Rat Uterine Stromal Cells: Thrombin Receptor and Growth Stimulation by Thrombin*

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ABSTRACT

The estrogen-stimulated maturation of the immature rat uterus is mediated by peptide growth factors whose expression is regulated by estradiol. We present evidence that thrombin is a uterine growth factor.

Thrombin is generated in situ by the activation of prothrombin through the coagulation cascade. It acts as a growth factor through the proteolytically activated thrombin receptor.

Thrombin's role as a growth factor in uterine stromal cells is proven by two lines of evidence: demonstrations that the proteolytically activated thrombin receptor is present and that cultured cells are stimulated to grow by thrombin.

Thrombin receptor in the uterus is demonstrated by reverse transcription-PCR for receptor messenger RNA by specific [125I]peptide labeling of a membrane-bound binding protein of about 60 kDa and by Western blot with a thrombin receptor antipeptide antibody.

Thrombin's effectiveness as a growth factor is shown by thrombin-stimulated growth of primary stromal cell cultures, with maximum stimulation at 100 nM. That the effect is mediated by the proteolytically activated thrombin receptor is shown by the inhibition of growth by hirudin, a highly specific inhibitor of thrombin, the absence of enhanced growth with Pro-Phe-Arg-chloromethyl ketone-thrombin, an active site-inhibited thrombin derivative; and the stimulation of growth by the thrombin receptor-activating peptide. (Endocrinology 137: 3744–3749, 1996)

EXTENSIVE studies of the estrogen-stimulated maturation of the immature rat uterus have shown that the process is mediated by peptide growth factors whose expression is regulated by estradiol (E₂). All cell layers of the organ grow in response to E₂, but the intermediary peptide growth factors provoke different responses in the different layers (reviewed in Ref. 1). We have postulated that thrombin is one such growth factor.

Thrombin is generated in situ by the activation of prothrombin through the coagulation cascade beginning with tissue factor (TF). In the unstimulated immature uterus, TF and prothrombin are present at low levels. A single injection of 30 µg E₂/kg administered to 19- to 21-day-old rats results in a 3- to 4-fold increase in the amount of prothrombin (2,3) and the expression of TF (4,5) in the uterus within 3 h. The combination of the two permits the generation of thrombin in the uterus at a time when estrogen-stimulated growth is beginning. We have postulated that thrombin can act as a growth factor under these circumstances (6).

Thrombin stimulates growth in a wide variety of cells. The extent of stimulation and the most effective thrombin dose are highly variable. In some types of cells, thrombin acts directly as a mitogen. In other cells, thrombin has been found to potentiate other growth factors or to cause increases in the amounts of such growth factors as basic fibroblast growth factor (7,8).

Thrombin acts as a growth factor through a G protein-coupled thrombin receptor (THREC) (9). The mechanism of activation of the receptor is unique: thrombin binds to the amino-terminal of the receptor and by proteolytic cleavage at R41-S42 exposes a new amino-terminal (the tethered peptide), which then binds to an independent site that may be in the second extracellular loop of the receptor (10), causing activation. An additional region, residues 83-94, may also be involved in activation (11).

The effects of thrombin mediated by THREC can be elicited by thrombin receptor-activating peptides (TRAPs) of the same sequence as the tethered peptide (10,12,13). The minimum requirement for a response is the 6-residue sequence SFLRN (rTRAP-6). Most studies have been performed with the 14-residue TRAP; for the rat, that sequence (rTRAP) is SFLRNPSEDTFEQ (14). Thousand-fold higher concentrations of rTRAP-6 or rTRAP are generally required to give responses equivalent to those to thrombin.

We report here that THREC is present in the immature rat uterus before estrogen stimulation, and we show that thrombin is a growth factor for isolated uterine stromal cells in primary culture. That thrombin acts through the proteolytically activated THREC is shown by the growth response to TRAP; that catalytically active thrombin is required is shown by the prevention of the growth response by hirudin, a specific inhibitor of thrombin's proteolytic activity, and by the failure of active site-inhibited thrombin to elicit the growth response. This evidence demonstrates the presence of the...
uterus of a complete functional thrombin growth factor receptor system that can mediate estrogen-stimulated growth.

**Materials and Methods**

Human thrombin (1324 NIH U/mg) and hirudin (9000 U/mg) were supplied by Drs. Thomas J. Ryan and William Lawson of the Wadsworth Center (Albany, NY). The rabbit antihuman prothrombin antibody was obtained from Dako (Carpenteria, CA). It reacted equally well with prothrombin and thrombin. Western blot antibody reagents were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

RNA was isolated from uteri by the procedure of Chirgwin et al. (15). RNA from cultured stromal and epithelial cells was prepared by the RNAzol method (Bio101, La Jolla, CA) and was previously described (16). Twenty-five to 30 μg RNA were treated with ribonuclease-free deoxynucleotidase I to remove contaminating DNA. Complementary DNA was synthesized from 5 μg RNA using AMV reverse transcriptase (Promega Corp., Madison, WI) and random hexamer primers (Pharmacia, Piscataway, NJ). One fifth of the complementary DNA was amplified by PCR using primers corresponding to 2068–2087 (GCGGGCAAGCGGCAAG-CAACCTTGCTT) and 2547–2566 (GCAAACCTTACCTGATAGGC-CTGGCTT) of the rat thrombin receptor (GenBank accession no. M 81642). Amplification (32 cycles) consisted of 94 C for 1 min, 55 C for 2 min, and 72 C for 3 min. Reverse transcription-PCR (RT-PCR) products were analyzed by digestion with PvuI and fractionation on a 1.2% agarose gel.

Pro-θ-Arg-chloromethyl ketone (P(家装)-thrombin was prepared by the addition of 100 nmol aqueous PPACK to 35 nmol thrombin in water (2). After 1 h of incubation at room temperature, total inactivation of the thrombin was confirmed by the addition of 1 μl of the PPACK-thrombin solution to 30 μl 2.5 mm tosyl-Gly-Pro-Arg-4-nitroanilide (Chromozyme TH, Boehringer Mannheim, Indianapolis, IN). Total inactivation of thrombin was indicated by the absence of yellow color after 24 h at room temperature.

The human thrombin receptor-activating peptide hTRAP (SELRNP选NPKYKEIF) rTRAP, and rTRAP-6 were synthesized at the Wadsworth Center Stable Synthesis Facility and purified by reverse phase HPLC chromatography at the Biochemistry Core Facility. The molecular weight and sequences of the products were confirmed by mass spectrometry at the Wadsworth Center Biological Mass Spectrometry Core. Purified peptides were used for growth experiments and as substrates for iodination. Crude peptide was used for the generation of antibodies.

hTRAP was iodinated by the lactoperoxidase procedure. In a typical reaction, 25 μg peptide were incubated with 2 μCi [125I]Na and 0.5 U lactoperoxidase in the presence of 25 mm sodium ascorbate and 1% H2O2 for 2 h. The iodinated peptide was separated from free [125I] by use of a Sep-Pak C18 cartridge (Waters Co., Milford, MA) and elution with 0.1% trifluoroacetic acid in 70% acetic acid. The DNA was solubilized by a 30-min incubation at room temperature in 0.3 M NaOH-1% SDS. An aliquot of each sample was transferred to a counting vial, 50 μl glacial acetic acid was added, and the samples were counted in Aquasol (Packard, Meriden, CT). At least six replicates were used for each experimental point. Data for growth curves were analyzed by ANOVA followed by the Tukey-Kramer multiple comparisons test to detect differences between individual means.

**Results**

**Demonstration of thrombin receptor in uterine cells**

RT-PCR for THREC. To demonstrate the presence of messenger RNA for THREC in the uterus, total RNA was prepared and reverse transcribed as described in Materials and Methods. The PCR reaction was performed with primers designed to give a 497-bp fragment of THREC from the 3’-end of the complementary DNA, residues 2068–2566 (14). This fragment contained two sites for the restriction endonuclease PvuII; reaction with PvuII resulted in the generation of fragments of 255, 148, and 94 bp. As shown in Fig. 1, a DNA fragment of the predicted size was obtained in the PCR reaction; after digestion with PvuII, the three predicted bands were seen. The experiment was repeated with isolated stromal and epithelial cells that had been cultured to confluence. The reaction was positive for both types of cells, also shown in Fig. 1.

[125I]TRAP labeling of uterine membranes. To identify a specific TRAP-binding protein, we used uterine membrane preparations and [125I]hTRAP. After a 1-h incubation, the reaction was cross-linked and electrophoresed on SDS-acrylamide gels. The gels were dried and exposed to x-ray film. As shown in Fig. 2, a band of about 70 kDa was labeled. The labeled band was somewhat larger than the expected size for THREC (19). Two experiments are shown in Fig. 2, in lanes 1–4 and lanes 5–7. In lane 1, labeling of the protein by 1.75 μM [125I]hTRAP is shown. Successful competition with 17.5 or 1.75 μM hTRAP, in lanes 2 and 3, respectively, reduced the labeling. Lane 4 shows that no labeling occurred when the

Primary cultures of stromal and epithelial cells were prepared from the uterus of immature rats (18–20 days old). All animal care followed an international animal care and use committee-approved protocol. Stromal and epithelial cells were isolated by the procedure of McCormack and Glasser (18). Cell cultures were maintained in a 1:1 mixture of DMEM and Ham’s F-12 containing 1 mM pyruvate, 2 mM glutamine, 0.25 μg/ml fungizone, 100 μM penicillin, 100 μg/ml streptomycin, and 10% FBS (complete medium). Stromal cells were plated directly into 24-well plates at a concentration of 1 x 10^5 cells/well. Although stromal cultures frequently contained epithelial cells immediately after plating (as determined by visual inspection), the epithelial cells did not attach or grow well on the uncoated plates and disappeared from the cultures well before stromal cells reached confluence. Epithelial cells were plated in collagen-coated T-25 flasks (2 flasks for cells from 30 uteri) and grown to confluence in complete medium.

Growth experiments were initiated with stromal cells that were 70–80% confluent. Complete medium was replaced with DMEM-Ham’s F-12 that contained 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenious acid, and 0.1% BSA instead of FBS (115 μg/ml). The other medium supplements were the same as those in complete medium. After 24 h, the cultures were used to determine growth curves in ITS medium plus the indicated additions. One microcurie of [125I]thymidine was added per well with the growth supplements. After 48 h, medium was removed, and methanol was added to the wells. After a 30-min incubation on ice, the methanol was removed, and the cells were washed twice with ice-cold PBS. Ice-cold 10% trichloroacetic acid (TCA) was added to inactivate the trichloroacetic acid after 20 min. The wells were washed once with 10% TCA, and the DNA was solubilized by a 30-min incubation at room temperature in 0.3 M NaOH-1% SDS. An aliquot of each sample was transferred to a counting vial, 50 μl glacial acetic acid was added, and the samples were counted in Aquasol (Packard, Meriden, CT). At least six replicates were used for each experimental point.

Data for growth curves were analyzed by ANOVA followed by the Tukey-Kramer multiple comparisons test to detect differences between individual means.
FIG. 1. RT-PCR for THREC. RT-PCR was performed on uterine RNA preparations. Aliquots of samples were digested overnight with PvuII, then electrophoresed on a 1.2% agarose gel. Lane 1, 4X 174/HaeIII molecular mass standards; lanes 2 and 3, uterine RNA, intact and cut; lanes 4 and 5, stromal cell RNA, intact and cut; lanes 6 and 7, epithelial cell RNA, intact and cut.

membranes were omitted. In the second experiment, the similarly labeled protein is shown in lane 7; 175 and 17.5 μM rTRAP-6 successfully competed with the label, as shown in lanes 5 and 6.

**Immunological demonstrations of THREC**

**rTRAP antibody.** A rabbit antibody, X167, was generated by immunizing rabbits with rTRAP. The antibody titer was followed by enzyme-linked immunosorbent assay with the peptide and with preparations of THREC expressed in a baculovirus-insect cell system. Details of the expression of THREC in this system will be published elsewhere. The antibody reacted specifically with THREC in Western blots of the expressed protein.

The specificity of protein A-purified antibody X167 for THREC in freshly prepared rat platelets and the reaction of the antibody with a protein in uterine membrane preparations are both shown in a Western blot in Fig. 3. Aliquots of 30 μg of a freshly prepared rat platelet extract and of stromal cell membrane preparations were electrophoresed and transferred to nitrocellulose. Antibody X167 gave a single faint band for the platelet receptor (Fig. 3, lane 2) at about 65 kDa, similar to the band for THREC in human platelets shown in a Western blot by Brass et al. (19). The rat uterine cell extract also gave a positive band in the Western blot, shown in lane 1. The apparent molecular mass (~60 kDa) is slightly smaller than that of platelet protein. Lane 1 also shows a very faint band at about 45 kDa that is probably a breakdown product of the receptor. When the antibody was competed with TRAP peptide (lanes 3 and 4), only nonspecific staining of high molecular mass aggregates was seen.

**Comigration of thrombin and THREC in uterine membranes.** The faint bands in Fig. 3 led us to seek additional confirmation of the presence of THREC in uterine membranes. If thrombin were generated in the uteri of E2-treated animals, it would bind to THREC, and the thrombin-THREC complex should comigrate to a higher molecular mass position in a gel if the two were cross-linked. Such a complex from uterus would be predicted to have a molecular mass between 90-100 kDa (37 kDa for thrombin and 55-65 kDa for THREC). Treatment of the rats with E2 would result in an influx of plasma proteins, including prothrombin, into the uterus, where thrombin could be generated. Thus, if E2-treated rats were used, no exogenous thrombin would be required in this experiment.
Fig. 4. Antibodies to thrombin react with a band of 90 kDa. Membranes were prepared from estrogen-treated immature rat uteri. Samples of 42 μg membrane protein were cross-linked and then electrophoresed and transferred to nitrocellulose. Individual lanes were separated and incubated for 24 h at 4 C with primary antibody to thrombin, competed or not with PPACK-thrombin. The blots were developed with alkaline phosphatase-coupled second antibody. Lane 1, Antihuman thrombin preabsorbed with PPACK-thrombin; lane 2, antihuman thrombin, 1:1000 dilution. The positions of the molecular mass standards are indicated on the left; the arrow on the right indicates the position of a band specifically labeled by the antibody. The high background in lane 1 is due to the tendency of thrombin to adhere nonspecifically to protein-covered surfaces.

Accordingly, membranes were prepared from the uteri of rats treated with E₂. Samples were cross-linked with bis[2-sulfosuccinimidyl] suberate, electrophoresed on a SDS gel, and transferred to nitrocellulose. The blot was incubated with an antithrombin antibody (Binding Site, San Diego, CA). Figure 4 shows the results. In lane 1, the primary antithrombin antibody was competed with PPACK-thrombin; in lane 2, the antibody was not competed. A specific band was clearly visible at 90-100 kDa.

Thrombin-stimulated cell growth

Thrombin as a growth factor. The stimulation of stromal cell growth by thrombin was shown in nearly confluent cultures of stromal cells that were depleted of serum for 24 h and then grown in ITS medium with human thrombin and [³H]thymidine for 48 h. Analysis of the TCA-precipitable DNA showed that the addition of thrombin resulted in a 2-fold stimulation of growth of the stromal cells. The thrombin concentration curve shown in Fig. 5 reveals a maximum response at 50-150 nM thrombin.

The nature of the thrombin growth response was further defined in additional growth experiments, shown in Fig. 6. The relative growth rates of the cultures, compared to their growth rates in ITS medium, are given. Only 100 nM thrombin produced a significant increase in [³H]thymidine incorporation. The addition of 350 U of the highly specific thrombin inhibitor hirudin to the medium prevented thrombin-stimulated growth, whereas hirudin alone had no effect on growth. The catalytically inactive PPACK-thrombin did not stimulate growth.

Growth with rTRAP. Thrombin stimulation mediated by THREC was also obtained with TRAP. Stromal cell cultures were stimulated to grow by 50-100 μM rTRAP. Figure 7 shows the rTRAP concentration curve for growth; the maximal response was achieved with concentrations higher than

50 μM. The magnitude of the response was similar to that of the response to 50 nM thrombin.

Discussion

The presence of THREC in immature rat uterine membrane preparations and the stimulation of growth of stromal cells in culture by thrombin and rTRAP are good evidence that thrombin is a growth factor for this organ (13, 20). There are three lines of evidence for the presence of THREC in the uterus: RT-PCR, specific binding of radiolabeled rTRAP, and immunoreactivity with a specific rTRAP-directed antibody.

The RT-PCR experiment shows that THREC messenger RNA is expressed in the uterus. Because of the very high sensitivity of the RT-PCR procedure, this positive result with total uterine RNA could have come from the amplification of RNA from monocytes, macrophages, eosinophils, or endo-
Our earlier immunohistochemical and enzymatic studies (3) showed that after estrogen stimulation, thrombin levels in the stroma are increased. Thrombin must bind to the receptor if it is to signal mitogenesis. After an appropriate incubation and cross-linking step, a thrombin-receptor complex was indicated on the Western blot by the specific staining of a band of 90–100 kDa. The indicated molecular mass of the receptor was 53–63 kDa, in the same size range as the human receptor (19) and the rat receptor shown in Fig. 3.

An early dramatic effect of estrogen stimulation in the immature rodent uterus is a 50% increase in wet weight within 3 h, which is the result of the imbibition of water and plasma proteins (24). It is this imbibition that increases the prothrombin concentration in the uterus. The concomitant induction of tissue factor (3) permits thrombin to be generated in situ.

The stimulation of stromal cell growth by catalytically active thrombin and TRAP is the final proof that thrombin is a growth factor for these cells. The growth rate was doubled with 50–150 nm thrombin. The optimum thrombin concentration for growth stimulation is generally in the 1–100 nm range, but varies widely in different types of cells. Mitogenesis in CCL39 hamster fibroblasts is a maximum at 100 nm thrombin (8); rat vascular smooth muscle cells respond to 140 nm thrombin (12); the growth of human umbilical vein endothelial cell is stimulated 10-fold by 1000 nm thrombin (7). Delloisi et al. (25) found that microvascular cells from mouse brain, lung, and liver all responded to thrombin; cell number increased 2- to 3-fold in 4 days with 1 4–270 nm thrombin. Among cells that are not part of the circulatory system, rat astrocytes have an EC50 of 0.5 nm for growth stimulation (26); T47D epithelial breast tumor cells are maximally stimulated by 4–5 nm thrombin (27).

Stimulation of growth by rTRAP confirmed that the proteolytically activated thrombin receptor was involved in the growth response. Growth was doubled at 10 25 μM TRAP and was increased nearly 3-fold at 100 μM rTRAP. Growth stimulation by TRAP occurred at similar concentrations in vascular smooth muscle cells (12, 28), human umbilical vein endothelial cells (7), and fibroblasts (29).

Our initial hypothesis (6) was that thrombin action in the uterus involved multiple steps; E2-stimulated induction of TF and infiltration of prothrombin would permit the local uterine generation of thrombin, which could act as a growth factor. This hypothesis is now shown to be correct. The remaining question is whether the growth stimulatory effect of thrombin is significant, in view of all of the growth factors known to be present in the uterus after estrogen stimulation. These include epidermal growth factor (EGF), transforming growth factor-α, and insulin-like growth factors (IGFs).

EGF is clearly very important as an estrogen-mediated growth factor in uterine epithelial cells (30) in rodents (31, 32). However, the effects of EGF are directed primarily toward the epithelial cells rather than the stromal cells (32). Transforming growth factor-α, which acts through the EGF receptor, is increased in the immature mouse uterus by E2 and, like EGF, affects primarily the epithelial cells (33).

IGF-I and its receptor are estrogen inducible in the immature rat uterus; they are found in stromal cells (34), but are primarily myometrial (35). In rat uterine slices, IGF-I in-
creased DNA synthesis 1.5- to 1.6-fold in the stroma compared to that in untreated controls, but only if a high level (10^-6 M) of E2 was present (35).

Fibroblast growth factor is also present in the uterus (1) and may play a role in angiogenesis there (36), although its role in uterine development is much less studied. It is unlikely that any single growth factor would be both necessary and sufficient for E2-stimulated uterine growth; extensive redundancy is much more likely. A very recent report (37) studied transgenic mice that overexpress IGF-binding protein-1. These mice had reduced uterine DNA synthesis and weight gain in response to E2; they also had a 20-30% reduction in litter size. These results indicate that although IGF-I is an important factor in uterine development and fertility, development can occur when the IGF-1 concentration is greatly reduced.

Thrombin could fill a particular niche in uterine development, as it could be available in the uterus by generation from prothrombin as rapidly as the E2-stimulated imbibition of plasma. We thank Ms. Antonella Restivo for technical assistance, and Dr. James Catalfamo for providing platelet-rich rat plasma.

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