Proteinases involved in implantation initiation in the rabbit: microdisc electrophoretic studies

Proteinasen und Einleitung der Implantation beim Kaninchen: Mikro-Disc-Elektrophoreseuntersuchungen

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Abstract

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Blastocyst and uterine secretion proteinases which, according to previous investigations, play a central role in initiation of implantation (attachment of trophoblast onto the uterine epithelium) are studied in the rabbit using micro disc electrophoresis. A specifically designed procedure using a highly sensitive gelatin substrate film technique and longitudinal sections of micro disc gels is described. Comparison is made with zymograms obtained with synthetic amide substrates (BANA and GPNA). Rabbit implantation stage (62½ d.p.c.) trophoblast as well as disintegrating blastocyst coverings are shown to contain high activity of a gelatin-dissolving proteinase of identical electrophoretic mobility. BANA-splitting activity of this enzyme is barely detectable. Uterine flushings from the same stage exhibit a gelatinolytic proteinase which shows slightly different migratory properties in micro disc electrophoresis. Considerable BANA-splitting activity (optimum pH in the range of 8.0) is found in the same fraction and is tentatively ascribed to the same enzyme which, by its catalytic properties, resembles trypsin more closely than the blastocyst-derived proteinase. Both enzymes are separable from β-glycoprotein. Chymotrypsin-like (GPNA-splitting) amidase activity is found predominantly in a slowly migrating fraction of pregnant uterine flushings. Indications for a regulation of proteinase activity of trophoblast and uterine secretion by proteinase inhibitors present in these sites, or by proenzyme activation, are discussed.

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Introduction

Recently, increased evidence has been presented for a central role of certain proteinases in initiation of implantation of the mammalian embryo in the uterus [3, 4, 5, 7, 9, 10, 13, 14, 15, 21, 25]. Specific inhibition of proteinases in vivo efficiently prevents the dissolution of extracellular blastocyst coverings (zona pellucida etc.) and subsequent attachment of the trophoblast onto the uterine epithelium [13, 14]. Most interest is being centered, in this context, on a peculiar gelatin-dissolving proteinase activity found in the disintegrating blastocyst coverings and at the interface between attaching trophoblast and uterine epithelium in the rabbit [5 to 15]. Controversial views have been presented, however, on the probable site of origin (trophoblast or uterine secretion) of this enzyme and on the respective role of trophoblast and endometrium in initiation of implantation [5, 7, 9, 10, 12 to 15, 18 to 21, 25]. By some authors, uterine secretion has been assumed to be the only or at least the principle source of proteinases [18, 21, 25], and β-glycoprotein, one of the major protein fractions of rabbit uterine secretion, has been assumed to represent the gelatinolytic enzyme of rabbit implantation sites [18, 19]. On the other hand, a number of experiments and observations provide evidence for dependence of rabbit blastocyst surface proteinase on trophoblast tissue but it was not possible, so far, to decide whether the trophoblast provides the enzyme (proenzyme) molecules or an essential activator [9 to 15]. It was found puzzling, however, that, in the previously published studies based on gelatin substrate film tests performed with tissue sections, most activity was seen in the extracellular blastocyst coverings rather than in trophoblast tissue.

In the present investigations, micro disc electrophoresis was used in an attempt to separate and to identify proteinases of rabbit implantation stage trophoblast tissue, disintegrating blastocyst coverings and uterine secretion, and to distinguish between enzymes of these different sources.

Materials and methods

Sexually mature, virgin female rabbits (mixed bred or Alaska strain) were mated to 1 to 2 fertile bucks. At 6½ days post coitum (d.p.c.), i.e. 8 hours before onset of blastocyst attachment, they were killed by stunning and exsanguination, and the uteri were quickly removed. Each uterine horn was flushed with 5 ml of ice-cooled 0.9% NaCl solution or Hank's balanced salt solution. Blastocysts were immediately removed from this uterine flushing which was, afterwards, separated from any spontaneously sedimenting debris by decanting. Blastocysts were washed twice in 0.9% NaCl or Hank's solution. Using fine forceps, blastocyst coverings were removed and the trophoblast was punched so that the blastocyst fluid was released while the blastocyst tissues (trophoblast and embryonic disc) collapsed. In some cases, trophoblast and embryonic disc were separated under a dissecting microscope with the aid of needles and fine scissors. By this it is possible to obtain pure trophoblast but the embryonic disc (ectoderm, entoderm and mesoderm) remains contaminated with trophoblast cells derived from both the margin of the embryonic disc and from the remnants of RAUBER's layer. Uterine flushings, blastocyst coverings and blastocyst tissues (trophoblast and embryonic knot) were separately frozen with liquid nitrogen or dry ice, and stored between −28 and −60°C.

Homogenization of the materials and subsequent micro disc electrophoresis were performed as previously described [23, 24], using 5 µl Drummond microcaps; collecting gel: 5% polyacrylamide (PAA) pH 6.7; separating gel: 20% PAA pH 8.8. After electrophoresis, the gels were cut at the buffer front.

Some of the gels were stained with amido black for demonstration of protein bands as described before.
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For demonstration of proteinase activity, both a protein substrate and synthetic amide substrates were applied. As in previous histochemical studies, gelatin was chosen as a protein substrate using a highly sensitive substrate film test (for details see [8]). Differing from the procedures described in the cited paper we now prefer to wash fixed gelatin films in deionized water rather than tap water (step A 5) and to freeze-store them after step A 6 (rather than after A 8). Alkali treatment (step A 7) is performed at the day when the test is to be done, which increases sensitivity further. Control of pH was attempted by impregnating ready-made films, after step A 8, with 0.1 mM phosphate buffer followed by air-drying.

Micro disc electrophoresis gels were exposed to so pretreated gelatin substrate films, either unsectioned or longitudinally sliced. Unsected gels were put immediately on the gelatin membranes followed by incubation in wet chambers (petri dish, sealed with vaseline) at 38°C for periods of 1 to 48 hours. Afterwards, gels were removed and stained in amido black solution, while gelatin membranes were fixed in saturated aqueous HgCl₂ solution at 4°C, washed and stained with 0.2% toluidine blue in borate buffer pH 10.0 at 4°C. After staining, micro disc gel tracks can be seen on the gelatin membrane, and local lysis zones mark proteinase-containing fractions.

Most gels, however, were sliced longitudinally in order to obtain, from each of these gels, both protease zymograms and protein stain. For this purpose, gels were put on a piece of cardboard and were quickly frozen with dry ice and mounted on a cryostat stage for longitudinal sectioning. 14 μm thick sections were taken and mounted on the gelatin substrate films. Incubation was performed as described above except that sections were not removed from the gelatin membranes after incubation as unsectioned gels were. The described procedure offers the opportunity to perform, with each micro disc gel, at least 3 individual proteinase tests (at least 2 sections each test), while the remainder of the PAA gel is still sufficient for staining of protein bands with amido black.

Amidase activity of endopeptidase-type enzymes was visualized by applying β-napthylamide substrates and demonstrating liberated β-napthylamine by simultaneous coupling to the diazonium salts Fast Blue B (·BF₄)₂, Serva, Heidelberg, No. 21269) or Fast Garnet GBC (·1/2 ZnCl₂, Serva, Heidelberg, No. 21290). Unsectioned PAA gels were incubated, after electrophoresis, in a medium containing 1 mg/ml of the respective substrate (first dissolved in N,N-dimethyl formamide, 0.04 ml/ml end volume) in veronal acetate buffer (after Michaelis) pH 6.0, 7.0 or 8.0, to which 0.5 mg/ml diazonium salt were added immediately before use. Incubation was performed for 60 minutes at 38°C. The following substrates were used: Na-benzoyl-L-arginine-β-napthylamide·HCl (BANA) (Serva, Heidelberg, No. 14 622) for demonstration of trypsin-like activity; glutaryl-L-phenylalanine-β-napthylamide (GPNA) (E. Merck, Darmstadt, No. 4245, or K & K Laboratories/ICN Pharmaceuticals No. 24820) for demonstration of chymotrypsin-like activity [1, 4, 16].

Microphotographs were taken of all zymograms as well as all protein stains, and were compared after correcting for size differences (as caused by differing degrees of swelling of gels and slices) by appropriate magnification of the prints.

Results

Longitudinal sectioning of PAA micro disc electrophoresis gels on a cryostat was found to be by far the most reliable and successful procedure when it was attempted to study, on the same single gel, both proteinase localization and protein bands. The latter were well preserved in the unsectioned rest of each gel as shown after amido black staining (Fig. 1). The highly sensitive gelatin substrate film test performed with sections of the gels revealed halos indicating the localization of proteinase-positive fractions, the size of the halos depending on both the duration of incubation and the load of the gel. Since several sections can be taken from each gel, it is possible to apply, in each case, a series of varying incubation periods, so that it is possible to obtain zymograms with optimized sharpness of localization.
Fig. 1. Micro disc electrophoresis of rabbit uterine flushings (a, b) and trophoblast homogenate (c, d) 6/3 d p.c. Zymograms obtained from 14 μm longitudinal sections of the gels and produced by the gelatin substrate film test are shown in (a) and (c); white lysis zones indicate fractions with gelatinolytic activity. The unsectioned rest of each gel is stained with amido black to show protein bands (b, d).

Fig. 2. Schematic summary of micro disc electrophoresis zymograms of gelatinolytic proteinases of rabbit blastocyst and uterine material, 6/3 d p.c. Approximate Rf values (mean values, number of electrophoretic runs indicated in parenthesis): blastocyst tissues (trophoblast) Rf 0.30 (15); blastocyst coverings Rf 0.33 (11); uterine flushings Rf 0.24 (19).

Figure 2 summarizes the results obtained in these tests, comprising 15 electrophoresis runs performed with blastocyst tissue material, 11 with blastocyst coverings and 19 with uterine flushings. Gelatin hydrolyzing proteinase activity is found in all three types of material, the activity being highest in blastocyst coverings zymograms, lower in case of trophoblast (blastocyst tissues) and lowest in case of uterine flushings, as judged from
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Proteinase activity is always found to migrate in the area between the β-glycoprotein and albumin fractions. In micro disc gels, the enzyme is in each case clearly separated from β-glycoprotein. In zymograms of uterine flushings, the enzyme activity seems also to be separable from albumin, while with blastocyst tissue and blastocyst coverings, material, proteinase is always found very close to and not clearly distinct from the albumin fraction. A slight difference in migratory properties seems to exist between uterine secretion proteinase on one hand and blastocyst tissue and blastocyst coverings proteinase on the other hand, although clear separation of these two categories of enzymes cannot be achieved by the used procedure.

Proteinase zymograms of isolated trophoblast were found identical with those of whole blastocyst tissues. Isolated embryonic disc, on the other hand, shows only little gelatinolytic activity, most of which appears in the same fraction as in the case of trophoblast tissue. Most probably this proteinase derives from contaminating trophoblast cells (see Materials and methods).

The above described results are in general confirmed by experiments with unsectioned micro disc gels. However, this procedure was found less reliable because localization of protein bands is difficult to evaluate in this case due to the fact that, during incubation of the PAA gels on the gelatin membranes, protein fractions diffuse out of the gels and bands tend to merge (most obvious in case of uteroglobin and albumin). Localization of proteinase activity also is less sharp than when sections of gels were used, obviously as a result of insufficient contact between the gel and the substrate film. When long incubation periods are used, the enzyme tends to spread along the gel. In such cases, uterine flushings zymograms show minimum gelatinolysis in the region of uteroglobin.

Fig. 3. Micro disc electrophoresis zymograms of rabbit uterine flushings 6½ d p.c. showing amidase activity. – a. Substrate BANA, pH 7.0, flushing from a blastocyst-containing uterus. – b. Same as (a), but flushing from a uterine horn without blastocysts. – c. Substrate GPNA, pH 8.0, flushing from a blastocyst-containing uterus.

A number of PAA gels were transversely sectioned in order to avoid artifacts possibly produced by diffusion along the longitudinal axis. The segments were incubated separately on the gelatin membrane. These experiments, too, confirm the results obtained with longitudinally sectioned gels.

When the synthetic trypsin type substrate BANA is applied, flushings of pregnant uteri show high activity in a fraction which migrates between β-glycoprotein and
albumin, more close to the latter than to the former, forming a dense single band (Figs. 3, 4). The reaction is much stronger at pH 8.0 than at pH 6.0. Considerably less activity was found in uterine horns which incidentally had no blastocysts. Some but not all uterine flushings (pregnant as well as pseudopregnant) exhibited an additional but more faint band in the region of β-glycoprotein (or just cathodically of it) which was also more prominent at pH 8.0 than at pH 6.0. Zymograms of both blastocyst tissue or blastocyst coverings show only very little BANA splitting activity, most of which is detectable in the β-glycoprotein region although even this reaction is only faint. Usually an additional but still less prominent band is seen close to the start point. Very little activity is demonstrable, in these gels, in those fractions which exhibit high gelatinolytic activity.

When the chymotrypsin-type substrate GPNA is applied, uterine flushings from pregnant uteri show one single band just cathodically from β-glycoprotein (Figs. 3, 4). This seems to be the same fraction which sporadically gives a weak reaction with BANA, but staining is much stronger with GPNA. The reaction is much more intense at pH 8.0 than at pH 6.0. This activity was not clearly detectable in flushings from the blastocyst-free uterine horns studied. Blastocyst tissue and blastocyst coverings material show only two very faint bands, one close to the start point and the other one in the β-glycoprotein area (or just cathodically of the later), but no GPNA-splitting activity is detected in the gelatinolytically active fractions.

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**Fig. 4.** Schematic summary of micro disc electrophoresis zymograms showing amidase activity of rabbit blastocyst and uterine material 6½ d p.c. Uterine flushings represented here are taken from blastocyst-containing uteri.
Discussion

Previous studies have shown that implantation-associated gelatinolytic proteinase activity is found, in the rabbit, almost exclusively at the interface between attaching trophoblast and uterine epithelium, mainly concentrated in the disintegrating blastocyst coverings, if sections of blastocysts and uteri were assayed using a histochemical substrate film test [5 to 13, 18]. Extensive studies of interaction with active site-directed specific proteinase inhibitors, performed with the same test, indicate that the active center of the enzyme(s) is very closely related to that of trypsin [12, 13]. A certain degree of inhibition by chymotrypsin-specific inhibitors was also noted but was assumed to result from accompanying proteinases with chymotrypsin-like activity possibly present at the same histologic sites. In fact, the experiments described in this communication demonstrate the presence of GPNA-splitting, chymotrypsin-like enzyme activity in the studied material, although more prominent in uterine secretion than in trophoblast or blastocyst coverings. These enzyme fractions are clearly separable electrophoretically from the main gelatin dissolving activity and they will not be discussed in detail here.

As pointed out before, rabbit implantation stage trophoblast shows only little gelatinolytic proteinase activity if tissue sections are essayed using the gelatin substrate film technique, while high activity is found at the interface between trophoblast and uterine epithelium and in disintegrating blastocyst coverings [8 to 15, 18]. In the present investigations, on contrast, it is shown that the trophoblast does possess high gelatinolytic proteinase activity which becomes demonstrable after electrophoresis. The enzyme seems to be identical with the one found in disintegrating blastocyst coverings. Both exhibit identical electrophoretic mobility and high gelatinolytic activity while hydrolysis of the synthetic trypsin-type substrate BANA is barely detectable and GPNA is not being split at all.

The gelatin dissolving proteinase of uterine secretion which is demonstrable by the same method, on the other hand, seems to differ in electrophoretic mobility, although these differences are only slight in micro disc electrophoresis. Considerable differences in electrophoretic mobility are observed, however, in agar gel electrophoresis [13, 18]. A comparison of BANA and gelatin zymograms as done in the present study reveals considerable BANA-splitting activity in the gelatin dissolving fraction of uterine flushings. We conclude that the uterine secretion of rabbit uteri contains, around the time of implantation, a remarkable activity of a gelatin and BANA hydrolyzing proteinase with a pH optimum around 8.0. It therefore resembles trypsin as far as its catalytic properties are concerned. Studies of interaction with specific proteinase inhibitors (not communicated here) confirm that its active site is very similar to that of trypsin. Interestingly, we found more activity in normally pregnant (blastocysts present) than in blastocyst-free uteri. This observation requires confirmation through investigation of a larger series of uteri. The possibility that blastocyst-dependent activation of a proenzyme might be involved will be investigated further. Based on PAA disc electrophoretic experiments in the usual macro scale, rabbit uterine secretion proteinase had previously been assumed to be identical with β-glycoprotein [18, 19]. To us it seems improbable, however, that an enzyme is present in secretions in as large quantities as β-glycoprotein which represents one of the predominant protein fractions of late preimplantation rabbit uterine secretion. In addition, the present study shows that under the conditions of micro disc electrophoresis with its higher sieve effects (due to a concentration of PAA of 20 % as compared to 6 to 7.5 % in macro
disc electrophoresis) and with a zymogram technique which allows better resolution, the proteinase activity can clearly be separated from β-glycoprotein.

It seems interesting that the actively gelatin-hydrolyzing proteinase of trophoblast and blastocyst coverings, although also closely related to trypsin according to inhibition studies (see above), does not markedly split the synthetic trypsin substrate BANA. A series of other synthetic low molecular weight trypsin substrates as well as casein have been tried in biochemical test systems but were likewise found to be poor substrates for this peculiar enzyme [12, 13]. This proteinase therefore seems to have more narrow substrate specificity than trypsin and the trypsin-like proteinase from uterine secretion (see above). As far as the physiological significance is concerned, this blastocyst-derived enzyme might be involved in weakening of the blastocyst coverings by partial hydrolysis rather than in complete breakdown, or it might act as an activator of a uterine secretion proteinase proenzyme. The trypsin-like uterine secretion proteinase is more likely to be responsible for complete hydrolysis of remnants of the blastocyst coverings to form small diffusible peptides as trypsin does [13].

For the discussion of the probable ways of physiological regulation of blastocyst proteinase it seems interesting that the trophoblast material exhibits its high activity only after electrophoresis (see above). This might reflect the presence, in trophoblast, of proteinase inhibitors separable from the enzyme by electrophoresis under the used conditions, or it might be due to activation of a proenzyme present in the trophoblast. Both assumptions are consistent with the hypothesis of a dependence of this enzyme on the trophoblast, as also suggested by a number of physiological experiments [9, 10, 15]. Recently evidence has been presented for trypsin inhibitor activity of uteroglobin, the progesterone-dependent major protein fraction of preimplantation uterine secretion in the rabbit [2, 17]. Our observations described in this paper give a first evidence that uteroglobin might in fact inhibit uterine secretion proteinases (observations made on unsectioned gels, see above). The system of proteinases and proteinase inhibitors found at the interface between attaching trophoblast and uterine epithelium, also present in other species [1, 3, 4, 13, 21, 25], seems to be of great relevance for establishment of cellular contact between embryo and mother, and might be in some way comparable to proteinase systems recently assumed to be involved in cell contact formation and invasive growth of tumor cells [22].

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References


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