Growth Factors and Components for Extracellular Proteolysis Are Differentially Expressed during In Vitro Maturation of Bovine Cumulus-Oocyte Complexes

Bettina Bieser, Miodrag Stojkovic, Eckhard Wolf, Heinrich Meyer, and Ralf Einspanier

ABSTRACT

In the present study, the time-dependent collagenolytic potential and mRNA transcription of extracellular matrix (ECM)-degrading components, transforming growth factor β1 (TGFβ1), and both basic fibroblast growth factor (bFGF) and FGF receptors (FGFR) in bovine cumulus-oocyte complexes (COCs) were investigated during 24 h of in vitro maturation (IVM). COCs were collected from 2- to 6-mm follicles, cultured in maturation medium, and sequentially removed at 3-h intervals for analysis. From 285 oocytes matured under these conditions, 114 (40.0%) developed to blastocysts on Day 9 after fertilization. Gelatin zymograms revealed protease activity at about 55 kDa for COCs matured for at least 3 h and two additional proteolytic zones at about 70 kDa after at least 9 h of IVM. The mRNAs of ECM-degrading components urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor 1 (PAI1), matrix metalloproteinase 1 (MMP1), and tissue inhibitor of metalloproteinases 1 (TIMP1), as well as TGFβ1, bFGF, and FGFR, were detected during IVM in a factor-specific pattern: all transcript levels found at COC 0 generally increased after 3 h of maturation and either remained high or decreased thereafter. On the basis of the chosen reverse transcription-polymerase chain reaction technique, one could suggest relative higher mRNA concentrations for TIMP1, PAI1, and both growth factors compared to uPA, MMP1, and FGFR.

Our results suggest a finely tuned extracellular proteolysis during IVM of bovine COCs, for which the concerted action of modulating growth factors like bFGF and TGFβ1 may be essential.

INTRODUCTION

Immature oocytes of eutherian mammals are tightly surrounded by cumulus cell layers, forming cumulus-oocyte complexes (COCs). Both the preovulatory surge of gonadotropins in vivo and the separation from the follicular environment and culture in suitable media in vitro initiate the resumption of meiosis coinciding with cumulus expansion. This process has been implicated in the control of meiotic arrest [1], oocyte transport [2], and fertilization [3] and is characterized by deposition of hyaluronic acid-anchored mucleoelastic material into the extracellular matrix (ECM). It changes the compact cumulus into a glistening mass increasing 20- to 40-fold in volume [4].

Other developmentally regulated processes associated with reconstruction and reshaping of the ECM, such as ovulation or uterus involution, have been reported to involve members of the matrix-degrading cascade [5, 6]. These include elements of the plasminogen/plasmin converting system (plasminogen activators: PA; plasminogen activator inhibitors: PAI), matrix metalloproteinases (MMP) for which PA are presumably activators, and tissue inhibitors of metalloproteinases (TIMP) (for an overview see [7, 8]).

Studies on oocyte physiology hitherto have focused on tissue-type PA (tPA) and its inhibitors. Transcription of the tPA gene and tPA activity have been shown to increase in rat oocytes during in vivo maturation [9]. Transcription of PAI1 and PAI2 genes has been described for human cumulus and granulosa-luteal cells [10].

Growth factors are known to play a central role in the regulation of matrix-degrading components. The heparin-binding basic fibroblast growth factor (bFGF) has been suggested as an intraovarian inducer of rat granulosa cell tPA gene transcription [11] and has been found to stimulate gene transcription and production of TIMP1 in cultured bovine granulosa cells [12]. Expression of bFGF transcripts has been reported for human granulosa and cumulus cells [13]. Our previous study revealed the presence of bFGF-specific mRNA and protein in the bovine oviduct [14]. Transforming growth factor β1 (TGFβ1) is generally considered to exert positive effects on the accumulation of ECM. This occurs as the net result of enhanced matrix protein synthesis, diminished overall MMP synthesis with the exception of MMP2, and augmented production of TIMP1 and other specific inhibitors of matrix degradation [15, 16]. For example, TGFβ1 has been reported to stimulate the production of hyaluronan in mouse mural granulosa and cumulus cells [17] and to enhance cell locomotion [18]. Specific mRNAs encoding TGFβ1 and TGFβ2 have been detected in human granulosa and cumulus cells [19]. Generally, TGFβ isoforms have been implicated as intraovarian regulators of cell functions in many species [20–22] including cattle [23].

To our knowledge, the potential roles and interactions of members of the ECM-degrading cascade and growth factors during in vitro maturation (IVM) of COCs have not been investigated. Therefore, we studied time-dependent changes of proteolytic activities and mRNA levels during a 24-h IVM of bovine COCs. We focused on transcription of MMP1, urokinase-type plasminogen activator (uPA), PAI1, and TIMP1, as well as TGFβ1, bFGF, and FGFR receptors (FGFR).

MATERIALS AND METHODS

Recovery of COCs and IVM

COCs were obtained by aspiration of 2- to 6-mm follicles of ovaries from slaughtered German Simmental cows. Oocytes with compact, multilayered cumulus investment were collected under a stereomicroscope, washed twice in Ringer solution (Fresenius, Bad Homburg, Germany), and washed once in maturation medium (MI199 [Sigma, De-

801
TABLE 1. Gene transcripts, number of cycles used, resulting fragment sizes, and primer sequences.

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<td>Rev: GCA AAC CAA GGC TGG</td>
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* For, forward; Rev, reverse; R: A or G; M: A or C; Y: C or T; S: G or C.
1 EMBL accession number or reference of published sequence.

senhorn, Germany), containing 10 mM NaHCO₃ and adjusted to pH 7.4, 10% heat-treated fetal calf serum, 2 μg/ml Enrofloxacin [Bayer, Leverkusen, Germany], and 10 ng/ml highly purified sheep FSH, which was kindly provided by Dr. H. Papkoff [San Francisco, CA]. Groups of about 80 COCs were pooled, added to a 0.5-ml drop of maturation medium under paraf®n oil, and cultured for up to 24 h at 39°C in a humidified atmosphere of 2.5% CO₂ in air.
To evaluate the efficiency of oocyte maturation in this system, 285 COCs were fertilized in vitro and cultured to the blastocyst stage as described previously [24]. For in vitro fertilization, oocytes were maintained in Tyrode’s-albumin-lactate-pyruvate medium containing 6 mg/ml BSA, 10 μg/ml heparin, and frozen-thawed semen (10⁶ spermatozoa/ml) that had been subjected to a swim-up procedure. After 18 h, cumulus cells from presumptive zygotes were removed by vortexing (120 sec). Groups of about 30 zygotes were cultured in tissue culture medium 199 supplemented with 10% estrous cow serum at 39°C in a humidified atmosphere of 5% CO₂:5% O₂:90% N₂. The cleavage rate of 5- to 8-cell stages was evaluated 66 h after in vitro fertilization. Blastocyst rates were recorded 168 h (Day 7), 192 h (Day 8), and 216 h (Day 9) after in vitro fertilization. Proportion of oocytes that progressed to 5- to 8-cell stages was 66.7%. Blastocyst rates ranged from 31.6% on Day 7 to 40.0% (based on total oocytes) on Day 9, respectively.
Two independent trials were performed for gelatin zymography. Two COCs were recovered at random at the beginning of each trial (COC 0) and then at intervals of 3 h (COC 3 to COC 24). They were washed in Ringer solution and stored at −20°C until processed for zymography.
For transcription analysis, 4 COCs were removed at the times indicated and stored at −60°C until RNA isolation. Data were collected from five independent maturation experiments.

Zymography

The collagenolytic capacity of maturing COCs was monitored using gelatin zymogram gels. The gels were prepared and processed according to the method of Fisher and Werb [25]. Gelatin (0.3 mg; Difco Laboratories, Detroit, MI) was added to the polycrylamide separating gel (12.5%) before polymerization. COCs were solubilized in 10 μl of the sample buffer indicated [25] and loaded, and their proteins were separated. Collagenolytic activity occurred overnight during incubation at 37°C in 50 mM Tris-HCl (pH 7.8) containing 150 mM NaCl and 5 mM CaCl₂. Gels were stained in Coomassie blue for 1 h and destained in 45% methanol, 10% acetic acid.

RNA Isolation and Reverse Transcription

Total RNA from each sample was extracted separately using the RNA easy total RNA kit (Quiagen, Hilden, Germany) as recommended by the manufacturer. RNA was eluted with 30 μl sterile water, and reverse transcription was performed in a total volume of 60 μl by use of hexanucleotides as primers according to the protocol for Expand reverse transcriptase (Boehringer, Mannheim, Germany). The resulting cDNA was stored at −20°C until polymerase chain reaction (PCR) amplification.

PCR Amplification

PCR analysis of cDNAs for growth factors and components of the ECM-degrading cascade was performed using Primezyme (Biometra, Göttingen, Germany). Two microtiter cDNA was amplified in a total reaction volume of 25 μl by use of 10 pmol each of the sequence-specific primer pairs, synthesized commercially (Pharmacia, Freiburg, Germany), and 0.5 U of Taq polymerase. All amplifications were done at 94°C and 60°C, 1 min each, except those for uPA and the FGFR, which were cycled at 94°C and 55°C, 1 min each. Number of cycles, resulting fragment sizes, primer sequences, and the source from which they were taken or according to which they were designed are shown in Table 1. The number of cycles used led to levels of amplification slightly above detection level when a cDNA, pooled for all maturation stages analyzed, was tested. Except for those for FGFR, all primer pairs were designed according to known bovine sequences. For FGFR, primers from consensus cDNA portions of highly conserved regions within the cytoplasmatic tyrosine kinase domains of all four FGFR types were used [26]. For uPA, primer sequences were synthesized according to Krätzschmar et al. [27]. The identity of each PCR product was confirmed by cDNA sequencing after subcloning or by use of direct PCR product sequencing (TopLab, München, Germany). Water blanks were negative.

Five microtiter of each PCR reaction was run on an ethidium bromide-stained 1.5% agarose (SeaKem Le; Biozym, Oldendorf, Germany) gel and documented using a Video Documentation System (Pharmacia). Band intensities
were analyzed by the corresponding software (Image Master 1D; Pharmacia). Peak transcription during each experiment was fixed at 100%, and the other intensities were expressed relatively.

**Statistics**

Reverse transcription-polymerase chain reaction (RT-PCR) data of each factor under study were tested for normal distribution by the Kolmogorov-Smirnov test and for equal variances (Levene median). If data passed both tests, pair-wise comparisons were performed by one-way ANOVA using the Tukey test. Data for TGFβ1 showed unequal variances and had to be tested by a Kruskal-Wallis ANOVA on ranks in combination with the Tukey test.

**18S rRNA as Internal Control**

The 18S rRNA primer and competitor technology (QuantumRNA module, containing 18S rRNA primers and competitors in separate stocks; Ambion, Austin, TX) is designed for duplex PCR and allows the 18S rRNA to be used as an internal control for target mRNA of any abundance. 18S competitors are 18S primers modified at their 3’ ends to block extension by DNA polymerase. By mixing 18S rRNA primers with increasing amounts of competitors, the 18S rRNA amplification efficiency, i.e., the yield of the specific 488-base pair (bp) product, can be lowered without affecting the target cDNA amplification. We used this technology to demonstrate that the changes in mRNA abundance for the individual factors monitored in our study were due to the maturation stage and nearly independent of tube-to-tube variations during RT-PCR.

As target cDNAs, those of all ECM-degradation components and growth factors analyzed were used. Only duplex PCR with FGFR had to be omitted since the two PCR products could not be separated due to their similar sizes.

The cDNAs of one IVM experiment were pooled and used to determine suitable 18S competitor and primer ratios. Those leading to equal amounts of 18S rRNA and target amplifications while PCR reactions were still in the exponential phase of amplification were judged to be ideal. They were subsequently used to screen the various cDNAs separately.

For all duplex PCR amplifications, cocktails and conditions were identical to those described above. Only polymerase quantities and dNTP concentrations were doubled. MetaPhor agarose (Biozym) was used for 18S-uPA duplex PCR, as it allowed a higher-resolution performance.

**RESULTS**

In gelatin zymograms, protease activity changed during the maturation process. Immature COCs showed almost no gelatinolysis. A cleared zone at about 55 kDa could be observed for COC 3 to COC 24. COC 9 and the subsequent stages demonstrated two additional, neighboring protease bands at about 70 kDa (Fig. 1). Although gelatin zymography used in this study is a semiquantitative technique, the activity in the 70-kDa size range seemed to increase during the last third of the maturation period.

Quantitative transcription analysis was hampered by the fact that the amounts of RNA that could be isolated from COCs at individual stages of IVM were too small for spectroscopic quantification. Instead, the amount of 18S rRNA served as an internal control. 18S rRNA is much more abundant than any mRNA species. Before it was used in duplex PCR as an internal standard, its amplification efficiency had to be lowered by the competitor technology. The optimal ratios of 18S rRNA primers and competitors were determined to be 10:90 (if used in duplex PCR with uPA primers), 40:60 (with PAI1 primers), 7.5:92.5 (with MMP1 primers), 25:75 (with TIMP1 or bFGF primers), and 17.5:82.5 (with TGFβ1 primers) (data not shown). For FGFR, duplex PCR could not be applied because of the small difference in length of the expected products (FGFR: 471 bp; 18S rRNA: 488 bp). The controls demonstrated that the transcription pattern observed was almost independent of deviations in RNA isolation or tube-to-tube variations during RT-PCR (Fig. 2, open arrow on zymograms). Previous studies had shown, however, that amplifications did not reach a plateau but remained in the linear phase under the cycle conditions used (data not shown).

RT-PCR amplification was successful for all components analyzed, and changes in factor-specific mRNA abundance were observed during the IVM period. Statistical analysis, while the relatively low number of experiments is noted, revealed that changes were significant except in the case of FGFR and TGFβ1. For uPA, mRNA levels increased slowly from COC 0 to COC 9, suddenly increased twofold by COC 12, and then continuously decreased until the end of the maturation period (Fig. 2A). PAI1-specific mRNA was merely detectable for COC 0 but increased until COC 15 and declined thereafter (Fig. 2B). The abundance of MMP1 transcripts was low at the beginning of IVM, increased markedly by COC 6, stayed at a plateau until COC 15, and then slowly declined to about half maximum levels by COC 24 (Fig. 2C). TIMP1 mRNA levels were low until COC 3 but then continuously increased to reach a maximum by COC 12, which was—in contrast to observations for the other ECM regulatory components—maintained at almost the same level until the end of the maturation period (Fig. 2D). Transcription of bFGF-specific mRNA was at the detection limit by COC 0 but increased during the next stages and peaked at COC 9; this was followed by a marked decline until COC 24 (Fig. 2E). Steady-state levels of FGFR mRNA were low by COC 0, increased markedly within the first 6 h of IVM, and remained at a plateau until COC 9. Subsequently, the relative amount of FGFR mRNA declined to reach about 50% of the maximum level by COC 15 and stayed there until the end of IVM (Fig. 3). For TGFβ1, mRNA levels corresponded to about 25% of maximum by COC 0 to COC 3, subsequently increased until COC 12, and then slightly declined to a relative abundance of 70% by COC 24 (Fig. 2F).

Overall, the abundance of mRNAs specific for PAI1, TIMP1, and both growth factors seemed to be higher during IVM than those of uPA-, MMP1-, and the FGFR-specific transcripts.
FIG. 2. Duplex RT-PCR analysis of A) uPA, B) PAI1, C) MMP1, D) TIMP1, E) bFGF, and F) TGFβ1 mRNA levels of bovine COCs matured for the time intervals indicated as compared to 18S rRNA levels. Closed arrows indicate 463, 363, 347, 380, 289, and 223 bp, respectively; open arrows, 488 bp. Marker bands represent A) 400 and 800 bp; B-F) 200, 400, and 800 bp. Graphs depict a statistical analysis of the RT-PCR-derived mRNA levels. Data represent percentage mean ± SE of four additional experiments. Values lacking a common letter are significantly different (p < 0.05).
likely that the cumulus itself represents the predominant contacts described for cumulus expansion [30]. It seems limited degradation could assist in diminishing cell-cell effects necessary for well-regulated ECM remodeling. Such dynamic may guarantee a finely balanced net effect. The component-specific transcription level and transcript dynamics could be related directly to the stage of maturation. The proteolytic potential may be active during IVM. The protease activities during IVM and their levels of mRNAs specific for components involved in ECM turnover.

Along with PA, MMP play a key role in extracellular proteolysis. The gelatin zymography technique was used as a well-established screening method for MMP detection. Our data indicate that at least two proteases with gelatinolytic potential may be active during IVM. The protease activity at about 55 kDa, seen for COC 3 and later stages, is in accordance with the reported molecular mass of pro-MMP1 (52 kDa) [28]. Identification is not definite, as other pro- and active MMP share comparable molecular weights. However, the RT-PCR results argue for an involvement of this protease during IVM. The double-banded gelatinase activity at around 70 kDa, which appeared first by COC 9 and seemed to increase during the following stages, matches solely with the pro- and active forms of MMP2 (72 and 66 kDa, respectively) [28]. The latent forms were active in the zymography assay without a reduction in molecular weight. This is consistent with their activation by SDS during gel electrophoresis [29]. The different timing of the two protease activities during IVM suggests control by complex regulatory mechanisms.

RT-PCR analysis of uPA, PAI1, MMP1, and TIMP1 mRNA levels revealed that, during IVM, all elements necessary for a proteolytic cascade were expressed in an individual manner. In addition, the almost constant 18S rRNA signals seen during duplex PCR indicated that transcription dynamics could be related directly to the stage of maturation. The component-specific transcription level and transcription dynamic may guarantee a finely balanced net effect necessary for well-regulated ECM remodeling. Such limited degradation could assist in diminishing cell-cell contacts described for cumulus expansion [30]. It seems likely that the cumulus itself represents the predominant location of mRNA synthesis at this stage of development. For COC 0, however, contribution by the oocyte to these and the growth factor/FGFR mRNA levels observed cannot be ruled out and is a subject for future studies.

We demonstrated a strong transcription for TGFβ1 and bFGF and a striking time-dependent regulation for the latter. Furthermore, preliminary studies revealed bFGF-like immunoreactivity in expanded cumuli. The FGFR-specific mRNA transcription observed supports a receptor-mediated concept for this bFGF. Two modes of growth factor action should be considered in this regard. Firstly, cumulus-derived growth factors could exert an influence on the oocyte. Basic FGF was reported to induce maturation of follicle-enclosed rat oocytes [11], and FGF showed a small but significant effect on germinal vesicle breakdown in the mouse [31]. Acceleration of both rat follicle-enclosed and isolated COC maturation has been shown for TGFβ [32]. Secondly, growth factors could represent a mechanism controlling ECM degradation. In cultured bovine microvascular endothelial cells, the proteolytic balance has been reported to tilt toward enhanced proteolysis in response to bFGF and toward antiproteolysis in response to TGFβ1, and to behave similarly to that of untreated cultures when the two agents were added simultaneously [33]. The situation might be similar during cumulus expansion. Basic FGF may support the induction of proteolysis when expressed during the very early hours of IVM, while TGFβ1 could modulate and limit its action in the second half of the maturation period. In this context, it is interesting to note that the activities of both growth factors depend on limited extracellular proteolysis [34, 35] favoring multiple feedback mechanisms.

Our findings suggest a finely tuned extracellular proteolysis during IVM of bovine COCs, possibly facilitating cumulus expansion. Growth factors such as bFGF and TGFβ1 may be possible modulators of such matrix turnover, leading to COCs ideally prepared for fertilization, as well as influencing meiotic arrest.

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REFERENCES