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Production of Plasminogen Activators (PAs) in Bovine Cumulus-Oocyte Complexes during Maturation In Vitro: Effects of Epidermal Growth Factor on Production of PAs in Oocytes and Cumulus Cells[1]

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ABSTRACT

We examined whether plasminogen activators (PAs) are produced by bovine cumulus-oocyte complexes (COCs) during maturation in vitro. The effects of epidermal growth factor (EGF) on production of PAs in oocytes and cumulus cells were also examined. When COCs were cultured for 24 h with 30 ng/ml EGF, three plasminogen-dependent lytic zones (58.5 ± 3.5 kDa, 79.0 ± 3.0 kDa, and 113.5 ± 6.5 kDa) were observed. Addition of amiloride, a competitive inhibitor of urokinase-type PA (uPA), to the zymogram eliminated the activity of the 58.5 ± 3.5-kDa zone, suggesting that this band is a uPA. However, since the activity of the remaining two bands was not eliminated, it was suggested that the 79.0 ± 3.0-kDa band is a tissue-type PA (tPA) and the 113.5 ± 6.5-kDa band is possibly a tPA-PA inhibitor (tPA-PAI) complex. In COCs before culture, however, no activity of PAs was detected. At 6 h of culture, the same level of uPA activity was detected in COCs cultured both in the absence and in the presence of EGF. The uPA activity was increased at 12 h of culture but without further increase at 24 h of culture, with higher activity in the presence than in the absence of EGF. The activity of tPA and tPA-PAI was first detected at 24 h of culture in the absence of EGF. In the presence of EGF, however, some activity of tPA-PAI was detected at 12 h of culture. At 24 h of culture, the activity of all PAs was detected in cumulus cells, but only uPA activity was detected in oocytes, with higher activity in the presence than in the absence of EGF. The uPA activity in oocytes was not detected when they were cultured without cumulus cells in either the presence or absence of EGF, although cumulus expansion was stimulated by EGF, exhibiting a time-course similar to that observed in PA production. These results suggest that uPA, tPA, and tPA-PAI are all produced by bovine COCs, but only uPA by oocytes, during maturation in vitro. However, cumulus cells play an essential role or roles in the production of uPA by oocytes, and EGF enhances the roles of cumulus cells.

INTRODUCTION

Plasminogen activators (PAs) are serine proteases, known to be secreted by a large number of cell types, that convert plasminogen to plasmin. PAs are classified into two groups on the basis of molecular mass: urokinase-type PA (uPA), which is secreted as an inactive single-chain molecule of 31–54 kDa, and tissue-type PA (tPA), which is secreted in an active form with a molecular mass of around 70 kDa [1]. PAs play roles not only in fibrinolysis but also in various reproductive processes including ovulation and implantation [2].

Although PA activity in rat [3], mouse [4, 5], porcine [6], ovine [7, 8], and bovine [9–12] embryos, and trypsin-like activity in hamster [13] and mouse [14] oocytes have been reported, reports describing proteases in the mammalian oocyte itself are limited. The first identification of an oocyte-produced protease was reported by Huarte et al. [15], who found that resumption of meiosis in mouse and rat oocytes triggers the production of tPA. The production of tPA and uPA in cultured cumulus-oocyte complexes (COCs) has also been reported in rats [16, 17] and pigs [18]. The activity of tPA, but not of uPA, in rat oocytes and cumulus cells is stimulated by FSH and forskolin [16]. It has been suggested that the stimulation of PA activity in rat COCs by FSH and GnRH is through protein kinases A and C, respectively [19, 20]. It was also reported that PA production by porcine COCs is influenced by protein kinases A and C and kinase inhibitors [18].

Epidermal growth factor (EGF) has been demonstrated to stimulate in vitro maturation of mouse [21, 22], rat [23], porcine [24–26], bovine [27–29], and human [22] oocytes. The development of in vitro-penetrated bovine oocytes was also promoted when oocytes were matured in medium with EGF [28, 30, 31] even in the absence of serum and gonadotropin [32, 33]. These positive effects of EGF on maturation may be mediated through the cumulus cells.

Although it has been reported that EGF stimulates tPA activity and mRNA levels in cultured rat granulosa cells [34], little attention has been paid to the effects of EGF on production of PAs by mammalian COCs, oocytes, and cumulus cells during maturation. Furthermore, to our knowledge no information on the production of PAs by bovine oocyte is available. Therefore, the present study was undertaken 1) to identify PAs in bovine COCs matured in culture, 2) to determine the effects of EGF on PA production in COCs, and 3) to clarify the effects of cumulus cells on PA production by oocytes during maturation.

MATERIALS AND METHODS

Medium

The medium used for maturation of oocytes was tissue culture medium (TCM) 199 (with Earle's salts and buffered with 25 mM Hepes, no. 12340–034; Gibco, Grand Island, NY) supplemented with 0.1% (v:v) polyvinylalcohol, 60 μg/ml sodium penicillin G, and 100 μg/ml streptomycin sulfate.

Collection and Culture of COCs

Ovaries were collected from Japanese black and Holstein heifers or cows at a local abattoir and transported to the

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laboratory in 0.9% (w:v) NaCl solution at 30–35°C within 2 h. COCs were aspirated from antral follicles of 2–5 mm in diameter with an 18-gauge needle fixed to a 10-ml disposable syringe and washed four times with maturation medium. Depending on the experiments, some COCs were freed from cumulus cells by repeated passage through a fine pipette in maturation medium without EGF (experiment 3) or by vortex for 2 min in TCM-199 (no. 31100–35; Gibco) supplemented with 10 mM Hepes, 2 mM $NaHCO_3$, 0.3% (w:v) BSA (A-4378, Sigma Chemical Co., St. Louis, MO), and 0.1% (w:v) hyaluronidase from bovine testis (Sigma; experiment 4). Ten to fifteen COCs with a compact cumulus or cumulus-free (denuded) oocytes with uniform ooplasm were transferred to 100 μl maturation medium with or without 30 ng/ml EGF (Sigma), which had been previously covered with paraffin oil in a polystyrene culture dish (35 × 10 mm, Falcon No. 1008; Becton and Dickinson, Franklin Lakes, NJ) and equilibrated at 39°C in an atmosphere of 5% CO_2 in air overnight. The COCs or denuded oocytes were then cultured for various periods according to the different experiments at 39°C under the same atmospheric conditions. After culture, COCs, denuded oocytes, or cumulus cells, depending on the experiments, were separately put into microtubes containing 20 μl of sample buffer (5.0% [w:v] SDS, 20% [v:v] glycerol, and 0.0025% [w:v] bromophenol blue in 0.125 M Tris-HCl buffer) and frozen at −80°C until used for zymographic analysis.

Electrophoresis and Zymography

SDS-PAGE and zymography were carried out by the procedures described by Dyk and Menino [11] with a slight modification. Frozen samples were thawed and homogenized with a sonicator. As a standard of tPA, stock solution of 0.5 ng/ml tPA from human melanoma cell culture (Sigma) was prepared in sample buffer. Each homogenized sample (20 μl) being compared in each experiment, a stock solution of human tPA (8 μl), and molecular mass markers (7 μl; Bio-Rad Lab., Hercules, CA) were placed in a castellated well in a 4.5% stacking gel with a 10% separating gel. Electrophoresis was conducted at 20 mA for 2 h.

After electrophoresis, the polyacrylamide gels were gently shaken in 2.5% Triton X-100 for 30 min, rinsed with distilled water three times, and incubated for 30 min at 39°C in PBS. Each gel was carefully laid on a casein-agar gel (zymogram) containing purified human plasminogen (Sigma) supported in a plastic chamber, and the other zymogram without human plasminogen was overlaid as a control to detect nonspecific proteases. For preparing zymograms, 0.8 g of nonfat dry milk was dissolved in 19 ml of buffer containing 0.0013 M $CaCl_2$, 0.1 M glycine, 0.038 M Tris, and 0.005 M sodium azide. The mixture was heated to 55°C and combined with 19 ml of 2% (v:v) melted agarose maintained at 55°C. One milliliter of purified human plasminogen stock solution was added to 19 ml of the warmed mixture to yield a final plasminogen concentration of 50 μg/ml. For zymograms without human plasminogen, 1 ml water was added to 19 ml of the warmed mixture. Each 10 ml of these mixtures was cast onto a warmed dish (100 × 15 mm; Falcon 1012; Becton and Dickinson) and allowed to cool. Each polyacrylamide gel exposed simultaneously to the two zymograms, i.e., casein-agar gel with and without plasminogen, was incubated at 39°C for 12–18 h to allow the development of lytic bands. After the distance from the edge of the separating gel to the center of the clear lytic bands in each lane was measured, incubation of zymograms was terminated by separating the gels. Then the zymograms were fixed with 3% (v:v) acetic acid for 10 min, rinsed under tap water, dried, and stained with 0.1% (w:v) Amido Black 10B dye (Bio-Rad) in acetic isopropanol (25% [v:v] isopropanol and 10% [v:v] acetic acid in water) for permanent storage [35]. Polyacrylamide gels were stained with 0.05% Coomassie Brilliant Blue R-250 dye (Bio-Rad) in acetic isopropanol overnight without fixing and rinsing. The molecular mass of PAs was determined from the regression calculations of log molecular mass and relative mobility. Protease activity of the lytic zone (height × width) was measured by a densitometric scanner (Gel Doc 1000; Bio-Rad) with Multi-Analyst software (Bio-Rad). PA activities were expressed relative to the activity in a fixed sample, which was different in each experiment.

Experimental Design

In experiment 1, to identify the presence and the type of PAs of bovine COCs, zymograms containing or not containing 10 mM amiloride, a competitive inhibitor of uPA [36], were used. Twenty COCs cultured in the presence of EGF for 24 h were used for analysis of PAs.

In experiment 2, to determine the changes of PA activity and cumulus expansion in COCs during maturation in the presence or absence of EGF, COCs were cultured in maturation medium with or without EGF. At 0, 6, 12, and 24 h after the start of culture, 20 COCs were sampled for determination of PA activity, and some remaining COCs were used for assessment of cumulus expansion. The degree of cumulus expansion was assessed under a dissecting microscope at ×50 and classified into four categories scored as 0 (no expansion), +1 (separation of only the outermost layer of cumulus), +2 (moderate expansion involving the outer half of cumulus), and +3 (complete expansion including or except the corona radiata). These classifications were originally described by Downs [21] in mouse oocytes.

In experiment 3, to clarify the PA production by cumulus cells and oocytes, COCs were cultured in the presence or absence of EGF. At 0 and 24 h after the start of culture, oocytes were separated from cumulus cells (denuded). After washing 4 times, 40 denuded oocytes with a polar body were sampled for determination of PA activity. Cumulus cells from 40 COCs were washed once by centrifugation at 2000 × *g* for 10 min. After the supernatant was discarded, 20 μl sample buffer was put on the pelleted cumulus cells.

In experiment 4, to examine the effects of cumulus cells on PA production of oocytes, COCs or denuded oocytes were cultured for 24 h with or without EGF. After culture, COCs were freed from cumulus cells, and each group of 40 denuded oocytes with a polar body was sampled for determination of PA activity.

Statistical Analysis

Statistical analysis of the data obtained from three replicates was carried out with ANOVA and Fisher's protected least-significant-difference test using the STATVIEW (Abacus Concepts, Inc., Berkeley, CA) program. When ANOVA revealed a significant treatment effect, the treatments were compared by Fisher's protected least-significant-difference test. A probability of $p < 0.05$ was considered to be statistically significant.

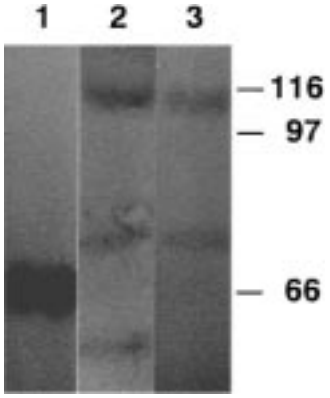

FIG. 1. Zymographic analysis of bovine COCs cultured for 24 h in the presence of 30 ng/ml EGF (lanes 2 and 3). Twenty COCs were used for the analysis. Lane 1 contained human tPA. Lanes 2 and 3 contained 0 and 10 mM amiloride, respectively. Molecular weight markers ($\times 10^{-3}$) are on the right.

RESULTS

Detection of PA Activity in COCs Cultured for 24 h with EGF

Three plasminogen-dependent lytic zones (58.5 ± 3.5 kDa, 79.0 ± 3.0 kDa, and 113.5 ± 6.5 kDa) were detected in COCs cultured for 24 h in the presence of 30 ng/ml EGF (Fig. 1). Addition of amiloride to the zymogram eliminated activity in the zone of 58.5 ± 3.5 kDa, suggesting that this band was a uPA. However, the activity in the other two zones, of 79.0 ± 3.0 and 113.5 ± 6.5 kDa, was not eliminated, suggesting that these represented different forms of PA. Since PA inhibitors (PAI) are involved in the regulation of plasma PA activity and form an SDS-stable complex with a high molecular mass retained in the zymogram [1], it is suggested that the 113 ± 6.5-kDa band was a tPA-PAI complex.

Change of PA Activity in COCs during Culture with or without EGF

No PA activity was observed in COCs just after aspiration from follicles (Figs. 2 and 3). As shown in Figure 3, irrespective of the presence of 30 ng/ml EGF, the activity of uPA was observed at 6 h of culture and significantly ($p < 0.05$) increased by 12 h of culture, but without further increase until 24 h. The uPA activity at 12 and 24 h of culture was significantly ($p < 0.05$) higher in the presence than in the absence of EGF. On the other hand, the activity of tPA was not detected until 24 h of culture, showing significantly ($p < 0.05$) higher activity in the presence than in the absence of EGF. The activity of tPA-PAI was detected at 12 h of culture and was significantly ($p < 0.05$) increased at 24 h of culture in the presence of EGF. However, significantly ($p < 0.05$) lower tPA-PAI activity was observed at 24 h of culture in the absence of EGF than in the presence of EGF.

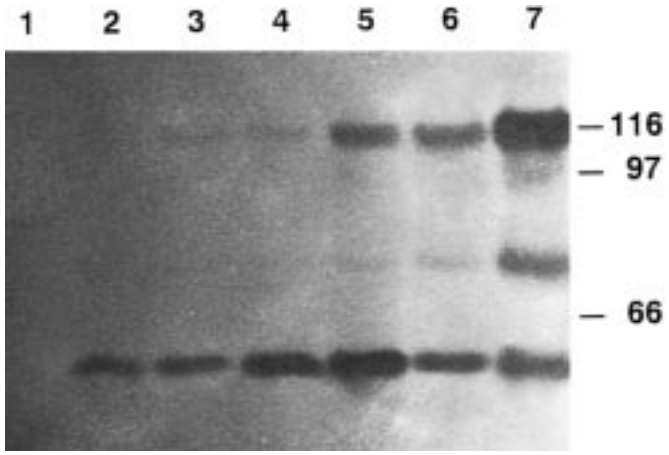

FIG. 2. Zymographic analysis of bovine COCs at 0 (lane 1), 6 (lanes 2 and 3), 12 (lanes 4 and 5), and 24 h (lanes 6 and 7) of culture in the presence (lanes 3, 5, and 7) or absence (lanes 2, 4, and 6) of 30 ng/ml EGF. Twenty COCs were used. Molecular weight markers ($\times 10^{-3}$) are on the right.

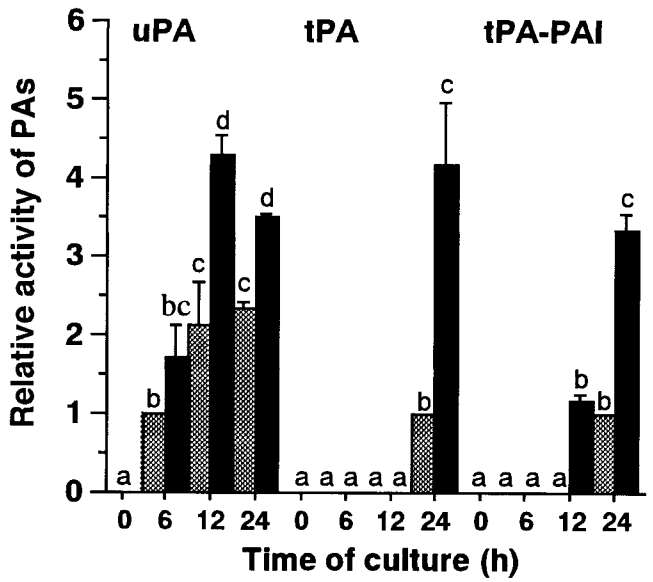

FIG. 3. PA activities of bovine COCs at various times of culture in the presence (solid bars) or absence (gray bars) of 30 ng/ml EGF. Results are shown as mean ± SE of 3 trials using 20 COCs each. PA activities are expressed relative to the activity at 6 h of culture without EGF for uPA and at 24 h of culture without EGF for tPA and tPA-PAI complex. Values with different letters are significantly different ($p < 0.05$).

Cumulus Expansion in COCs during Culture with or without EGF

As shown in Table 1, when COCs were cultured in the presence of 30 ng/ml EGF, cumulus expansion proceeded rapidly from 12 h to 24 h after the start of culture, showing full expansion (category +3) in 86% of COCs examined at 24 h of culture. However, in the absence of EGF, only 23% of COCs showed a slight expansion (+1) even 24 h after the start of culture.

Production of PAs in Cumulus Cells and Oocytes after Culture of COCs for 24 h with or without EGF

No PA activity was detected either in cumulus cells or in oocytes just after aspiration of COCs from follicles (Figs. 4 and 5). Irrespective of the presence of 30 ng/ml EGF, 24 h after the start of culture, activities of uPA, tPA, and tPA-PAI were all detected in cumulus cells, but only that of uPA in oocytes. The activities of these PAs in both cumulus cells (Fig. 5A) and oocytes (Fig. 5B) were significantly ($p < 0.05$) higher when COCs were cultured for 24 h in the presence compared to the absence of EGF.

TABLE 1. Cumulus expansion in bovine COCs cultured in the presence (+) or absence (−) of EGF.

Time of culture (h)	EGF	No. of COCs examined	No. (%) of COCs with cumulus expansion at score of			
			0	+1	+2	+3
0	+	51	51 (100)	0	0	0
	−	60	60 (100)	0	0	0
6	+	51	51 (100)	0	0	0
	−	60	60 (100)	0	0	0
12	+	51	2 (4)	49 (96)	0	0
	−	60	60 (100)	0	0	0
24	+	51	0	2 (4)	5 (10)	44 (86)
	−	60	46 (77)	14 (23)	0	0

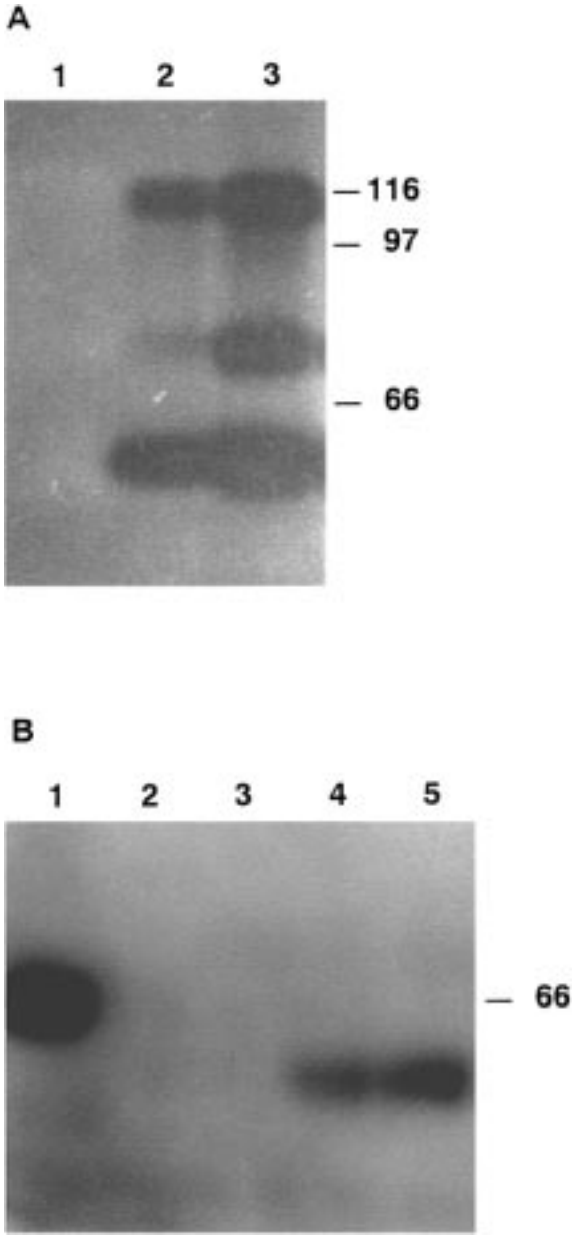

FIG. 4. Zymographic analysis of cumulus cells (**A**) and oocytes (**B**) that were separated from bovine COCs at 0 h (lane 1 in **A** and lane 3 in **B**) and 24 h (lanes 2 and 3 in **A** and lanes 4 and 5 in **B**) of culture in the presence (lane 3 in **A** and lane 5 in **B**) or absence (lane 2 in **A** and lane 4 in **B**) of 30 ng/ml EGF. Forty COCs were used for the analysis. At 24 h of culture, only oocytes with a polar body were used. Lanes 1 and 2 in **B** contain human tPA and no samples, respectively. Molecular weight markers ($\times 10^{-3}$) are on the right.

Effects of Cumulus Cells and/or EGF during Culture on Production of uPA in Oocytes

When COCs were cultured for 24 h in the presence or absence of 30 ng/ml EGF, extrusion of a polar body was observed in about 92% and 72% of oocytes, respectively, while only about 66–67% of oocytes cultured without cumulus cells had a polar body, irrespective of the presence of EGF (data not shown).

No uPA activity was detected in oocytes cultured without cumulus cells either in the presence or absence of EGF (Figs. 6 and 7). However, when COCs were cultured, the activity of uPA was detected in oocytes, with activity significantly ($p < 0.05$) higher in oocytes cultured in the presence than in the absence of EGF (Fig. 7).

DISCUSSION

The present study demonstrates, for the first time, the following: 1) Bovine COCs do not show PA activity just after collection from antral follicles; however, different types of PAs are produced during maturation in vitro. 2) Both PA production and cumulus expansion are enhanced by EGF and exhibit similar time-courses. 3) Cumulus-enclosed, but not denuded, oocytes produce only uPA during maturation in vitro, and this is also enhanced by EGF, in-

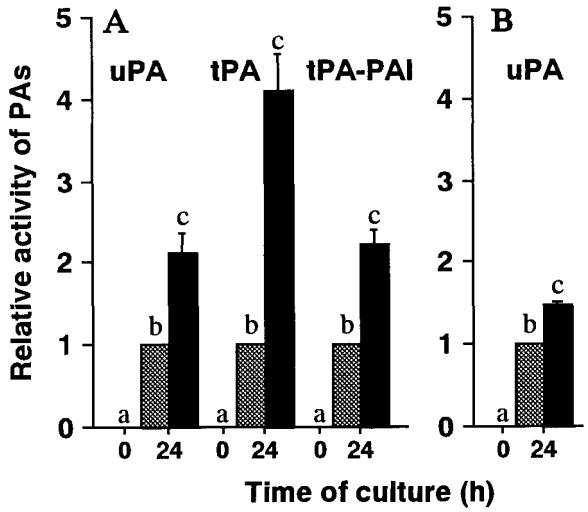

FIG. 5. PA activities of cumulus cells (**A**) and oocytes (**B**) that were separated from bovine COCs at 0 and 24 h of culture in the presence (solid bars) or absence (gray bars) of 30 ng/ml EGF. At 24 h of culture, only oocytes with a polar body were used for the analysis. Results are shown as mean ± SE of 3 trials using 40 COCs each. PA activities are expressed relative to the activity at 24 h of culture without EGF. Values with different letters are significantly different ($p < 0.05$).

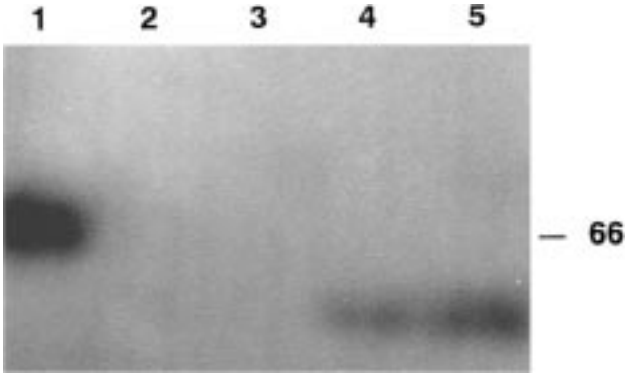

FIG. 6. Zymographic analysis of bovine oocytes cultured for 24 h with (lanes 4 and 5) or without (lanes 2 and 3) cumulus cells in the presence (lanes 3 and 5) or absence (lanes 2 and 4) of 30 ng/ml EGF. Each of 40 denuded oocytes with a polar body was used for the analysis. Lane 1 contains human tPA. Molecular weight marker ($\times 10^{-3}$) is on the right.

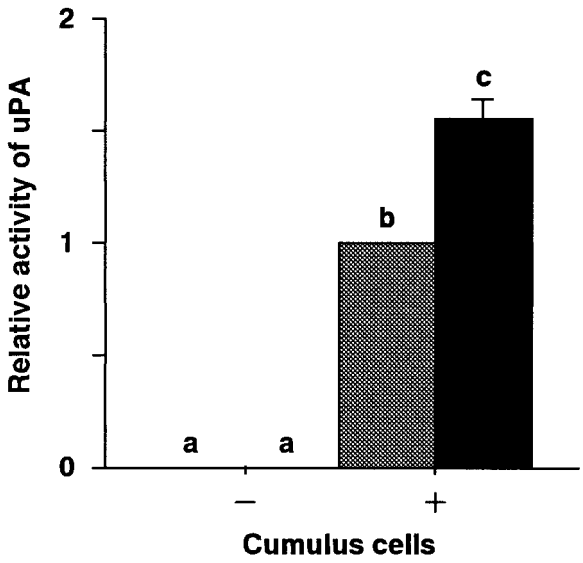

FIG. 7. The activities of uPA of bovine oocytes cultured for 24 h with (+) or without (−) cumulus cells in the presence (solid bar) or absence (gray bar) of 30 ng/ml EGF. Results are shown as mean ± SE of 3 trials using 40 oocytes with a polar body. PA activities are expressed relative to the activity in oocytes cultured with cumulus cells in the absence of EGF. Values with different letters are significantly different ($p < 0.05$).

dicating that cumulus cells play an essential role or roles in production of uPA by oocytes.

In the present study, it has been found that three plasminogen-dependent proteases are produced by bovine COCs during maturation in vitro. The resistance to amiloride treatment in zymographic analysis and molecular mass suggested that the 58.5 ± 3.5-kDa, 79.0 ± 3.0-kDa, and 113.5 ± 6.5-kDa species that were detected in COCs are uPA, tPA, and tPA-PAI, respectively. Although the activity of these PAs was not detected in COCs freshly collected from follicles, it increased time-dependently during maturation in vitro until 12–24 h of culture. Although fibrinolytic activity has been observed only in cumulus cells of bovine COCs before and after maturation [37], no analytical identification of PAs has been done. The increase of PA activity in COCs during maturation in vitro or in vivo is also reported in rats [16, 38] and pigs [18]. However, the type of PAs detected in COCs seems to be different according to the different species: in rats, low amounts of tPA are detected in freshly obtained COCs, but both tPA and uPA activity increases during maturation in vivo [38] and in vitro [16]; whereas in pigs, uPA activity is not detected in COCs before and after maturation, but both tPA and tPA-PAI activity increases during maturation in vitro [18]. Although the precise role of PAs in COCs has not yet been elucidated, their possible involvement in the last period of maturation of oocytes and ovulation [17, 39, 40], or cumulus expansion or dispersion [16, 17] is suggested. However, although PA production in COCs seemed to be temporally correlated with oocyte maturation in the present study, it has been shown that reagents which stimulate PA activity in porcine COCs inhibit oocyte maturation, suggesting no correlation between these two processes [18]. Regarding cumulus expansion, time-courses and responses to EGF similar to those exhibited in PA production were observed in the present study.

It is reported that rat denuded oocytes freed from cumulus cells just after collection from follicles do not contain tPA activity [15] or contain [16, 38, 41] low amounts, but the activity was time-dependently increased during maturation in vivo [38, 41] or in vitro without cumulus cells [15, 16]. An increase of tPA activity during maturation in vitro is also reported in cumulus-free mouse oocytes [15]. In these species, however, uPA and tPA-PAI activity are not detected in maturing oocytes. In rats and mice, resumption of meiosis triggers the production of tPA by oocytes, probably by translation of stored mRNA [15, 42]. It has been also demonstrated that rat oocytes contain mRNA for tPA, suggesting that oocytes synthesize tPA themselves and do not simply take up tPA from the extracellular space [41].

However, these findings in rats and mice are not consistent with those of the present study using bovine oocytes. In the present study, no PA activity was detected in oocytes just after collection from follicles; but after maturation in vitro, only uPA, but not tPA and tPA-PAI, activity was detected only when they were matured with cumulus cells, suggesting that uPA production in bovine oocytes may not be of oocyte origin but is acquired from cumulus cells, which also produce uPA during maturation in vitro. It is not clear how oocytes acquire the ability to produce uPA from cumulus cells, but it is possible that uPA produced by cumulus cells is transported into oocytes through gap junctions between both cells, since cumulus expansion is not extensive, and thereby gap junctions may still be kept firmly, during the period (6–12 h after the start of culture) at which large amounts of uPA are produced. However, there is no direct evidence for production of uPA by cumulus cells without the influence of the oocyte because cumulus cells from which oocytes had been removed were not cultured in the present study. As another possibility, some unknown signal(s) from cumulus cells may stimulate oocytes to produce uPA. It is also possible that uPA is produced in an inactive form by the oocyte and requires a cumulus cell factor to convert it to an active form. However, further studies are required to confirm these possibilities.

Huarte et al. [15] have suggested that spontaneous increases in oocyte tPA activity correlated with germinal vesicle breakdown in rats and mice. These results are consistent with further reports showing the presence of tPA activity in rat oocytes [16, 41] but again are not consistent with results of the present study using bovine oocytes. In the present study, nuclear maturation of bovine oocytes was not directly examined, but about 66–67% of cumulus-free oocytes cultured for 24 h with or without EGF had a polar

body, indicating that possibly these oocytes completed meiotic maturation reaching metaphase II. Nevertheless, absolutely no production of uPA was observed in these oocytes, suggesting that there is no correlation between meiotic maturation and production of uPA in bovine oocytes. However, at present, the reason for the discrepancy between our results using bovine oocytes and others using rat and mouse oocytes is not known.

It has been demonstrated in a great number of reports that EGF stimulates in vitro maturation of oocytes and thereby the development of the oocytes after in vitro penetration is promoted in various mammalian species including cattle. In the present study, EGF (30 ng/ml) added to maturation medium stimulated not only nuclear maturation and cumulus expansion in bovine COCs, but also PA production by oocytes and cumulus cells. However, in denuded oocytes, the proportion (about 66–67%) of oocytes having a polar body 24 h after the start of culture was not different in the presence and absence of EGF, but no uPA activity was detected in either group of oocytes. These results strongly suggest again that bovine oocytes have no ability to produce uPA themselves, at least under the experimental conditions used here; EGF stimulates uPA production by cumulus cells, and thereby uPA activity in oocytes increases. Although the role of uPA found in maturing bovine oocytes is not clear, uPA may play a role in cytoplasmic maturation of oocytes, which results in normal fertilization and development of oocytes.

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