Interaction of Proteinase Inhibitors with Blastocyst Proteinases involved in Implantation

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RABBIT blastocysts exhibit a remarkable proteinase activity during the first (abembryonic) phase of implantation in the uterus. The activity becomes detectable around 6 days post-coitum (d p.c.) and rises rapidly until 7–7⅓ d p.c. when syncytial trophoblastic "knobs" penetrate the extracellular blastocyst coverings and establish contact to the uterine epithelium; thereafter blastocyst proteinase activity declines rapidly until 8–8½ d p.c.[4,6] Blastocyst coverings are composed of both protein and sialic acid- and sulfate ester-rich glycosaminoglycan components.[5] Presumably a number of enzymes including glycosidases and proteinases are involved in the breakdown of this "barrier" between trophoblast and uterine epithelium,[5,8] blastocyst proteinase activity apparently playing a major role as suggested by the following observations. This enzyme activity is always found maximal at the abembryonic pole where lysis of blastocyst coverings starts, which holds true also in the case of abnormal orientation of the blastocyst in the uterus.[10] Blastocysts that show signs of beginning lysis of their coverings have never been found to lack high proteinase activity. Furthermore, hormonal regulation of blastocyst proteinase activity is reflected by hormonal regulation of lysis of the coverings: in ovariectomy and progesterone replacement therapy experiments both respond in a parallel way.[7]

So far the histologic site of synthesis of the proteinase molecules is unknown (trophoblast? endometrium?),[11,16] but there is strong evidence that the trophoblast provides at least an essential cofactor (activator) if not the enzyme (proenzyme) molecules.[10–12] According to the apparent physiological importance of this proteinase, biochemical characterization seems to be very desirable but has still not been achieved yet due to the small quantities of enzyme present in an embryo. So far only gelatin membranes have been successfully used as a substrate.[4,6–12,16] Therefore, until purified preparations are obtained and/or a specific substrate has been found, we decided to study the action of a number of purified and well-characterized inhibitors on this proteinase using a highly sensitive histochemical gelatin substrate film test[9] (Fig. 1).

MATERIAL AND METHODS

Pure gelatin substrate films were prepared as previously described[9] using 0.15 ml of the gelatin solution per slide. Immediately before use, fixed, alkali-treated and dried films were impregnated with inhibitors in the following manner:

1. Water-soluble inhibitors were dissolved in 0.1 M Na-, K-phosphate buffer pH 7.0. Half of the area of the substrate film was layered with 0.2 ml of this solution, left for 5 min,

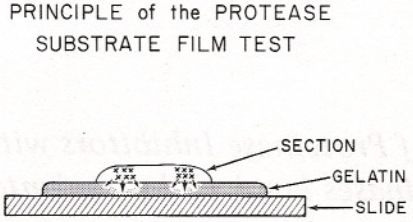

FIG. 1. Principle of the proteinase substrate film test. The enzyme diffuses, from its site in the cryostat section, into the gelatin substrate film which either contains or (in controls) does not contain proteinase inhibitor.

drained and air-dried. The other half of the slide was treated with pure buffer in the same way to serve as a control. Inclusion of crystallized bovine serum albumin into the control test buffer at the same molar concentration as the inhibitors was shown to be without any influence on the results.

2. In case of water-insoluble inhibitors, the whole substrate film was first impregnated with 0.4 ml of phosphate buffer; after air-drying, half of the slide was layered with inhibitor solution in organic solvent, the other half was similarly treated with solvent alone. Solvents used were: *N,N*-dimethyl formamide, methanol and ethanol.

Pieces of rabbit uteri containing blastocysts at the stage 7 d p.c. were frozen with liquid nitrogen and cut on a cryostat. 14-μm native sections were mounted on the gelatin films, incubated in wet chambers at 38°C for 1¾ hr, fixed in saturated aqueous $HgCl_2$ solution, washed and stained (see ref. 9). All tests were run at least in duplicate.

RESULTS

The appearance of control tests is shown in Fig. 2. The minimal concentrations of inhibitors (concentrations as present in the solutions used for impregnating the gelatin membrane) which still caused definite reduction in size of lysis zones are given in Table 1. Higher concentrations of inhibitors No. 1–9 (Table 1) caused complete inhibition. The specificity of the inhibitors is indicated in the table only with respect to trypsin and chymotrypsin. The value given for diisopropylphosphofluoridate is probably too low because this inhibitor is volatile and it is therefore probably not possible to maintain a sufficient concentration on the slide.

Table 1 refers to the main proteinase activity of the blastocyst which is found predominantly in the blastocyst coverings and abembryonic-lateral trophoblast. In addition, there is another much lower gelatin dissolving activity in certain embryonic pole cells which is resistant to 10^{-4} M soybean trypsin inhibitor.

DISCUSSION

Strong inhibition of the main blastocyst proteinase by inhibitors No. 1–8 (Table 1), especially by the trypsin-specific inhibitors No. 3–6 and the active site titration reagent NPGB (No. 8), clearly proves that its active center exhibits striking similarities to trypsin. In addition, there is also a definite effect of the chymotrypsin-specific inhibitors α_1-antichymotrypsin and chymostatin. Both charges have been assayed for trypsin-inhibiting impurities and there were

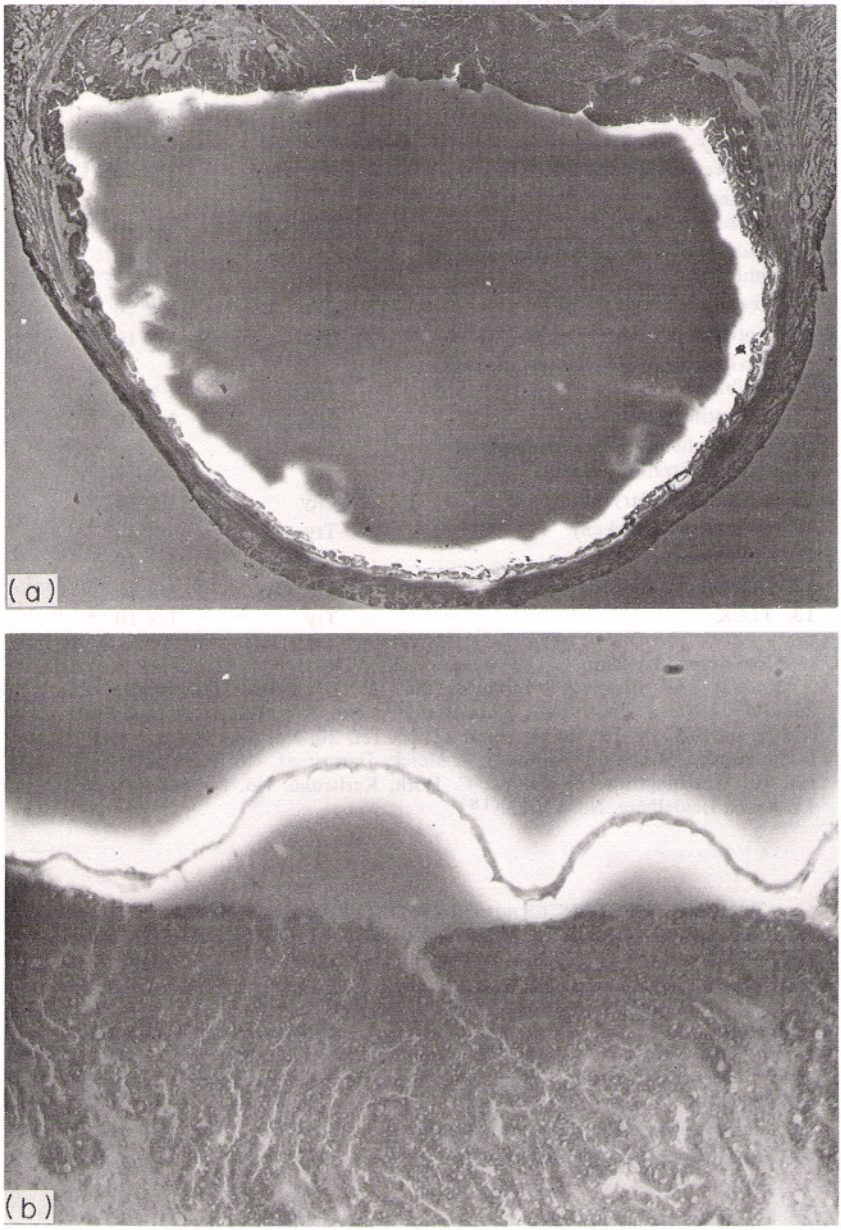

FIG. 2. Results of control tests (no inhibitor included). (a) × 14, (b) × 90. The dark-stained background represents the gelatin substrate film, white areas indicate sites of proteinase activity. Fig. 2a shows the pattern typical for the 7–7⅓ d p.c. stage with high activity predominantly in the antimesometrial and lateral regions of the large attaching blastocyst. After ovariectomy (see ref. 7) (Fig. 2b), reduced activity permits attributing the protease activity to the wall of the blastocyst (above) rather than the endometrium (below).

TABLE 1. MINIMAL EFFECTIVE INHIBITOR CONCENTRATIONS ACTING ON RABBIT BLASTOCYST PROTEASE (GELATIN SUBSTRATE FILM TEST)

Inhibitor	Specificity	Molarity
1. Soybean trypsin inhibitor (Kunitz)	Try, Chy	1×10^{-6}
2. Bovine basic trypsin–kallikrein inhibitor (Trasylol)	Try, Chy	1×10^{-6}
3. Boar seminal plasma trypsin–acrosin inhibitor	Try	5×10^{-6}
4. Leupeptin	Try	5×10^{-6}
5. Antipain	Try	5×10^{-6}
6. Bovine pancreatic secretory trypsin inhibitor	Try*	1×10^{-5}
7. α_1-Antitrypsin	Try, Chy	1×10^{-5}
8. *p*-Nitrophenyl-*p*-guanidinobenzoate (trypsin titration reagent)	Try	5×10^{-5}
9. α_1-Antichymotrypsin	Chy	1×10^{-4}
10. Ovomucoid (chicken)	Try	5×10^{-4}
11. Chymostatin	Chy	1×10^{-3}
12. Diisopropylphosphofluoridate	Try, Chy	1×10^{-3}(?)
13. TLCK	Try	1×10^{-2}

*Not human trypsin.

Sources and literature: Inhibitor No. 1: E. Merck, Darmstadt, No. 24020;[21] No. 2: Bayer AG, Wuppertal;[21] No. 3: a mixture of fractions I and II, see refs. 14, 19; No. 4, 5 and 11: see ref. 20; No. 6:[21]; No. 7 and 9: Behringwerke, Marburg;[15] No. 8: E. Merck, Darmstadt, No. 10562;[18,p.21] No. 10: Worthington;[13] No. 12: C. Roth, Karlsruhe, No. 1–5465;[18] No. 13: Serva, Heidelberg, No. 17013.[18]

TABLE 2. SUBSTANCES *not* INHIBITING RABBIT BLASTOCYST PROTEASE AT HIGH CONCENTRATION (GELATIN SUBSTRATE FILM TEST)

Inhibitor	Specificity	Molarity (m)
1. Pepstatin		5×10^{-2}
2. Glutaryl-phenylalanin-β-naphthylamide	Chy	1×10^{-2}
3. Benzoyl-arginine-β-naphthylamide	Try	1×10^{-2}
4. TPCK	Chy	1×10^{-2}
5. ϵ-Aminocaproic acid	(Try)	1×10^{-2}
6. 2-Hydroxy-5-nitro-α-toluenesulfonic acid sultone (chymotrypsin titration reagent)	Chy	1×10^{-2}

Sources and literature: Inhibitor No. 1: see ref. 20; No. 2: K. & K. Laboratories, Plainview, N.Y., No. 24820; Nos. 3–5: Serva, Heidelberg, Nos. 14630, 17016 and 12548.[18]

essentially none. On the other hand, the enzyme does not hydrolyze trypsin and chymotrypsin substrates like benzoylarginine ethyl ester (BAEE), benzoylarginine-β-naphthylamide (BANA), benzoylarginine-*p*-nitranilide (BAPA), carbobenzoxy-di-glycyl-arginyl-β-naphthylamide, acetyl-tyrosine ethyl ester (ATEE) and carboxypropionyl-phenylalanine-*p*-nitranilide in measurable rates, and activity against casein and dimethylcasein is low (Denker and Fritz, in preparation and unpublished data). The blastocyst enzyme therefore might be more related to elastase although not identical with pancreatic elastase or known elastase-like enzymes which are not or only weakly inhibited by soybean trypsin inhibitor or Trasylol.[17, p. 113, 844; 17] Other possibilities like, for example, being a collagenase are excluded by the spectrum of inhibitors shown to interact with this enzyme.

The rabbit blastocyst proteinase also has to be considered different from trypsin-like (BANA-splitting) and chymotrypsin-like (glutaryl-phenylalanine-β-naphthylamide-splitting) enzymes found in mouse blastocysts and uteri.[1–3] Further experimentation is needed before the question can be answered whether there might be a relationship to fractions seen in PAA-gel electrophoresis which split hemoglobin and casein but not BANA.[1,2]

Experiments on isolation and further biochemical characterization of the rabbit blastocyst proteinase as well as studies on interaction of inhibitors with implantation *in vivo* are in progress.

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