay). The fluorescence images were then analysed with

AQUACOSMOS system (Version 2.6, Hamamatsu Photonics, Hamamatsu, Japan). The TUNEL index was calculated as total intensity of TUNEL staining divided by that of Hoechst staining per CEO. Thirty CEOs in each group from three replicates were examined.

Statistical analysis

The statistical analyses of data were performed using the StatView4.02 (Abacus Concepts, Berkeley, CA, USA). Developmental percentages were arcsine transformed to stabilize the variances and subjected to one-factor ANOVA followed by *post hoc* Fisher's PLSD (experiment 1) or Tukey's test (experiment 2) to detect significant differences among the treatments. In experiment 1, data were further subjected to 2-factor ANOVA to detect the overall effect of each factor (presence or absence of MK and cumulus cells) and interaction between them. In experiment 3, apoptotic and TUNEL indexes were subjected to *t*-test. Significance was accepted at $P < 0.05$. All data were expressed as mean $\pm$ S.E.M.

Results

In experiment 1, DOs were matured in IVM medium supplemented with or without 200 ng MK/ml in the presence or absence of cumulus cell mass and subjected to IVF and IVC. The percentages of embryos that developed to the cleavage and blastocyst stages in each treatment group are shown in Table 1. More than 60% of the oocytes were cleaved and the rates were not significantly different between the treatments. In the absence of cumulus cells, the blastocyst rates were low regardless of MK addition, but the rates increased by MK addition in the presence of cumulus cells. The overall effects of MK and cumulus cells during IVM on each developmental rate and the interaction between the two factors (MK and cumulus cells) were not significant with regard to cleavage rates, but significant regarding blastocyst rates.

In experiment 2, DOs were matured in IVM medium containing various dosages of CMMK+ or CMMK- and in 40% (v/v) of CMMK- containing IVM medium supplemented with 200 ng MK/ml, followed by IVF and IVC. The developmental rates are shown in Fig. 2. When the DOs were matured in basic IVM medium, their developmental rates to blastocysts after IVF were very low (1.5% for IVM/IVF oocytes and 3.0% for cleaved oocytes). Forty percent (v/v) of CMMK+ significantly enhanced the blastocyst development compared with the same dose of CMMK-. The most effective dose of CMMK+ was 40% (12.3% for IVM/IVF oocytes and 23.4% for cleaved oocytes). The 40% (v/v) CMMK- supplementation showed no significant effect on the blastocyst yield, even if MK was added. From the results of experiment 1 and 2, it was suggested that the enhancing

Table 1 Effects of MK in combination with the presence or absence of isolated cumulus cells during IVM of bovine DOs on post-fertilization development.

MK	CC	IVM/F oocytes (replicates)	Cleavage (% $\pm$ S.E.M.)	Blastocyst (% $\pm$ S.E.M.)	
				Per IVM/F cleaved oocyte	Per cleaved oocyte
–	–	46 (4)	65.6 $\pm$ 3*	1.9 $\pm$ 2*	3.1 $\pm$ 3*
+	–	45 (4)	64.2 $\pm$ 2*	1.9 $\pm$ 2*	2.8 $\pm$ 3*
–	+	43 (4)	63.3 $\pm$ 7*	2.3 $\pm$ 2*	4.2 $\pm$ 4*
+	+	43 (4)	64.2 $\pm$ 5*	16.3 $\pm$ 5 [†]	24.3 $\pm$ 5 [†]

MK, midkine; IVM *in vitro* maturation; DO, denuded oocyte; CC, cumulus cells; IVF, *in vitro* fertilization. *, [†]Values in the same column without common superscripts differ significantly ($P < 0.05$).

effects of MK on developmental competence are mediated through its action on cumulus and granulosa cells. We therefore propose that MK may enhance the viability of cumulus cells in CEOs by its anti-apoptotic property.

In experiment 3, the MK action on the apoptosis of cumulus cells was investigated. Figure 3 shows the results of LM-PCR using genomic DNA from bovine CEOs matured in serum-free IVM medium with or without MK addition. The ladder-like PCR products in CEOs were hardly detectable at 0 h of IVM, while they increased after 12 h of culture and toward the end of IVM (24 h), irrespective of MK addition. The sizes of the ladders observed were, as expected, 185 bp multiples with additional 24 bp oligonucleotides at each DNA end corresponding to the apoptotic internucleosomal fragments (Ikeda *et al.* 2003). When compared at each time point, the apoptotic index was significantly lower in the MK-treatment group at 18 and 24 h of IVM than in the control. No ladder could be obtained from the oocytes

that were freed from CEOs after 24 h of culturing (data not shown).

The effects of MK on apoptosis of cumulus cells were further examined *in situ* by means of TUNEL (Fig. 4). TUNEL index was calculated as intensity of TUNEL staining divided by that of all DNA staining (Hoechst) in CEOs, which had been IVM cultured in the presence or absence (control) of MK for 24 h. MK added into IVM medium significantly reduced apoptotic index compared with no additive control (0.226 ± 0.00236 vs 0.181 ± 0.00143 , $P = 0.0035$).

Discussion

MK was first identified as a product of a retinoic acid-inducible gene in a teratocarcinoma cell line (Kado-matsu *et al.* 1988), which forms a unique family with pleiotrophin, also known as HB-GAM (Rauvala 1989, Li *et al.* 1990). MK was reported to be rich (125 ng/ml) in

Figure 2 Effects of conditioned media of granulosa cells cultured with or without MK (CMMK+ or CMMK- respectively) on the IVM of denuded oocytes in terms of (A) development to the cleavage stage, (B) blastocyst yield from tested oocytes and (C) blastocyst yield from cleaved embryos after *in vitro* fertilization. Data are presented as mean with the S.E.M. from three replicates. Numbers of oocytes examined per group are indicated in the column. Values without common superscripts differ significantly ($P < 0.05$). NONE and MK mean no additive control and midkine respectively.

Figure 3 (A) LM-PCR analysis of internucleosomal DNA fragmentation in CEOs during IVM culture with or without MK (200 ng/ml) addition. PCR products derived from adaptor-ligated DNA corresponding to half of a CEO were subjected to electrophoresis and then photographed. The lower panel shows a control PCR assay for the β -actin gene to confirm that equivalent amounts of DNA were analysed. 1–3 nuc: the PCR ladder derived from 1–3 nucleosomal units of DNA fragments. Ctrl. and MK mean no additive control and MK-treated group respectively. (B) Apoptotic index calculated from the results of the LM-PCR during IVM culture. Numbers in parentheses indicate the number of replications for each value. Different letters (a and b) depict significant differences between the treatments at the same time point ($P < 0.05$).

bovine follicular fluid (Ohya *et al.* 1994). Furthermore, it was reported that follicular granulosa cells produced MK under the control of gonadotrophin (Karino *et al.* 1995, Minegishi *et al.* 1996), suggesting its role in follicular development, including oocyte growth and maturation.

We previously reported that the recombinant MK added into the IVM medium of bovine oocytes enhanced their developmental competence to the blastocyst stage after IVF (Ikeda *et al.* 2000a, 2000b). In the previous study, since the enhancing effects of MK on the developmental competence of DOs were exerted only in the presence of isolated granulosa cells, we suggested that the MK effects are mediated by granulosa cells rather than by a direct action on oocytes (Ikeda *et al.* 2000b). In experiment 1, in the present study, we investigated the effects of MK on the IVM of DOs in the presence or absence of isolated cumulus cells that are a subpopulation of granulosa cells and demonstrated that MK exerted its developmental competence-enhancing effects only under the presence of cumulus cells (Table 1). This finding confirmed the involvement of follicular cumulus and granulosa cells in MK action on oocytes during the maturation period.

Furthermore, in experiment 2, the granulosa cell-conditioned medium prepared in the presence of MK (CMMK+) also enhanced the developmental competence of DOs (Fig. 2). The inability of MK added into CMMK– containing IVM medium to promote the developmental competence indicates that the enhancing effect of CMMK+ during IVM is not due to MK included in CMMK+ in the preparation of the conditioned medium. In experiment 1, the addition of cumulus cells into IVM medium did not affect the developmental

competence of DOs to the cleavage stage (Table 1). On the contrary, even with no statistical significance, the granulosa cell-conditioned medium at 40% (v/v) increased the cleavage rate compared to the no addition group, consistent with the previous study, in which, addition of granulosa cells into IVM culture of DOs had a significant effect on the cleavage rate (Ikeda *et al.* 2000b). The difference in the cleavage rates of DOs between the effects of cumulus cells and of granulosa cells may be due to the mutually unique function of these cells (Gilchrist *et al.* 2004). Whatever the uniqueness, the results of experiment 2 further confirmed the MK action via cumulus and granulosa cells.

These findings prompted us to explore the action of MK on the cumulus and granulosa cells. We previously showed that apoptosis occurs in cumulus cells during IVM of CEOs (Ikeda *et al.* 2003). We tentatively refer to this phenomenon as IVM-induced apoptosis because apoptosis is unlikely to occur in cumulus cells during natural (i.e. *in vivo* and not artificially stimulated) oocyte maturation (see the discussion in the reference, Ikeda *et al.* 2003). It was also found that apoptosis during IVM was induced irrespective of serum addition, although serum has been shown to protect cultured granulosa cells from apoptosis (Hu *et al.* 2001, Johnson *et al.* 2001). On the other hand, MK has been reported to have anti-apoptotic effects on certain types of cells, including neuronal (Owada *et al.* 1999a, 1999b) and tumour (Qi *et al.* 2000, Ohuchida *et al.* 2004) cells. In the present study, we demonstrated for the first time that MK could suppress the apoptosis that occurred in cumulus cells during the IVM of bovine CEOs (Figs 3 and 4). It was revealed from the results that the enhancing effects of MK

Figure 4 (A) A representative image of bovine cumulus enclosed oocytes (CEOs) after IVM (24 h) subjected to TUNEL (green label) to detect DNA fragmentation. All cell nuclei are also stained by Hoechst (blue). Scale bar represents 50 μ m. (B) TUNEL index calculated as intensity of TUNEL staining divided by that of Hoechst staining in the control and MK-treatment group. Numbers in parentheses indicate the number of CEOs subjected to the analysis.

on the developmental competence of bovine CEOs could be exerted in part by its effect of improving the viability of cumulus cells. After we reported that cumulus cells undergo apoptosis during the IVM period and proposed the negative correlation between the degree of cumulus cell apoptosis during IVM and the developmental competence of CEOs (Ikeda *et al.* 2003), supportive results were successively published (Alisch *et al.* 2003, Corn *et al.* 2005, Leroy *et al.* 2005, Yuan *et al.* 2005).

The results from experiment 2 also suggest that MK exerts its enhancing effects via soluble factor(s) from cumulus and granulosa cells. Recently, Luciano *et al.* (2005) found that the addition of intact CEOs during IVM of DOs could restore their developmental competence to the blastocyst stage and reached the presumption of diffusible factor(s) from cumulus cells, which enhances the developmental competence.

So far, many reports have suggested the importance of the presence of cumulus and granulosa cells during IVM culture in terms of the developmental competence of oocytes (Zhang *et al.* 1995, Kim *et al.* 1997, Hashimoto *et al.* 1998, Luciano *et al.* 2005), and the importance may at least be attributed to soluble factor(s), as mentioned earlier. It is therefore suggested that MK promotes the production of such soluble factor(s) in cumulus and granulosa cells. MK has been reported to activate a cell-surface receptor, resulting in the activation of a transcription factor, STAT1 α (Ratovitski *et al.* 1998). The activation of such transcription factor(s) by MK signal transduction may be directly involved in the expression of the developmental competence enhancing factor(s) at transcriptional levels. Alternatively, MK may promote the production of the enhancing factor(s) as a consequence of inhibition of the cumulus cell death by its anti-apoptotic effects elucidated in this study.

In conclusion, data from previous and the present study indicate that MK enhances the developmental competence of oocytes via surrounding cumulus and granulosa cells and suppresses the cumulus cell apoptosis that occurs spontaneously in bovine CEOs during the IVM period. The putative soluble factor(s) from cumulus and granulosa cells was suggested and MK may promote the production of such factors in part by its anti-apoptotic effects on cumulus cells.

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