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The Biology of the Fluids of the Female Genital Tract

INHIBITORS OF TROPHOBLAST PROTEINASES

H.-W. Denker

Why with the time do I not glance aside
To new-found methods, and to compounds strange?
(Shakespeare, Sonnet 76)

SUMMARY

Certain proteinases of the trophoblast and of uterine secretions play an important role in the initiation of embryo implantation as suggested by investigations done in the rabbit and mouse. Blastolemmase, a trophoblast-dependent endopeptidase studied in detail in the rabbit, appears to be a major factor in the disintegration of the blastocyst coverings during the initiation phase of implantation. Regulation of this proteinase activity may involve the action of proteinase inhibitors present in the endometrium and the uterine secretion, of the human as well as in some animal models although nothing is known about proteinases of early implantation stage human trophoblast. A first attempt at studying a model system was made by investigating the interaction between rabbit blastocyst proteinase (blastolemmase) and plasma inhibitors present in human endometrium and uterine secretion. Examples of other proteinase inhibitors from animal tissues were also included for comparison. Recently developed biochemical tests based on the hydrolysis of synthetic tripeptide p-nitroanilide substrates were used in order to obtain quantitative data on enzyme-inhibitor interactions, and the results were com-

Abteilung Anatomie, Rheinisch-Wesfaelische Technische Hochschule, Aachen, FRG

pared with those obtained with gelatin substrate film tests. Biochemical and physiological aspects of the relationship between blastolemmase and other proteinases of the trypsin family present in implantation stage trophoblast are discussed.

INTRODUCTION

Proteinase activity associated with implantation, in particular with its first phase, i.e. attachment of the trophoblast to the uterine epithelium, has recently received increased interest (for review see Denker 1977, 1978). There is good evidence that certain enzymes of the trypsin family, which are found in the trophoblast and the uterine secretion and which have been studied in detail in the rabbit, play a significant role in initiation of implantation since it was shown that inhibition of these enzymes by administration of specific proteinase inhibitors *in vivo* results in blockage of implantation (Denker 1977, 1978).

As far as other species are concerned, only few data on implantation associated proteinases are available, most of them for the mouse. A specific role in dissolution of the zona pellucida and in implantation initiation has been suggested for a uterine secretion proteinase in this species (''implantation initiating factor'', Mintz 1971, Pinsker et al., 1974). On the other hand, trypsin-like and chymotrypsin-like enzymes were shown to be present in late preimplantation stage mouse blastocysts (Dabich and Andary, 1976). Intrauterine administration of proteinase inhibitors caused embryonic loss also in this species (Dabich and Andary, 1974), although it has not been shown that this treatment interferes in fact with the process of implantation as in the rabbit. Cathepsin-like enzymes as demonstrated in the guinea pig (Owers and Blandau, 1971) and the cat (Denker et al., 1978) can be assumed primarily to play a role in intracellular protein degradation rather than in implantation initiation. Little is known about trypsin-like enzymes which may also be present in these species.

PROTEINASES

The most detailed data available so far have been presented for *blastolemmase*, a trophoblast-dependent enzyme which seems to play an important role in the process of dissolution of the extracellular blastocyst coverings (which is the prerequisite for attachment of the trophoblast to the uterine epithelium) in the rabbit. Blastolemmase shows enzymatic properties which are in many respects closely related to those of trypsin. However, it shows different electrophoretic mobility (Denker 1977, Denker and Petzoldt, 1977) and has more restricted substrate specificity: while there is a general specificity for arginyl bonds as in the case of trypsin, blastolemmase seems to be more selective with respect to the conformation and nature of the protein/peptide to be split: hydrolysis rates as measured in rabbit blastocyst extracts are strongly influenced by the type of amino acid present in subsite position P_2 and P_3 preceding the residue (P_1) which fits to the specificity pocket of the enzyme (Denker and Fritz 1979). Only little general proteolytic activity can be measured with conventional protein substrates like casein. It has been suggested, therefore, that the physiological function of this enzyme does not involve complete digestion of the blastocyst coverings but may lie more in hydrolyzing a limited number of peptide bonds. This may result in a change of the physicochemical properties of the blastocyst coverings at the abembryonic pole of the blastocyst, i.e. the region of maximal blasto-

lemmase activity. These changes may have physiological significance in the following processes: 1. Increased stickiness of the blastocyst coverings at the abembryonic pole has been suggested to provide the mechanism for ensuring the correct orientation of the blastocyst with its abembryonic pole facing the antimesometrial endometrium (Böving, 1963) (for a hypothesis concerning the possible mechanism, taking into account the alkaline pH optimum of blastolemmase and higher alkalinity of the antimesometrial as compared to the mesometrial endometrial surface, see Denker 1978). 2. Softening of the blastocyst coverings, resulting from limited hydrolysis of certain peptide bonds by blastolemmase, may be sufficient to allow the trophoblastic knobs to penetrate them, with the vis a tergo provided by the continuing expansion of the blastocyst. 3. The so altered glycoproteins of the coverings may become more susceptible to the action of other enzymes present (like glycosidases, see Denker, 1970b, 1971a, 1977). 4. Limited proteolysis might also change properties of cell surface-bound receptors possibly involved in the attachment of the trophoblast to the uterine epithelium, although experimental support for this hypothesis is lacking so far.

The presence, in the implanting trophoblast of the rabbit, of additional proteinases has been deduced from experiments with various substrates and inhibitors (Denker and Fritz, 1979) and has been shown electrophoretically (Denker and Petzoldt, in preparation). In particular, a possibly kallikrein-like enzyme and a plasminogen activator have been demonstrated in the non-gelatinolytical fractions. More detailed studies of these enzymes are in progress.

Proteinase Inhibitors

The presence of various *proteinase inhibitors in the endometrium and in the uterine secretion* is well known. It seems reasonable to assume that such inhibitors act as physiological regulators of the described proteinase activities. In fact, this concept had already been developed around the turn of the century when it was found that human decidua had antitryptic activity able to counteract the proteolytic activity of the trophoblast (Gräfenberg, 1909, 1910; Halban and Frankl, 1910). This was confirmed and extended by Schmidt-Matthiesen (1967). More recently, numerous investigations have identified various proteinase inhibitors in the endometrium and the uterine secretion in the human as well as in laboratory animals (Schumacher, 1970, Beier, 1970, Somerville and Dabich, 1974). Two fractions have been demonstrated by agar gel and starch gel electrophoresis, in rabbit uterine fluid, particularly after treatment of the animals with large doses of estradiol-17β and/or with progesterone (Beier 1970). Purified preparations of uteroglobin, the predominant protein in rabbit uterine secretion in the preimplantation phase, were recently shown to possess trypsin-inhibiting activity (Beier 1977). It is still not completely sure, however, whether uteroglobin can be identified on this basis as a proteinase inhibitor, and further investigations are certainly needed in order to clarify this. Interestingly, the uteroglobin-like protein from lung tissue does not show trypsin inhibition in the same assay (fibrin agar gel electrophoresis). Uteroglobin binds more or less strongly a number of different compounds like progesterone (Beato 1977) so that it is not completely excluded so far that it may perhaps also carry a low molecular weight proteinase inhibitor. Since the quantities of uteroglobin applied in published trypsin inhibition tests (using the fibrin agar gel electrophoresis technique) appear relatively high, it remains to be determined whether the kinetic constants for trypsin binding by this protein are in the range typical for true proteinase inhibitors.

As far as the *human* endometrium and uterine fluid are concerned, the presence of various plasma proteinase inhibitors (α_1-antitrypsin, α_1-antichymotrypsin, α_2-macroglobulin) has been described by several authors using semiquantitative radial diffusion tests (Schumacher, 1970, Tauber, 1979 and this volume) and qualitative immunological tests (Beier and Beier-Hellwig, 1973). The concentration of α_1-antitrypsin found in the uterine mucosa from the corpus was, however, much lower than in the cervix, and only minor cyclical variations have been shown in the corpus (Schumacher, 1970, Tauber 1979 and this volume).

Unfortunately, detailed biochemical data on the counterpart, i.e. on individual proteinases of the trophoblast and of the uterine secretion which might play a role in initiation of implantation are totally missing for the *human*. Recently, biochemical investigations of various proteinases found in mature human placentae have been performed (Unger and Struck, 1977, 1978); however, it is questionable whether they are at all related to those postulated enzymes which might have a physiological function in the phase of implantation initiation.

In this situation, it may appear justified to take a somewhat speculative look at the problem, discussing the interaction of: 1. Trophoblast-dependent proteinase (blastolemmase) available so far only from the rabbit, and 2. inhibitors known to be present in the endometrium and uterine secretion in the human as discussed above.

RESULTS AND DISCUSSION

Experiments of this type will be described below. Naturally, they cannot give any information on the proteinase: proteinase inhibitor system present *in vivo* in the two species, but they may help to develop some ideas and concepts useful for further investigations. These experiments are based primarily on quantitative photometric assays developed since synthetic chromogenic tripeptide p-nitroanilide substrates became available (Denker and Fritz, 1979). This technology will improve the investigation of blastocyst proteinases (previously based primarily on qualitative (or, at the best, semi-quantitative) gelatin substrate film tests (Denker, 1971, 1972, 1974, 1976). As long as purification of trophoblast proteinase(s) has not been achieved, non-purified extracts of late preimplantation rabbit blastocysts are used (for the methodological details, see Denker and Fritz, 1979). This material contains, in addition to blastolemmase, two other enzymes electrophorectically distinguishable enzymes (see above) which are able to split arginyl and lysyl bonds and which can, therefore, contribute to the photometer readings obtained with the tripeptide p-nitroanilide substrates used. Therefore, interpretation of the photometric tests has to be done cautiously if interest is focused on blastolemmase alone. On the other hand, the relative homogeneity of results of kinetic investigations (Denker and Fritz, 1979) and experiments with highly specific inhibitors (like BSTI-II, see below) suggest that one enzyme seems to dominate in the hydrolysis rate, if the substrate N-tosyl-glycyl-L-prolyl-L-arginine-p-nitroanilide (*TosGlyProArgPNA*) is used. Since the accompanying proteases can be easily distinguished from blastolemmase due to the fact that they do not digest gelatin membranes (Denker and Petzoldt, in preparation) we are not basing our investigations only on tests with the synthetic substrates but are also including qualitative tests with the gelatin substrate film method.

The results obtained with various proteinase inhibitors from human plasma are summarized in Table I. Notice that the inhibitor concentrations indicated can

TABLE I. Inhibitors of Rabbit Blastocyst Proteinase

Inhibitor	Lowest effective concentration[1]	
	Biochemical test system[2] (substrate: TosGlyProArgPNA)	Histochemical test system[3] (substrate: gelatin)
Human plasma inhibitors:		
α_1-antitrypsin	$3 \cdot 10^{-6}$ M[4]	$1 \cdot 10^{-5}$ M
α_1-antichymotrypsin [5]	$3 \cdot 10^{-5}$ M	$1 \cdot 10^{-4}$ M
inter-α-trypsin inhibitor	($>1 \cdot 10^{-7}$ M) [6]	$1 \cdot 10^{-6}$ M
α_2-macroglobulin	($>1 \cdot 10^{-7}$ M) [6]	($>1 \cdot 10^{-5}$ M)
Other inhibitors: (selected examples)		
boar seminal plasma trypsin-acrosin inhibitor (BSTI-II)	$7 \cdot 10^{-8}$ M	$5 \cdot 10^{-6}$ M
aprotinin (basic trypsin-kallikrein inhibitor from bovine organs, Trasylol)	$1 \cdot 10^{-10}$ M	$1 \cdot 10^{-6}$ M

1) In the range of ~50% inhibition
2) Test performed as described by Denker and Fritz (1979). Preincubation of enzyme with inhibitor: 5 min. at 37° C if not indicated otherwise.
3) For details of the test see Denker (1974,1976). This basically qualitative test gives no information on kinetic constants. Inhibitor concentrations indicated are as present in the solution used for soaking the gelatin, not identical with actual concentration during the test.
4) Progressive inhibition; preincubation 2 hours.
5) Contains 1-2% α_1-antitrypsin.
6) At higher concentrations, TosGlyProArgPNA-splitting activity present as an impurity in the inhibitor preparation influenced photometer readings.

Human plasma inhibitors were obtained from Behringwerke Marburg; purity given as determined by immunological test; α_1-antitrypsin: ca. 98% pure; no contamination by other proteinase inhibitors; α_1-antichymotrypsin: ca. 98% pure, contains 1-2% α_1-antitrypsin; inter-α-trypsin inhibitor: ca. 98% pure, no contamination by other proteinase inhibitors; α_2-macroglobulin: purity ca. 90% (values corrected accordingly), main contaminant IgA but no other proteinase inhibitors, 1/3 of dry matter glycin added. Other chemicals used were as indicated by Denker and Fritz (1979).

allow to draw conclusions on the kinetics of the interaction only in the case of the biochemical test system (substrate: TosGlyProArgPNA) and not in the case of the histochemical gelatin film test. The latter shows clearly that blastolemmase is inhibited by the inhibitors of trypsin-like enzymes, *inter-α-trypsin inhibitor*, *α_1-antitrypsin*, and, from the group of other inhibitors from animal tissues included for comparison, by *boar seminal plasma trypsin-acrosin inhibitor (BSTI-II)*

and by *aprotinin* (basic trypsin-kallikrein inhibitor from bovine organs, Trasylol[R]). No inhibition was obtained, in the range tested, with α_2-macroglobulin (concerning the specificity of the plasma inhibitors, see Heimburger, 1975; for BSTI see Fritz et al., 1975, 1976; for further references see Denker, 1976, 1977).

The results obtained in the biochemical test system show that the plasma inhibitors are less effective than the inhibitors from animal sources. This is particularly obvious when they are compared to aprotinin, the most effective inhibitor of blastolemmase known so far (K_i estimated below 10 pM, Denker and Fritz, 1979). The interaction with inter-α-trypsin inhibitor and α_2-macroglobulin could not be studied in the biochemical test system because the sensitivity of this system is so high that traces of proteinases present in the inhibitor preparations influenced the photometer readings at concentrations higher than $1 \cdot 10^{-7}$ M. The histochemical gelatin substrate film test, however, shows that the inter-α-trypsin inhibitor does inhibit blastolemmase efficiently.

The inhibitory effect seen with α_1-*antichymotrypsin* is somewhat surprising because this inhibitor is rather specific for chymotrypsin (Heimburger, 1975) while the blastocyst proteinases discussed here are trypsin-like enzymes. This phenomenon had already been observed (Denker, 1976) at a time when only the gelatin substrate film test was available. At that time it appeared possible that a chymotrypsin-like enzyme, perhaps present at the same sites as blastolemmase, would contribute to the results of the test and its inhibition could account for the effect seen with α_1-antichymotrypsin. It seems interesting, therefore, that inhibition is now also seen in the biochemical test system using TosGlyProArgPNA, a substrate which is being hydrolyzed quite specifically by trypsin-like enzymes. The preparation of α_1-antichymotrypsin used contains 1–2% α_1-antitrypsin. However, according to the inhibitor concentrations found effective (see Table), the presence of these impurities does not explain the observed effect satisfactorily. Further investigations are needed in order to prove whether α_1-antichymotrypsin is in fact able to interact with blastolemmase.

α_1-*antitrypsin* was found to inhibit rabbit blastocyst proteinase activity in a progressive manner (Figure 1). This has been shown likewise for the interaction of this inhibitor with acrosin (Fritz et al., 1972), kallikrein and plasmin.

Boar seminal plasma trypsin-acrosin inhibitor (BSTI-II) has been included in the study for comparison (Table, Figure 2) although this inhibitor is not a constituent of the implantation phase uterine milieu. As the biochemical test shows, a relatively strong inhibition is obtained with this compound. BSTI is known to inhibit trypsin and acrosin but not the related enzymes with very restricted substrate specificity, i.e. plasminogen activator and kallikreins. It inhibits blastolemmase strongly as seen in the gelatin substrate film test. Incomplete inhibition is seen, on the other hand, in the biochemical test system using TosGlyProArgPNA as a substrate, in the indicated range of inhibitor concentrations (Figure 2). The residual activity is, however, only low. This indicates that blastolemmase probably accounts for most of the activity measured with this substrate, and the other proteinases present in this material and mentioned above seem to influence the test only to a minor degree.

The described investigations show that proteinase inhibitors, known to be present in the plasma and in the endometrium and uterine lumen in the human, modulate trophoblast proteinase activity, as demonstrated using the rabbit as a model, *in vitro*. Since blastolemmase has previously been shown to play a major role in implantation initiation in the rabbit, we may hypothesize that uterine pro-

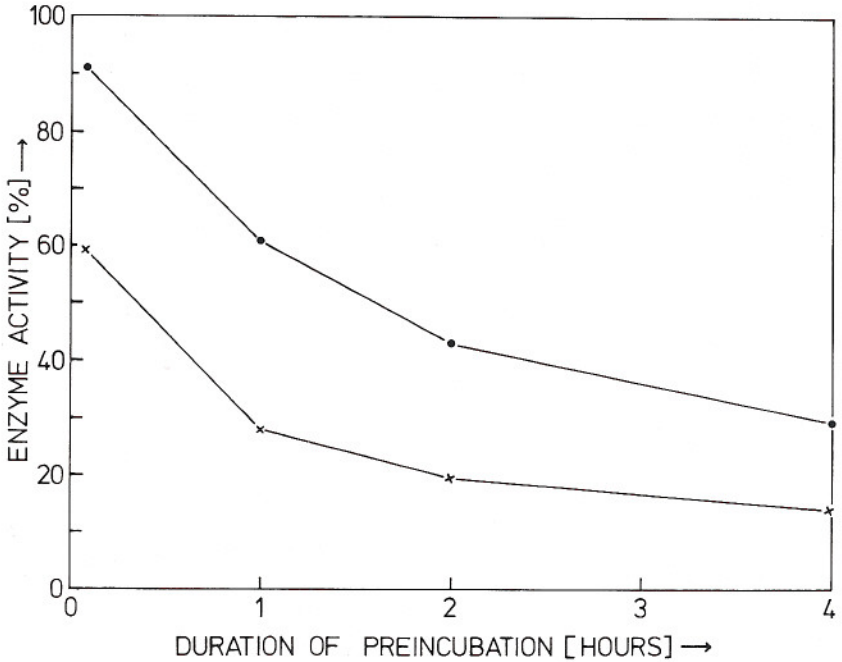

FIGURE 1. Progressive inhibition of rabbit blastocyst proteinase by α_1-antitrypsin. Biochemical test system as indicated in the Table. The inhibition concentration was maintained during preincubation as well as during the test: • —— • $3.3 \cdot 10^{-6}$ M; x —— x $1 \cdot 10^{-5}$ M.

teinase inhibitors are one of the factors which *regulate implantation events from the maternal side in vivo*. Regulation by proteinase inhibitors is well documented for other systems in which proteinases are being produced, activated, and play a biological role (like digestive enzymes in salivary glands and the intestinal tract, the blood clotting and fibrinolytic system, etc.). It can be regarded as a rule that, *in vivo*, proteinases are always controlled by a well-developed and often complicated system of activators and inhibitors.

Experimental evidence indicates that the uterine milieu is not particularly favorable for implantation but rather contains, at least in certain stages, implantation inhibiting factors (Weitlauf, 1978; Finn, 1974; McLaren, 1973; Psychoyos and Bitton-Casimiri, 1969). Implantation-like events, in particular invasive growth of the trophoblast, starts readily in ectopic sites, independent of the hormonal status of the host (Kirby, 1970). In rodents, the implantation-initiating effect of the estrogen surge is possibly based on depression of uterine inhibitor. After experimental delay of implantation, implantation can be induced by administration of actinomycin D, which is again thought to result from blockage of inhibitor synthesis (Finn, 1974). Although this view of the *uterus as an organ that regulates implantation* is not very new (for discussion and references see Denker, 1977), these recent experiments seem to accentuate this aspect. Identification of inhibitory factors of the uterus as proteinase inhibitors is speculative, at present. In a general sense, the following physiological functions of uterine proteinase inhibitors may

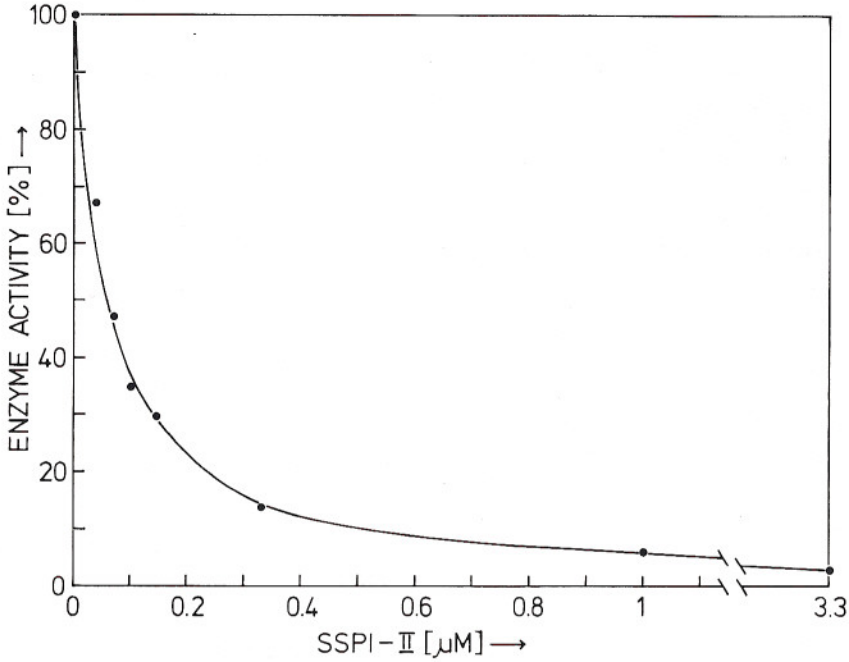

FIGURE 2. Titration of rabbit blastocyst proteinase with various concentrations of boar seminal plasma trypsin-acrosin inhibitor (BSTI-II). Biochemical Test system as indicated in the Table.

be envisaged: 1) a general protective role, i.e. prohibiting spreading of proteinase activity and localizing it in space and time where it is needed for implantation; 2) a more specific regulatory function in the initiation of implantation by influencing the equilibrium between trophoblastic (and uterine) protease on the one hand and uterine inhibitors on the other.

As mentioned above, in addition to blastolemmase, a number of different proteinases are present in the blastocyst and the uterine secretion—even the trypsin family is represented by a number of individual members for which different physiological functions must be envisaged. Such functions include partial dissolution of the blastocyst coverings (as an element of the implantation initiation process) by blastolemmase; digestion of remnants of the blastocyst coverings by trypsin-like (and a chymotrypsin-like ?) proteinase(s) of the uterine secretion; fibrinolysis (and promotion of invasion ?) by the plasminogen activator—plasmin system; liberation of biologically active peptides by kallikrein-like enzymes, etc. Far too little is known about the presumed differential effects of the present inhibitors on these different proteinases and proteinase systems, and we therefore feel that this field merits further investigations.

Finally, one aspect of practical relevance may be mentioned. At present, animal experiments as well as clinical trials with intrauterine administration of proteinase inhibitors into the uterine lumen are being performed in several institutions to prevent increased menstrual blood loss associated with IUD use (Tauber et al.,

1977, WHO 1978). In the light of the cited *in vivo* studies performed in the rabbit, it must be considered that one "side"-effect of such treatment is interaction with those proteinases (of the trophoblast and the uterine secretion) which are involved in implantation initiation. In fact, exactly those inhibitors used in the IUD studies, i.e. aprotinin (Trasylol [R]) (Tauber et al., 1977) and aromatic diamidines (WHO 1978) are effective in blocking the dissolution of the blastocyst coverings and implantation initiation in the rabbit experiments (Denker, 1977 and unpublished data). Therefore, more detailed investigation of proteinase systems involved in implantation and/or in various functions of the uterus seems desirable.

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