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Trypsin-Like Inhibitor Activity in Mouse Uteri during Early Gestation and Delayed Implantation (40015)

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Introduction. The invasiveness occurring at implantation sites in rodent uteri suggests that lytic mechanisms may be involved in implantation. Support for the participation of proteinases, in particular, during early embryonic development and implantation is based on the following observations: (a) Maximum uterine fluid proteolytic activity, which is believed to promote blastocyst attachment to the uterus, coincides with the initiation of implantation (1); (b) trophoblasts sequester (2, 3) and digest (4) particulate cellular material during the invasive phases of implantation; (c) proteolytic activities which may be involved in cellular digestion and/or cell surface transformation of blastocysts have been detected (5-7), and (d) proteinase inhibitors administered *in utero* impair implantation (8). The results of measurements of proteinase inhibitor activity in uteri during early gestation show species differences (9, 10). To establish relationships between proteinase activity associated with implanting embryos and uterine proteinase inhibitor activity, measurements of the respective biochemical activities must be made on a single species. The investigations in this report, therefore, pertain to quantification of trypsin-like inhibitor activity (TLI) in mouse uteri during early gestation and delayed implantation.

Materials and methods. Synchronization of ovulation and delayed implantation. The ovulation of virgin, female, Swiss-Webster mice of approximately 8 weeks of age was synchronized by injection of 5 IU of FSH (Equinex; Ayherst Labs., Inc., N.Y.) followed by 5 IU of chorionic gonadotropin (Ayherst Labs., Inc., N.Y.) (11). Following the latter injection, mice were mated and checked for copulation plugs the following morning, designated Day 1 of pregnancy. Control animals were treated with the same hormonal regimen, but not mated. Delayed implantation in some pregnant mice was induced by ovariectomy performed on the morning of Day 4 of pregnancy. These animals were treated with 1 mg of progesterone daily (12).

Preparation of uterine homogenates. After sacrificing mice by cervical dislocation, uteri were excised and placed into cold (4°) physiological saline until each experimental group was collected. All procedures during the preparation of the respective extracts were performed at 4°. Uteri were trimmed free of blood vessels and connective tissue, flushed with cold physiological saline to remove uterine fluid and unattached embryos, then weighed after blotting excess fluid. In cases where implantation had occurred (i.e., Day 6, 8, or 12 of pregnancy), the embryos were either left attached or gently removed from the uterine wall with forceps. The weighed uteri were minced well with scissors prior to homogenization (1 g wet weight/2 ml of 0.25 *M* sucrose in 0.05 *M* Tris-HCl buffer, pH 7.5) in a Potter-Elvehjem homogenizer, approximately 10 passes. The homogenate was centrifuged at 27,000*g* for 30 min. No inhibitor activity was found in the pellet before or after solubilization with various concentrations of Triton X-100. The supernatant solution (27,000*g*) contained inhibitor activity which did not sediment at 100,000*g* after 1 hr.

Fluorometric assay. Analysis of TLI activity in the uterine extracts was performed at 37° with a modified fluorometric kinetic assay (13). Each assay mixture contained: 0.20 ml of 0.0116 *M* BANA (Benzoyl-DL-arginine-β-naphthylamide,

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Schwarz/Mann, Orangeburg, N.Y.) dissolved in methyl cellosolve; 0.05 ml of a trypsin solution (sp act = 12,000 BAEE units/mg; Sigma Chemical Co., St. Louis, Mo.) containing 0.10 mg of trypsin/ml of 0.10 *M* phosphate-citrate buffer, pH 7.5 (assay buffer); aliquots of a 1:10 dilution of the 27,000*g* supernatant solution diluted with assay buffer; and sufficient assay buffer to adjust the final volume to 5.0 ml. The inhibitor was incubated with the enzyme at 37° for approximately 8 min before triggering the reaction with substrate. Hydrolysis of BANA was monitored with a Turner fluorometer, Model 111, equipped with thermospacers and attached to a recorder. A 7-60 primary filter and 50% interference filter were used in conjunction with a 2A-12 secondary filter.

A standard curve relating fluorescence measurements to β-naphthylamine concentration was used to determine the amount of BANA hydrolyzed. Pure β-naphthylamine, a white powder with a melting point of 110°, was obtained after numerous recrystallizations from water. One unit of inhibitor activity was defined as the amount of inhibitor required to inhibit the hydrolysis of 1 nmole of BANA/min. To obtain specific activities (units of inhibitor per milligram of protein), protein determinations were completed according to Lowry *et al.* (14).

Identical assays, but without trypsin, obviated possible interference by endogenous peptidases in the uterine extracts. Moreover, inhibition of trypsin by large amounts of albumin, as 500 μg (Fraction V, Sigma Chemical Co., St. Louis, Mo.), were considered and found to have no effect. Blood plasma was also analyzed for TLI activity to determine the extent to which possible blood contamination of homogenates might contribute to total inhibitor activity. The amount of contamination was ascertained by determining the amount of hemoglobin in homogenates compared to whole blood by the cyanomethemoglobin method (15) (Hycell, Inc., Houston, Tex.).

Results. Quantitative measurements of inhibitor activity. The specific activity of TLI in uterine extracts prepared at specific times of gestation is shown in Figs. 1 and 2. The histograms are based on the mean value for each group. Each individual experiment represented an analysis of an extract prepared from 5 to 10 uteri. Inhibitor activity due to blood contamination was found to be less than 2.6% of the total activity.

The elevated specific activity of TLI in uterine extracts prepared on Day 6 of pregnancy is different ($P \leq 0.05$) from values found for hormone-treated controls, Days 4 and 12 of normal pregnancy (Fig. 1), or Days 6 and 8 during implantation delay (Fig. 2). The data were analyzed by multiple comparison of means (16). Moreover, a P value of 0.055 was obtained when the specific activity of TLI of Day 6 pregnant uteri (Fig. 1) was compared to that of nontreated controls (Figs. 1 and 2) or Day 5 of implantation delay (Fig. 2).

One must consider the fact that embryos remaining *in situ* in uteri used to prepare uterine extracts on Day 6 of pregnancy (Fig. 1) might themselves contribute significantly to the observed increase in inhibitor activity relative to control samples. This is believed not to be the case for the

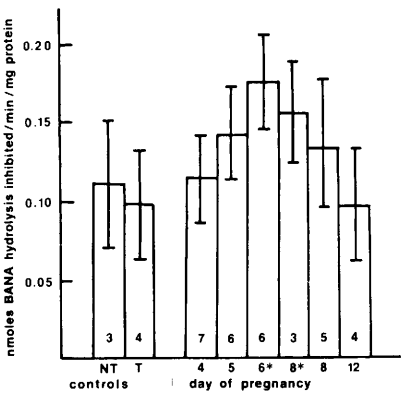

FIG. 1. Specific activities of tryptic-like inhibitor in uterine extracts prepared from nonpregnant or pregnant uteri. The control mice were either treated hormonally (T) to achieve cycle synchronization or not treated (NT). Numbers at the base of the histograms denote the number of independent experiments. The perpendicular lines at the top of each histogram indicate the 95% confidence limits. An asterisk beside the number indicating day of pregnancy denotes the fact that the embryos were not removed from these uteri.

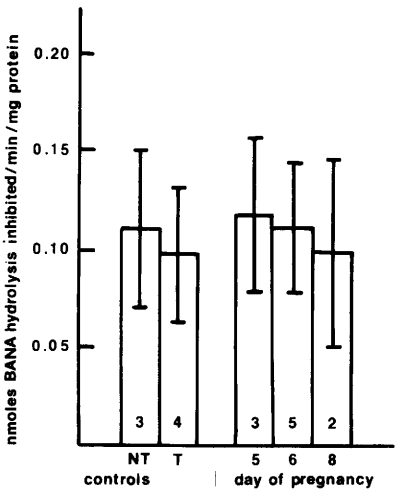

FIG. 2. Specific activities of tryptic-like inhibitor in uterine extracts prepared from nonpregnant or delayed implantation, pregnant uteri. The control animals were either treated hormonally (T) to achieve cycle synchronization or not treated (NT). Numbers at the base of histograms denote the number of independent experiments. The perpendicular lines at the top of each histogram indicate the 95% confidence limits.

following reasons. First, the embryos represent only a very small fraction of the total mass of the Day 6 pregnant uterus (17–19). Second, one can estimate that approximately 10% of the total embryonic protein which could be present in a Day 6 uterine extract must be in the form of an inhibitor, such as soybean trypsin inhibitor (SBTI), to account for the increase. This calculation is based on the following: (a) the efficacy of a purified preparation of SBTI in the assay described in this report; (b) the average number of embryos per uterus (19); and an assumption that there is a 100-fold increase in embryonic protein during development between Days 5 and 6 of pregnancy (17, 18). The calculated level of synthesis for a specific protein (approximately 10% in this case) is not usual, although exceptions in specialized tissues can be found (20–22). Finally, attempts in this laboratory to identify an isoinhibitor of possible embryonic origin and different in electrophoretic mobility from those in serum or extracts of control uteri were not successful. Consequently, the increased inhibitor activity appears to be the result of modulation of TLI levels by the uterus and/or transduction of serum proteins, among which are proteinase inhibitors, into uterine tissue. Reasons for serious consideration of the latter possibility are described below.

Effects of pH and temperature on inhibitor activity. Although acidification of uterine extracts to pH values of 6.0 or 5.0 prior to analysis did not result in loss of inhibitor activity, dropping the pH value to 3.9 led to complete loss of activity. Alternatively, a basic milieu (pH 8.5 or 10.5) had no effect on stability. Complete inactivation of inhibitor activity also resulted after heating extracts for 5 min at 60°. These results contrast with those obtained by Blackwood *et al.* (9) for rat uterine trypsin-like inhibitors, but correspond to the reported characteristics of serum (23–25) and oviductal fluid (26) trypsin-like inhibitors in terms of both pH and temperature labilities.

Discussion. On the basis of studies with a macromolecular dye and ^{125}I-labeled protein, Psychoyos (27) has suggested that the estrogen surge (28, 29) which occurs in rodents at implantation causes transudation of serum proteins into uterine tissue. Contributing to this effect may be estrogen from blastocysts (30). The presence of serum-like proteinase inhibitors in the reproductive tract has been reported in the case of oviductal fluid of both rabbits (26) and monkeys (31).

The following observations in this study appear to be in accord with the possibility of transudation and the presence of serum-like TLI activity in the extracts: (a) The time profile (Fig. 1) indicates maximum activity just subsequent to the estrogen surge (28, 29); (b) the temperature and acid pH lability of the inhibitor preparations is similar to the TLI activity in serum (23–25); and finally (c) the specific activities of TLI are similar in nonpregnant (control) and delayed implantation uteri. One cannot discount, however, the possibility that elevated inhibitor activity on Day 6 may be related, in part, to an inflammatory-like response of the uterus during the invasive stages of implantation.

Several physiological functions can be proposed for the TLI activity in the uterine extracts. (i) It may be necessary to balance proteinase and proteinase inhibitor activities of both embryo and uterine environment for implantation to occur. This is indicated by the fact that exogenous proteinase inhibitors administered *in utero* impair implantation (6), while addition of trypsin to mouse blastocyst cultures initiates further development *in vitro* (32). (ii) The uterine inhibitors may modulate the activity of trypsin-like proteinases of the blastocyst (6, 7) or uterine fluid (1). (iii) The inhibitors may control or restrict the activity of proteinases liberated by leukocytes which are found at implantation sites (33); without modulation of proteolytic activity, digestion of tissue beyond areas of implantation might also occur. Finally (iv) the inhibitors, like α_1-antitrypsin and α_2-macroglobulin, may act as antifibrinolytic agents (25, 34, 35) and prevent blood clotting in the capillary penetrated by the embryo during implantation.

Summary. Specific activities of tryptic-like inhibitors (TLI) in 27,000*g* supernatant solutions of mouse uterine homogenates were determined for the following conditions: nonpregnant, pregnant, and delayed implantation. Quantification of inhibitors was achieved by a fluorometric kinetic assay. Inhibitor levels in uteri from Day 4 pregnant and from delayed implantation mice were the same as nonpregnant uterine control values. A significant increase in inhibitor activity ($P = 0.05$) was found in uteri from Day 6 pregnant mice. This increased activity returned to control levels by Day 12 of pregnancy. Possible functions of TLI in regulating proteolytic activity associated with early development and implantation are discussed.

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