Role of cathepsins in blastocyst hatching in the golden hamster

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The mammalian embryo is encased in a glycoproteinaceous coat, the zona pellucida (ZP) during preimplantation development. Prior to implantation, the blastocyst must undergo ‘hatching’ or ZP escape. In hamsters, there is a thinning of the ZP followed by a focal lysis and a complete dissolution of the ZP during blastocyst hatching. Earlier studies from our laboratory have indicated a role for cysteine proteinases in the hatching phenomenon. In this study, we tested the effect of specific inhibitors of the three classes of cysteine protease on blastocyst hatching. Cystatin, an endogenous cathepsin inhibitor, blocked blastocyst hatching. Similarly, Fmoc-Tyr-Ala-diazomethane, a synthetic cathepsin inhibitor, blocked hatching. Both showed dose-dependent and temporal inhibition of hatching. However, Z-Val-Ala-Asp-fluoromethylketone, a synthetic caspase inhibitor, and calpastatin, an endogenous calpain inhibitor, had no effect on hatching. The cathepsins were localized to blastocyst cells. Exogenous addition of cathepsins L, P or B to cultured 8-cell embryos caused a complete ZP dissolution. The expression of mRNA and protein of cathepsins L and P was observed in peri-hatching blastocysts. Cathepsins L and P were detected in trophectodermal projections and in the zona pellucida. These data provide the first evidence that blastocyst-derived cathepsins are functionally involved as zonalytic factors in the hatching of blastocysts in the golden hamster.

Keywords: blastocyst; cathepsins; hatching; hamster

Introduction

The mammalian preimplantation embryo is encased in a glycoproteinaceous coat, the zona pellucida (ZP). The ZP is an acellular matrix, consisting of three glycoproteins (ZP-1, ZP-2 and ZP-3), and is involved in regulating sperm–egg interaction and protecting the embryo during preimplantation development (Wassarman et al., 2005). Prior to implantation, the blastocyst emerges out of the ZP by a critically important process called hatching. This is followed by the attachment, adhesion and invasion of blastocyst into the receptive uterine endometrium (Paria et al., 2002). Hatching of blastocysts in most mammalian species is characterized by shedding of the ZP, and has been observed in rodents (Bergstrom, 1972; Surani, 1975; Perona and Wassarman, 1986; Kane and Bavister 1988a; Seshagiri et al., 1999; Mishra and Seshagiri, 2000a; O’Sullivan et al., 2001), cattle (Massip and Mulnard, 1980), rhesus monkey (Seshagiri and Hearn, 1993) and humans (Sathananthan et al., 2003). In most cases, the fully expanded blastocyst exerts a mechanical pressure causing a nick in the ZP, probably facilitated by a protease. Through the nick, the blastocyst egresses out of the ZP, leaving the ZP intact.

During blastocyst hatching and implantation, a number of proteases have been shown to play important roles (Vu et al., 1997; O’Sullivan et al., 2001; Salamonsen and Nie, 2002; Tang and Rancourt, 2005). Expression of proteases of embryo–endometrial origin are regulated by various growth factors and cytokines, such as epidermal growth factor (EGF), heparin binding like-epidermal growth factor (HB-EGF), transforming growth factor-β and leukemia inhibitory factor (LIF) (Hardy and Spanos, 2002; Salamonsen and Nie, 2002; Seshagiri et al., 2002). In mice, two implantation serine proteases, ISP1 and ISP2, have been proposed to play a role in the hatching process (Perona and Wassarman, 1986; O’Sullivan et al., 2001; Sharma et al., 2006). The situation in human blastocyst hatching is less clear as these genes are not present in the human genome, and the closest related human enzyme is tryptase, a mast cell protease. Other proteases such as plasminogen activators (Aflalo et al., 2005) and matrix metalloproteases, and inhibitors such as tissue inhibitors of metalloproteases have been identified in mammalian embryos and endometrium but their role in hatching is not clearly established (Seshagiri et al., 2003; Wang et al., 2003). The cysteine proteases, cathepsins L, B, P and their natural inhibitor, cystatin C, are secreted by the embryo–endometrium during implantation and placentation in the mouse (Afonso et al., 1997, 2002; Nakajima et al., 2000; Sol-Church et al., 2002), although their role in hatching has not been investigated. More data are required to establish which proteases are critical for blastocyst hatching.

In the golden hamster, blastocyst hatching occurs in a more controlled fashion and thus is amenable to study the phenomenon of blastocyst hatching. The expanded blastocyst deflates and the ZP undergoes a gradual thinning, followed by a global dissolution leading to a complete disappearance of the ZP by Day 3.5 of...
and was either processed for RNA extraction or fixed for immunohistochemistry required, Day 14 pregnant females were used to recover placental tissue that were used as embryo donors. Females were mated overnight with proven fertile with pelleted feed and water 14 h light:10 h dark lighting schedule at ambient temperatures and provided 12 h for a total of 72 h. Embryos at different stages were either stored at

Sharma accompanied first by a nick and a hole in the ZP, presumably overlying process are not clear. In contrast, hatching of mouse blastocysts is Fig. S3). The Institutional Animal Ethics Committee approved procedures of Day 3 of pregnancy by flushing the excised uterine horns with 0.5 ml of HECM-2 h at 37

Female hamsters were superovulated with an i.p. injection of 30 IU pregnant m of HECM-2 h at 37

Effect of class-specific inhibitors of different cysteine proteases on blastocysts

Our earlier studies showed that hamster blastocysts undergo hatching by their intrinsic ability to produce zonalytic factor(s) that have cysteine protease-like activity and can cause complete dissolution of the ZP (Mishra and Seshagiri, 1998, 2000a). This study, however, did not clearly identify which cysteine proteases produced by blastocysts regulated the hatching process. Interestingly, the ZP of hamster blastocyst was shown generally to be more sensitive to protease treat ment than those from other species (Mishra and Seshagiri, 2000a). The present study aims to define which cysteine proteases function in blastocyst hatching, using class-specific inhibitors, and determine embryonic expression and cellular localization of key cysteine proteases during the peri-hatching period in the golden hamster.

Materials and Methods

Animals

Sexually mature golden hamsters (Mesocricetus auratus) were maintained on a 14 h light:10 h dark lighting schedule at ambient temperatures and provided with pelleted feed and water ad libitum. Six- to eight-week-old female hamsters were used as embryo donors. Females were mated overnight with proven fertile males. The following day, vaginal smears were checked for presence of sperm and assigned as Day 1 of pregnancy. In some experiments, when placenta was required, Day 14 pregnant females were used to recover placental tissue that was either processed for RNA extraction or fixed for immunohistochemistry and in situ hybridization, as described in Supplementary data (Methods and Fig. S3). The Institutional Animal Ethics Committee approved procedures for animal handling and experimentation, in accordance to guidelines on Use of Laboratory Animals for Research (INSA, New Delhi).

Recovery and culture of embryos

Female hamsters were superovulated with an i.p. injection of 30 IU pregnant mares serum gonadotrophin on the day of post-estrus discharge and mated 3 days later. In vivo developed, 8-cell, embryos were collected in the evening of Day 3 of pregnancy by flushing the excised uterine horns with 0.5 ml of equilibrated HECM-2 m (Ain and Seshagiri, 1997; Mishra and Seshagiri, 1998). Well-formed non-compact 8-cell embryos were used for all experiments. Embryos were cultured in 50 μl of HECM-2 h at 37 °C in an atmosphere of 5% CO2 in air and monitored for development and hatching. Hatched blastocysts were cultured on fetal bovine serum-coated dishes for trophoblast (TB) attachment. Embryo development was monitored microscopically every 12 h for a total of 72 h. Embryos at different stages were either stored at −70 °C for RNA isolation or fixed in 4% paraformaldehyde (PFA) for staining purposes. All reagents were procured from Sigma (St Louis, MO, USA) unless specified otherwise.

Influence of protease inhibitors on hatching

Effect of class-specific inhibitors of different cysteine proteases on blastocysts hatching was tested. These included cystatin, an endogenous cathepsin inhibitor; Fmoc-Tyr-diazomethane (FYAD) or peptideyl diazomethane, a synthetic cathepsin inhibitor; Z-Val-Ala-Asp-fluoromethylketone (Z-VD-FMK), a synthetic caspase inhibitor; calpastatin, an endogenous calpain inhibitor. Cystatin and calpastatin inhibitors were dissolved in HECM-2 h, while ethanol was used to dissolve FYAD and, DMSO was used to dissolve Z-VD-FMK. In control experiments, embryos were treated with similar concentrations of the appropriate diluents. Concentrations of the inhibitors tested were chosen based on their IC50 values for the respective enzymes. The experimental design to test various inhibitors on blastocyst hatching was scheduled as depicted in Supplementary data (Fig. S1). Freshly recovered 8-cell embryos or in vitro developed blastocysts (either expanded or deflated) were cultured in HECM-2 h with or without cystatin-C (0.01, 0.1 or 1.0 μg; Sigma Aldrich) or FYAD (0.5 or 1.0 μg; Bachem, King of Prussia, PA, USA) or Z-VD-FMK (20, 50 or 100 nM; Calbiochem, San Diego, CA, USA), or calpastatin (20, 50, or 100 nM; Calbiochem). Embryos were cultured continuously for 72 h (8-cells), 48 h (expanded blastocysts) or 36 h (deflated blastocysts). In separate experiments, cultured blastocysts were treated transiently with inhibitors for 6 or 12 h (expanded or deflated blastocysts) or for 24 h (expanded blastocysts). Following transient exposure, blastocysts were transferred to inhibitor-free medium and cultured for a further 12–42 h (Supplementary Fig. S1).

All cultured embryos were monitored for viability, hatching, TB attachment and outgrowth. The viability of the inhibitor-treated embryos was monitored by 0.5% Trypan blue staining. After Trypan blue staining, the number of blue cells and unstained cells were judged in each blastocyst and those with about ≤10% blue staining were considered viable. Representative examples of staining appearances of viable and nonviable embryos, judged by Trypan blue staining, are shown in Supplementary data (Fig. S2). The percent viability of embryos given in the Results section denotes the number of viable embryos out of the total embryo cultured in that particular treatment. As described elsewhere (Mishra and Seshagiri, 1998, 2000a; b), blastocysts hatching was microscopically assessed by zona dissolution. Blastocysts with the lysis of zonae were considered hatched and their percentages out of total embryos cultured determined. Experiments to determine attachment and outgrowth of TB were performed on serum-coated dishes (Mishra and Seshagiri, 1998). Blastocysts that did not move when the medium was gently swirled were considered to be attached. TB outgrowth was observed as the appearance of a monolayer of cells around the embryo and was monitored at every 12 h up to 72 h as described below (Mishra and Seshagiri, 1998). At least five embryos from the same donor were cultured for each treatment group and experiments were repeated three times with different embryo donors.

Morphometric analysis of TB outgrowth

Embryo images were captured using a CCD camera (JVC, TK 1085 E, Victor company of Japan Ltd, Yokohama, Kanagawa, Japan) and the area of TB outgrowth was determined using an image analysis software program, Image Pro Plus (Media Cybernetics, Silver Spring, MD, USA) as described elsewhere (Mishra and Seshagiri, 1998).

Localization of cathepsin inhibitor-interacting enzymes

To detect blastocyst localization of cathepsin, its biotinylated inhibitor, Biotin-Tyr- Ala-diazomethane (BYAD, Mason et al., 2004) was used. The enzyme-inhibitor complex could be detected with avidin Cy3 staining. Expanded blastocysts were treated with 0.5 μM BYAD, for 3 h in culture, washed with PBS-PVA (phosphate-buffered saline-polyvinyl alcohol) and fixed in 4% PFA for 30 min. Embryos were then permeabilized with 0.1% Triton-X 100 for 30 min and blocked with 2% bovine serum album (BSA) for 1 h. Finally, embryos were incubated with 1 μg/ml avidin-Cy3 for 1 h and 1 μg/ml DAPI for 10 min, after which they were washed in PBS-PVA and mounted in anti-fade solution. Control embryos, i.e. blastocysts not treated with BYAD, were also processed in a similar manner, to act as negative control. Fluorescence was monitored with a fluorescence microscope (IX70; Olympus Optical Co Ltd, Tokyo, Japan).

Influence of proteases on ZP dissolution

Freshly recovered hamster 8-cell embryos (n ≥ 15) and mouse 8-cell embryos (n ≥ 10), for comparison, were used as ZP substrates for testing with various enzymes. Embryos were treated with cathepsin L (4.5 μg/ml) or cathepsin B

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RNA isolation and RT–PCR analysis
Embryonic mRNA was isolated using Dyna beads-Oligo dT25 (Dynal Biotech, Oslo, Norway). Pooled embryos (n ≥ 20) were lysed in 100 mM Tris–HCl (pH 7.6) containing 500 mM LiCl, 10 mM EDTA and 1% SDS (pH 8.0) and loaded onto the beads. The RNA-bound beads were washed thrice in 10 mM Tris–HCl containing 150 mM LiCl, 1 mM EDTA (pH 8.0) with or without 0.1% SDS and mRNA was eluted into water by heating the beads at 65°C for 2 min and then snap chilling on ice. The mRNA was reverse transcribed in 20 μl of reaction mixture with MMLV reverse transcriptase (Promega Corporation, WI, USA). Water blanks were used for negative controls. One embryo equivalent cDNA was used for PCR analysis using Platinum Taq polymerase (Invitrogen Corporation, CA, USA). Day 14 placental RNA was isolated by GITC method (Chomczynski and Sacchi, 1987).

Primers used for PCR analysis of hamster cathepsins were designed from sequences identified from RT–PCR of hamster placental cDNA using redundant primers based upon sequences of mouse cathepsins M, P, Q, and R (K. Sol-Church, J. Frenck and R.W. Mason, unpublished results). Primers used for hamster cathepsin L were: F: 5′-TGCAATGTGCGCTGTAGTGG-3′, R: 5′-GAACCCATGGTCAAGGTC-3′ (from AF479267), for hamster cathepsin P: F: 5′-ACTGCGCAACCTGACACCTCTAG-3′, R: 5′-ATGGGAGGCATC AACCAGCAG-3′ (from AF479266) and for β-actin: F: 5′-TGAACTCTAAAG GCCAACCGT-3′, R: 5′-GCTCATAGCTTCTTCCAGG-3′ (from AF014363). Exon spanning primers were used to ensure that genomic DNA was not amplified. PCR conditions followed were as follows: denaturation at 95°C for 5 min, 35 cycles of: 30 s denaturation at 95°C, annealing at 55°C for 30 s and extension at 72°C for 30 s, and final extension at 72°C for 10 min. Hamster placental cDNAs were cloned into pGEMT-Easy vector (Promega Corporation), sequenced and identities verified by BLAST analysis.

For embryonic expression analysis of cathepsins L and P, gene-specific primers were designed against the mouse sequences. By RT–PCR, hamster cathepsin L (304 bp) and cathepsin P (298 bp) were amplified from Day 14 placenta. These products were sequenced and submitted to GenBank and the following accession numbers were obtained: DQ017755 (cathepsin L) and DQ017756 (cathepsin P). Both partial cDNA sequences were identical to the sequences that we identified previously (AF479267 and AF014363, K. Sol-Church, J. Frenck and R.W. Mason, unpublished results). Like mouse cathepsin P, hamster cathepsin P was only expressed in the placenta and not in any adult tissues (Sol-Church et al., 1999, data not shown).

Immunostaining
PFA-fixed embryos were washed in PBS-PVA, permeabilized with 0.2% Triton X-100 and blocked in 10% normal goat/rabbit serum along with 2% BSA-0.1% saponin-PBS-PVA for 1 h at room temperature. Embryos were incubated with monoclonal anti-cathepsin L antibody (Serotec Ltd, Oxford, UK) or polyclonal anti-cathepsin P antibody (Mason et al., 2004; Hassanein et al., 2007). Both were used at 1:100 dilutions in blocking solution overnight at 4°C. After washing thoroughly in 0.01% saponin-PBS-PVA, embryos were incubated in anti-rabbit-FITC (for cathepsin P) or anti-mouse-HRP (for cathepsin L) for 1 h. Specific antibodies used in the study were well characterized and were procured commercially or used earlier by us (Mason et al., 2004). Similarly, control embryos were treated with appropriate non-immune IgG which acted as negative controls. All Embryos were washed and mounted in anti-fade solution and viewed under Zeiss LSM confocal microscope (Carl Zeiss Microimaging, GmBH, Germany) for fluorescent signal (cathepsin P) or processed for DAB staining (for cathepsin L). TEPS were stained with phalloidin-TRITC.

Statistical analysis
Mean values in all experiments were calculated from the total number of embryos cultured during a particular treatment. To control between animal variations, a block design was used. For this, freshly recovered 8-cell embryos from each donor female were distributed randomly and in similar numbers among all treatments and this constituted one replicate experiment. This design considerably strengthens statistical analysis of data. Moreover, all replicate experiments were performed under identical experimental conditions. Likewise, a minimum of three replicate experiments (from three different animals) were performed. To assess statistical significance of treatment effects, one-way ANOVA, followed by a test for linear trend was performed to obtain P-values (Graph Pad Prism Software Version 4, CA, USA). Additional replicate experiments were also performed when necessary (Supplementary Tables S1–3).

Results
Influence of cysteine protease inhibitors on blastocyst hatching
We tested various categories of cysteine protease inhibitors in carefully designed embryo culture experiments which used three stages of embryos at the beginning of culture viz., non-compact 8-cell embryos and zona-intact expanded or deflated blastocysts (Supplementary Fig. S1). Cystatin and FYAD were used to inhibit cathepsins, Z-VAD-FMK was used to inhibit caspases and calpastatin to inhibit calpains. Moreover, inhibitors were tested either continuously during the entire period of embryo culture or transiently for the specified period, depending on developmental stage of embryos being cultured. Later, embryo cultures were continued in inhibitor-free medium and embryo development assessed.

Hatching of expanded blastocysts after 24 h treatment with 20–100 nM calpastatin or Z-VAD-FMK was close to 100% and it was not significantly different from the untreated blastocysts (Fig. 1A and B). In contrast, cystatin inhibited hatching of blastocysts in a dose-dependent manner; 71.0 ± 4.0% of blastocysts incubated with 0.01 μM cystatin hatched, whereas only 27.5 ± 2.5% of blastocysts treated with 0.1 μM cystatin hatched and no blastocysts treated with 1.0 μM cystatin hatched (Fig. 1C). When 8-cell embryos and expanded or deflated blastocysts were continuously exposed to 1.0 μM cystatin, there was a complete inhibition of blastocyst hatching. Also, there was a dose-dependent inhibition of hatching across different embryonic stages. In the continuous presence of 1.0 μM cystatin, none of the expanded blastocysts underwent hatching (Fig. 2A, top); however, 17.7 ± 1.5% of these blastocysts were found to be viable (Fig. 2A, bottom). Similarly, none of the deflated blastocysts hatched in the continuous presence of 1.0 μM cystatin, though 61.3 ± 0.8% of the deflated blastocysts were viable (Fig. 2B). With 0.1 μM cystatin, 22.2 ± 1.5% of expanded blastocysts underwent hatching in spite of the fact that 42.7 ± 1.6% of the total expanded blastocysts were viable (Fig. 2A). Of the 62.3 ± 1.5% deflated blastocysts that were viable, after continuous exposure to 0.1 μM cystatin, only 34.0 ± 2.8% exhibited hatching (Fig. 2B).

We tested the hatching ability of expanded or deflated blastocysts exposed to cystatin transiently for 12 or 6 h (Fig. 3). Treating expanded (Fig. 3A and B) or deflated (Fig. 3C and D) blastocysts with cystatin for 6 h (B and D) prior to returning to inhibitor-free medium less effectively blocked hatching compared with 12 h (Fig. 3A and B). Additional set of experiments to test the influence of continuous or transient exposure of the cathepsin inhibitors on hatching of cultured expanded and deflated blastocysts are shown in Supplementary Tables S1–3). Results obtained were consistent and similar to those observed in Figs 2–3.

Viability of cystatin-treated embryos was assessed by their ability to attach and develop through TB outgrowth (Fig. 4). We observed that cystatin-treated blastocysts that hatched also attached to serum-coated dishes and exhibited TB outgrowth and they were indistinguishable from control embryos (Fig. 4A). The area of TB outgrowth after 60 h of culture of cystatin-treated blastocysts (72.5 ± 3.1 ×
10^{-3} \mu m^2) was indistinguishable from that of control embryos (78.0 \pm 9.2 \times 10^{-3} \mu m^2), indicating that cystatin treatment did not affect embryo viability, in terms of their ability to develop through TB outgrowth (Fig. 4B).

A specific, membrane permeable inhibitor of lysosomal cathepsins, FYAD (Xing et al., 1998) showed a clear dose-dependent inhibition of hatching, when treated continuously (Fig. 5A) or transiently (Fig. 5B). Although only 18.0 \pm 1.2\% of expanded blastocysts continuously exposed to 1.0 \mu M FYAD hatched, 31.0 \pm 1.0\% proved to be viable after removal of the inhibitor (data not shown). The influence of the irreversible inhibitor was time-dependent. When expanded blastocysts were transiently treated with 1.0 \mu M FYAD for 6 h, 47.5 \pm 2.5\% of blastocysts underwent hatching (P < 0.05; Fig. 5B), and 62.5 \pm 2.5\% were viable, indicating that short-term exposure of embryos to inhibitor has a reduced effect on blastocyst hatching compared with continuous 48 h exposure to inhibitor. As observed with cystatin treatment experiment, deflated blastocysts were a little less sensitive to FYAD treatment, both continuous and short-term exposures (Fig. 5C and D). The hatching of blastocysts, after transient exposure to cystatin C or FYAD indicates that cysteine proteases that cause ZP lysis are secreted continuously by blastocysts.

**Influence of exogenous cathepsins on ZP dissolution**

To test cathepsin-induced dissolution of ZP, freshly recovered 8-cell embryos were used as ZP substrate. Embryos incubated with 4.5 \mu g/\mu l cathepsin L showed rapid ZP thinning within 6 s (Fig. 6Aii) followed by a complete dissolution within 30 s (Fig. 6Aiii). ZP thinning with cathepsin P was observed after 7 min of treatment and a complete dissolution by 10 min (Fig. 6B) and with cathepsin B, thinning was observed by 5 min and a complete dissolution by 8 min (Fig. 6C). Thinning of control ZP by endogenous proteases was not observed.
even after 50 min (Fig. 6D), and complete dissolution took up to 24 h. Cathepsin L was clearly the most effective ZP degrading enzyme, although both cathepsins P and B were also capable of degrading the ZP.

A complete dissolution of mouse ZP by cathepsin L required over 60 min, consistent with our previous studies showing that mouse ZP is generally more resistant to proteolysis than hamster ZP (Mishra and Seshagiri, 2000a, b). The thinner mouse ZP remained intact when cultured with cathepsin L for 20 s, whereas a hamster ZP cultured in the same experiment lysed (Fig. 6E). Mouse ZP was particularly resistant to cathepsin P (Table I). Effect of exogenous cathepsins was also tested on primate oocyte ZP from *M. radiata*. These enzymes failed to bring about ZP dissolution of primate oocytes within 2 h of treatment (results not shown). Control embryos of mouse took more time for ZP dissolution than that for hamster embryos (120 ± 2.0 versus 60 ± 2.0 min, respectively, *P* < 0.01).

**Characterization of embryonic cathepsins and their expression**

The enzyme(s) interacting with FYAD were detected in the all cells of the blastocyst. Most of the staining was observed in peri-nuclear region probably representing lysosomal compartments (Fig. 7A). Interestingly, control embryos, not treated with FYAD, showed negligible staining (Fig 7B).

Peri-hatching blastocysts were tested for the expression of cathepsins L and P by RT–PCR analysis. Cathepsin L (304 bp) and Cathepsin P (298 bp) transcripts were detected in hamster peri-hatching blastocysts (Fig. 8). The specificity of the amplicons was confirmed by Southern blot hybridization, using sequenced hamster placental products as radiolabeled probes (data not shown). In all reactions, water blank negative controls were used. The amplification of a housekeeping gene, β-actin (Fig. 8C), was also detected as a control for quantity and integrity of the mRNA.
Cathepsin L and cathepsin P proteases were localized to cells of expanded (Fig. 9Aa and Ba) and hatched (Fig. 9Ab and Bb) blastocysts. Cathepsin P was detected in granules within the cytoplasm and at the outer edge of the blastocyst TE cells close to the ZP (Fig. 9Ba). Cathepsin P immunoreactivity was not observed in 8-cell embryos (data not shown). Cathepsins P and L in TE showed significant co-localization with the TEPs that stained positive for β-actin and the immunostained TEPs were associated intimately with the ZP (Fig. 10).

Interestingly, we could show the mRNA and protein expression of Cathepsin P and L in placental derived TBs (Supplementary Fig. S3), which are derived initially from TE cells of blastocysts.

**Discussion**

This study demonstrates for the first time that blastocyst-derived cathepsins are involved in the lysis of ZP during hatching of golden hamster blastocysts. This is supported by the observations that (i) blastocyst hatching is blocked by cathepsin-specific inhibitors (cystatin C and FYAD), but not by inhibitors of the other major cysteine proteases, i.e. calpains and caspasces, (ii) FYAD-interacting cathepsins are detected in the blastocyst, (iii) ZP are sensitive to exogenously added peptidyl diazomethane (FYAD), and (iv) the mRNA and protein expression of cathepsins P and L are detected in placental derived TBs (Supplementary Fig. S3), which are derived initially from TE cells of blastocysts.
added cathepsins, (iv) cathepsin mRNA and protein are expressed in the peri-hatching blastocysts and (v) Cathepsin L and P are associated with TEPs, in intimate contact with the lysing zonae.

One of the striking observations made is the marked inhibition of blastocyst hatching by the specific inhibitors of cathepsins viz., cystatin C and FYAD, and the lack of inhibition of hatching by inhibitors of calpains and caspases viz., calpastatin and Z-VAD-FMK, respectively. This is consistent with our earlier observation indicating the involvement of cysteine proteases, but not other categories of proteases, in blastocyst hatching phenomenon (Mishra and Seshagiri, 2000a). Cathepsins play important roles throughout early development, including ovulation (Robker et al., 2000), fertilization (Ichikawa et al., 1985), implantation (Afonso et al., 1997) and placentation (Nakajima et al., 2000; Sol-Church et al., 2002), and are critical for correct fetal development (Grubb et al., 1991). Our study has added a critical role for cathepsins in late preimplantation development, i.e. blastocyst hatching to ensure successful implantation.

The hatching of hamster blastocysts is a highly time-dependent and embryo stage-dependent phenomenon (Kane and Bavister 1988a; Gonzales and Bavister 1995; Mishra and Seshagiri, 2000a). We observed that the effect of inhibitors was more pronounced with expanded blastocysts than with deflated blastocysts, indicating that ZP lysis may be initiated prior to deflation. Moreover, blastocysts transferred to inhibitor-free medium after initial treatment with an irreversible cathepsin-inhibitor, FYAD, were able to hatch, indicating that the ZP lysins are actively produced continuously by cultured blastocysts during the peri-hatching period. Similarly, treatment of blastocysts with cystatin C blocked hatching, but its removal overcame the inhibition and blastocysts hatched. It is significant that inhibitor treated-embryos, following their removal remained viable, blastocysts hatched and attached, exhibiting TB outgrowth. This indicates that protease inhibition specifically affected the hatching process and the observed inhibition of hatching is not due to loss of viability. Although calpains and caspases are reported to play important roles in preimplantation embryonic development (Zakeri et al., 2005; Dutt et al., 2006), our studies show that they are not critical for blastocyst hatching and that cathepsins are in deed critical for the hatching process in hamsters.

Studies in our laboratory have shown that blastocyst hatching is regulated by LIF, EGF and HB-EGF and these regulators are expressed in hamster embryos (Mishra and Seshagiri, 2000b; Seshagiri et al., 2002; Rao et al., 2008). How these growth factors regulate the expression, secretion and activity of the ZP lysins/cathepsins is unclear. Attempts however, are being made to understand the molecular characteristics, regulation and expression of hatching associated-cathepsins in hamster peri-implantation embryos. We showed that ZP of hamster embryos is generally more sensitive to exogenous proteases, i.e. cathepsins than that from the mouse or primate embryos. The increased sensitivity of hamster ZP may be due to differences in ZP matrix architecture between species (Fischer et al., 1991; Green, 1997; Keefe et al., 1997; Wassarman et al., 2005). Whether different hatching processes and/or hatching proteases are involved in different species is currently not clearly known (Mishra and Seshagiri, 2000a; O’Sullivan et al., 2001; Seshagiri et al., 2003). It is interesting to point out that different features exist in the hatching behavior of hamster vis-a-vis mouse blastocysts. While ZP of hamster completely disappeared and highly susceptible to cathepsins that of the mouse remains intact (Mishra and Seshagiri, 2000a) and it is relatively less susceptible to cathepsins (Table I; Fig. 6). The cellular and molecular basis of this pronounced difference in hatching behavior in mouse and hamster species remains to be investigated. In hamsters, we however,
demonstrated that hamster peri-hatching embryos express mRNA and protein of cathepsins B, L and P. FYAD and BYAD are able to react with all three of these cathepsins in vitro so each may contribute to the hatching process (Xing et al., 1998; Mason et al., 2004).

This is the first demonstration that any of the placentally expressed cathepsins (PECs) are expressed in blastocysts and TE cells. Blastocyst-derived placental TBs showed expression of mRNA and protein for cathepsin L and/or P (Supplementary Fig. S3). All studies of PECs prior to this report have focused on the expression in the post-implantation embryo and the expression of cathepsin P has been shown to be the greatest in term placenta of mice (Deussing et al., 2002; Sol-Church et al., 2002) and we have similar findings using Day 14 hamster placenta (Supplementary Fig. S3). Cathepsins 1 and 2 were shown to be expressed preferentially in Day 7.5 ectoplacental cone compared with term placenta in mice, but expression earlier in gestation has not been examined (Hemberger et al., 2001). Cathepsin P is the only currently known hamster homolog of any of the eight PECs found in the mouse genome. PCR amplification of

Figure 9: Embryonic expression of cathepsin proteins. (A) represents immunolocalization of cathepsin L in expanded (a) and hatched (b) blastocysts. Non-immune IgG controls for expanded and hatched blastocysts are shown in c and d, respectively. (B) represents immunofluorescent localization of cathepsin P in expanded (a) and hatched (b) blastocysts. The denser layer of cathepsin associated with the ZP of expanded blastocyst is highlighted with yellow arrow. Non-immune IgG control stained blastocyst is shown in (c). Corresponding bright field images are shown in (d–f). Magnification bar: 20 μm.

Figure 10: Localization of cathepsins to trophectodermal projections (TEPs) in blastocysts. (A) TEPs were stained positive for β-actin with phalloidin-TRITC (a). Penetration of TEPs into ZP is evident in the bright field image (b) and the overlay (c). (B) represents immunolocalization of cathepsin L in the blastocyst (a), TRITC staining of TEPs (b) and colocalization of cathepsin L with TEPs in the overlay (c). Corresponding bright field image is shown in d. Panel C shows immunolocalization of cathepsin P in the blastocyst (a), TRITC staining of TEPs (b) and colocalization of cathepsin P and TEPs in the overlay (c). Corresponding bright field image of the embryo is shown in (d). TEPs are indicated by arrow heads in all the panels. Magnification bar: 20 μm.
The manuscript.

Acknowledgements

Supplementary material is available at MOLHER journal online.

Supplementary data

Supplementary data is available at MOLHER journal online.

References


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