Expression of plasminogen activator genes and enzymatic activities in rat preimplantation embryos

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Plasminogen activator has been implicated in tissue invasion and remodelling because of its role in the degradation of the extracellular matrix. Its activity can be detected in mouse embryos as early as day 6 of pregnancy, suggesting that plasminogen activator is involved in the process of implantation. The present study determined the time course of expression of the genes encoding tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) during the preimplantation period in rats by the sensitive mRNA phenotyping procedure of reverse transcription–PCR. The tPA mRNA was present in rat oocytes and two-cell embryos, but was not detected between the four-cell and blastocyst stages. The uPA mRNA was first detected in two-cell rat embryos, and was present through to the blastocyst stage. In chromogenic assays, plasminogen activator activity was detected in oocytes and embryos between two-cell and blastocyst stages. Most plasminogen activator activity present in preimplantation embryos appeared to be uPA, as it could be inhibited by anti-uPA antibody and a specific uPA inhibitor, amiloride, but not by anti-tPA antibody. The present data demonstrate the expression of uPA gene and uPA activity in preimplantation rat embryos, suggesting that embryonic uPA may be involved in early embryo development and implantation.

Introduction

Plasminogen activator converts plasminogen, an inactive proenzyme, into the active protease plasmin, and has been implicated in many important biological processes that require proteolytic activity, such as tissue remodelling, tissue destruction, cell migration, and in dissolving blood clots (Dano et al., 1985; Saksela, 1985; Vassalli et al., 1989). Two types of plasminogen activator, tissue-type PA (tPA) and urokinase-type PA (uPA), have been identified. Plasminogen activators are thought to play an important role in many events of mammalian reproduction. During the process of ovulation, they are involved in the proteolytic cascade that leads to the rupture of the follicular wall to release the oocyte (Beers et al., 1975; Tsafriti et al., 1989). tPA is expressed during oocyte maturation (Huarte et al., 1985) and probably participates in preventing polyspermy and in other events during fertilization (Zhang et al., 1992; Huarte et al., 1993). uPA is probably involved in embryo implantation, as uPA activity is detected in mouse trophoblast cells as early as day 6 of pregnancy, coinciding with the beginning of trophoblast invasion of the endometrium (Strickland et al., 1976; Sappino et al., 1989). Plasminogen activator activity is also present in embryos from animals such as the cattle, in which embryo implantation is not as (or is less) invasive as in rodents and humans (Kaaekuhawi and Menino, 1990).

Implantation is a critical period in the establishment of pregnancy, as pregnancy failure frequently occurs before or during implantation in many species (Haéf, 1967; Cooke, 1988). Systematic studies of the expression and function of plasminogen activators during implantation and early development may further our understanding of the molecular mechanisms of implantation, thereby facilitating the development of strategies for preventing early pregnancy losses in humans and farm animals. For this purpose, the present study examined the time course of expression of genes encoding tPA and uPA, and the enzymatic activities of the two plasminogen activators in rat preimplantation embryos.

Materials and Methods

Collection of oocytes and embryos

Prepubertal Sprague–Dawley rats (Charles River Canada, St Constant, Quebec), weighing 65–70 g, were induced to ovulate by s.c. injections of 15 iu eCG followed, 56 h later, by i.p. injections of 15 iu hCG (Sigma, St Louis, MO). Rats were mated with fertile males during the night after hCG treatment.
Ovulated oocytes or zygotes, two-cell embryos, four-cell embryos, morulae and early blastocysts were collected from oviducts or uteri at 24, 48, 72, 92 and 120 h, respectively, after the hCG injection. The oocytes and embryos were rinsed in phosphate-buffered saline (pH 7.4) containing 0.3% polyvinyl pyrrolidone (Sigma) and frozen at −70°C until further processing.

**RNA extraction, reverse transcription and PCR**

These procedures were performed as described by Rappolee et al. (1989) with slight modifications. Briefly, 100–150 oocytes or embryos were pooled according to their stage of development and lysed in a denaturing solution containing 4 mol guanidine thiocyanate L⁻¹ to extract total RNA by centrifugation on a cushion of 5.7 mol CsCl L⁻¹ and 0.1 mol EDTA L⁻¹ at 250 000 g for 4 h at 20°C. The RNA pellet was then dissolved in a solution of 3 mol ammonium acetate L⁻¹ and precipitated at −20°C in the presence of two volumes of ethanol.

Single-stranded cDNA was synthesized from these RNA preparations in reverse transcription reactions in a final volume of 20 µl, using avian myoblastosis virus reverse transcriptase (Boehringer-Mannheim, Laval) as directed by the manufacturer. An aliquot of the reverse transcription reaction product (equivalent to 10–15 oocytes or embryos) was used for PCR. The PCR reaction proceeded for 30–40 cycles in a DNA thermal cycler (Perkin Elmer-Cetus, Norwalk, CT). Each amplification cycle consisted of denaturing at 94°C for 1 min, annealing primers to target sequences at 65°C for 1 min and primer extension at 72°C for 2 min. An aliquot (15–20 µl) of the PCR products was analysed by electrophoresis on 7% polyacrylamide gels and stained with ethidium bromide to visualize PCR products on a UV transiluminator. The authenticity of the PCR fragment in every PCR sample was verified by digestion with endonucleases, the cutting sequences of which were bracketed by the PCR primers (PstI for tPA and TaqI for uPA). To confirm the identity of the PCR fragment, PCR products in some samples were cloned into a pBluescript plasmid vector, using a blunt-end ligation method (Sambrook et al., 1989). The nucleotide sequences of the cloned PCR fragments were then determined by the dideoxynucleotide terminator method, using an automated DNA sequencing system (Applied Biosystems, Foster City, CA).

Every RNA sample was verified for the absence of genomic DNA contamination by the lack of any amplification product after an aliquot of the RNA preparation was subjected to standard PCR with the primers (made in a DNA synthesizer in the Dept of Biochemistry, University of Western Ontario) for tPA and uPA.

**PCR primers:**

For tPA:

Forward: 5'-TCCACCTGGGCGCTGAGGCAAT-3'  
(nucleotides No. 915-936)  
Reverse: 3'-GAGGGACTGACCTGCTCACAC-5'  
(nucleotides No. 1338-1359)

For uPA:

Forward: 5'-GTGGAGAACCGCTGCT-3'  
(nucleotides No. 620-638)

**Fig. 1.** Total RNA equivalent to approximately 20 oocytes (O), 20 embryos at one-cell (F) or two-cell (2) stages, or 15 embryos at four-cell (4), eight-cell (8) morula (M) or blastocyst (B) stages was analysed by reverse transcription–PCR. The medium in which the oocytes or embryos were rinsed before RNA extraction was subjected to the same reverse transcription–PCR procedures as a negative control (R). The desired PCR product for tPA mRNA (445 bp) and uPA mRNA (348 bp) was verified by cutting with PstI and TaqI, respectively, to yield two fragments of the expected sizes (355 and 90 bp for tPA; 178 and 170 bp for uPA; C = cut; U = uncut control). The representative digestion results from an oocyte sample (for tPA) and a morula sample (for uPA) are shown here. The values indicate the number of base pairs for some of the DNA fragments in the 1 kb ladder from Gibco-BRL (L). These gels are representative of at least three replicates. (a) tPA mRNA in rat oocytes or embryos; (b) uPA mRNA in rat oocytes or embryos.

Reverse: 3'-TATGTTCTGGTAGACGGACGG-5'  
(nucleotides No. 947-966)

These primers were chosen within rat tPA and mouse uPA cDNA sequences (tPA: Ny et al., 1988; uPA: Belin et al., 1985; Rickles et al., 1988) that are conserved between different species but divergent between the two PA genes. The forward and reverse primers for tPA and uPA are located on different exons within each gene, according to the human gene structures for tPA (Ny et al., 1984) and uPA (Verde et al., 1984), so that amplification products resulting from contaminant genomic DNA would be larger and thus could be easily distinguished from those from cDNA.

**Enzymatic assays for plasminogen activators**

A chromogenic assay, as described originally by Coleman and Green (1981) with slight modifications, was used to determine the presence of plasminogen activators in rat embryos. This assay was chosen to conserve experimental materials and reagents because of its high sensitivity and small assay volume (50 µl). It measures total plasminogen activator
activity without discrimination against tPA or uPA. Briefly, embryos or oocytes were lysed by repeated freezing and thawing in PBS containing 0.01% Tween 80, and mixed with, or without (negative control), 2.5 μg plasminogen and with, or without, neutralizing antibodies against pig tPA and human uPA (Biopool Canada, Hamilton, Ontario) or a uPA inhibitor, amiloride (Vassalli and Belin, 1987). The antibodies are known to crossreact with respective antigens in mice, according to the manufacturer. The mixture was incubated at 37°C for 60–90 min. At the end of this incubation, 1 ml of substrate solution, containing 220 μmol 5,5’-dithiobis(2-nitrobenzoic acid) 1⁻¹ and 220 μmol thiobenzyl benzyloxycarbonyl-l-lysinate 1⁻¹ (Sigma) in phosphate buffer pH 7.4, was added and the incubation was carried out for a further 60 min. The absorbance at 410 nm was indicative of plasminogen activator activity. uPA activity standards (Calbiochem, San Diego, CA), ranging from 0.02 mili U to 4 mili U, were included in each assay to estimate the PA activity from rat embryos. The sensitivity of the assay under the conditions described above was 0.02 ± 0.01 mili U. The intra-assay and interassay coefficients of variation were below 10% and 15%, respectively.

Expression of PA genes

A 445-base pair (bp) PCR product corresponding to tPA mRNA was detected in rat oocytes and one- and two-cell embryos (Fig. 1a). Thereafter, tPA mRNA was not detected until the early blastocyst stage. tPA mRNA was detected in day 5 rat blastocysts (data not shown). Digestion of the PCR product with PstI endonuclease confirmed the authenticity of the PCR product. The PCR product corresponding to uPA mRNA with an expected size of 348 bp was detected in rat embryos from the two-cell stage onwards, but not in oocytes or one-cell embryos (Fig. 1b). Digestion with TaqI confirmed that the PCR product is probably a true representation of uPA mRNA.

The identity of the amplified product was confirmed by cloning and sequencing PCR fragments from a one-cell embryo sample (for tPA) and from a blastocyst sample (for uPA). The nucleotide sequence was determined in two clones for each PCR fragment. The sequence of PCR fragments for tPA was compared with that of the published rat cDNA (Fig. 2). The variation between the published sequence (Ny et al., 1988) and that of the two clones of the PCR fragment is less than 0.5%. For uPA, the sequence of the cloned PCR fragment was compared with that of mouse cDNA (Belin et al., 1985; Fig. 3), as the rat cDNA sequence is not available. The variation in nucleotide sequence between the two species is less than 10%. A total of seventeen amino acids was altered as a result of the nucleotide differences, and almost all the amino acid variations occurred in regions where there is also heterogeneity between mouse and human sequences (Verde et al., 1984). The nucleotide sequences of the two clones of the PCR fragments for rat uPA are in good agreement with only one nucleotide variation.

Expression of plasminogen activator activity

A chromogenic assay was used to determine the possible presence of plasminogen activator activity in embryos (Table 1). No PA activity was detected in the absence of plasminogen (data not shown). PA activity in oocytes was inhibited by anti-tPA antibody, but not by anti-uPA antibody or amiloride. This finding indicates that oocytes contain tPA, which is consistent with previous findings and also verifies the specificity of the antibodies used. Activity of plasminogen activator was low in two-cell embryos, and increased to eight-cell and blastocyst stages. The activity of plasminogen activator in embryos between the two-cell and blastocyst stages was not affected by anti-tPA antibody, but was inhibited by anti-uPA antibody and amiloride, suggesting that most, if not all, of the PA activity found between the two-cell and blastocyst stages is uPA.
Fig. 3. Sequence of PCR fragment of rat uPA gene. The amino acid and nucleotide sequences of the target mouse uPA fragment (Belin et al., 1985) are shown in rows 1 and 2, respectively. The nucleotide sequences of the PCR primers are in italic. Rows 3 and 4 are the nucleotide and amino acid sequences of rat PCR fragment, respectively, and Row 5 is the amino acid sequence of the relevant human uPA fragment (Verde et al., 1984). Dots denote sequences identical to those of the mouse uPA. *Variation noted in only one of the two clones sequenced.

Table 1. Plasminogen activator activity in rat oocytes and embryos (µIU per embryo ± SD)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Control</th>
<th>tPA antibody (0.1 mg ml⁻¹)</th>
<th>uPA antibody (0.1 mg ml⁻¹)</th>
<th>Aminolide (100 µmol l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocyte</td>
<td>146 ± 21⁺</td>
<td>2 ± 0⁺</td>
<td>151 ± 32⁺</td>
<td>149 ± 26⁺</td>
</tr>
<tr>
<td>Two-cell</td>
<td>8 ± 2⁺</td>
<td>10 ± 2⁺</td>
<td>2 ± 0⁺</td>
<td>≤ 1</td>
</tr>
<tr>
<td>Four-cell</td>
<td>32 ± 5⁺</td>
<td>29 ± 3⁺</td>
<td>2 ± 0⁺</td>
<td>≤ 1</td>
</tr>
<tr>
<td>Eight-cell</td>
<td>50 ± 8⁺</td>
<td>52 ± 9⁺</td>
<td>2 ± 0⁺</td>
<td>≤ 1</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>86 ± 11⁺</td>
<td>81 ± 11⁺</td>
<td>3 ± 0⁺</td>
<td>≤ 1</td>
</tr>
</tbody>
</table>

Fifteen oocytes or embryos at each stage were analysed in the chromogenic assay in the absence (control) or presence of antibodies against tPA or uPA or a uPA inhibitor, aminolide. Each assay was carried out on three occasions.

⁺ versus ² in each row: significantly different (P < 0.01; 1 test); * versus ² versus ³ in each column (excluding oocytes): P < 0.05 (Duncan's multiple range test).

Discussion

The study reported here determined the time course of expression of the two PA genes during preimplantation development in rats. In mice, tPA mRNA accumulates in growing oocytes and remains dormant until the resumption of meiosis when the mRNA is translated and degraded (Fiuarte et al., 1987). Consistent with these findings, tPA mRNA was expressed in rat oocytes and one-cell and two-cell embryos, and was not expressed thereafter, probably as the result of the degradation of the maternal messengers. tPA mRNA was not expressed until the blastocyst stage. uPA mRNA was first expressed in rat embryos at the two-cell stage. The lack of expression of uPA mRNA in rat oocytes indicates that this mRNA in embryos is not derived from the maternal genome. The expression of uPA mRNA in rat two-cell embryos probably results from the activation of the embryonic genome. It is well established that the mouse embryonic genome is
activated after the first cell division (late two-cell, Telford et al., 1990), but there are no conclusive studies in the rat that establish the time when the embryonic genome is activated. The present data on gene expression were obtained by the use of the sensitive reverse transcription–PCR method. The authenticity of the PCR products was verified by restriction digestion of all the samples with specific endonucleases and by determination of the nucleotide sequence after the PCR fragment was cloned into a plasmid vector.

Plasminogen activator activity was present in ovaclt embryos, and it appeared to be tPA, in agreement with previous findings (Huarte et al., 1985; Zhang et al., 1992). Plasminogen activator activity decreased in two-cell embryos and increased until the eight-cell stage. The increase of plasminogen activator activity in blastocysts was relatively small. The activity in embryos between the two-cell and blastocyst stages was probably attributable to uPA. Thus, the activity was inhibited by anti-uPA antibody and amiloride, but not by anti-tPA antibody. This finding is consistent with the presence of uPA mRNA in embryos from the two-cell stage onwards. Our preliminary study using a zymography assay confirmed the presence of uPA activity in rat blastocysts and their secretions (X. Zhang, G. M. Kidder and D. T. Armstrong, unpublished).

In mice, uPA activity was detected in day 6 embryos, but not at earlier stages (Strickland et al., 1976; Sappino et al., 1989), coinciding with the beginning of trophoblast invasion during implantation. In rat embryos, uPA activity was present from the two-cell stage. This apparent discrepancy may be due either to possible differences in the sensitivity of the PA assays used in these two studies or to species variations in the onset of production of plasminogen activator. However, tPA activity has recently been found in four-cell and morula embryos from mice, and this tPA is probably from the oviduct and binds to the embryo through a cell surface receptor present in mouse embryos (Carroll et al., 1993). The presence of plasminogen activator activity in embryos before implantation suggests that it may be involved in early embryo development as well as in implantation. For example, plasminogen activator or plasmin may activate a latent growth factor by limited proteolysis (Vassalli et al., 1989). This is relevant to the findings that many growth factors or their mRNAs are present in preimplantation embryos and some of these growth factors can improve embryo development (Schultz and Heyner, 1993; Zhang et al., unpublished).

In summary, the present study confirms the expression of tPA mRNA in oocytes and zygotes as maternal messages and further demonstrates that uPA mRNA is present in rat preimplantation embryos as the result of the activation of the embryonic genome. Rat preimplantation embryos also possess plasminogen activator activity which appears to be uPA. Further studies are required to characterize the regulation of the expression of plasminogen activator activity in preimplantation embryos. Such studies should help to elucidate its role in implantation and early development, thereby improving our knowledge of the molecular mechanisms controlling these important developmental events.

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