Role of Proteinases in Implantation

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Introduction

During the last years, morphological, biochemical and experimental studies of the implantation process have provided increasing evidence that proteinases are involved in the initiation phase when contact is made between trophoblast and uterine tissues. Most of these studies were performed in the rabbit and the mouse [9–11, 13–15, 17–20, 25, 28, 30, 32, 38, 42]. Coincidentally, recent cell biological and oncological studies performed in other systems have provided ample data which indicate that the action of certain proteinases on cell surfaces has profound effects on various cell biological phenomena like cell adhesion, migration, secretion of other enzymes, and they can cause metabolic changes or mitogenic activation [39, 40, 45]. Such phenomena have most frequently been demonstrated for trypsin, but can interestingly also be elicited by highly specific endopeptidases causing limited proteolysis, like thrombin [8, 33].

Naturally, observations like these give strong impact to studies of the role of proteinases in implantation. In fact, the hypothesis that proteinase action is not only somehow related to but essential for implantation initiation, as postulated on the basis of studies of a more analytical type [1, 10–14, 18, 23, 26, 29, 32, 38] has received strong support from experiments on the influence of specific proteinase inhibitors on implantation: after administration in vivo, certain proteinase inhibitors can very

1 Dedicated to Professor Dr. Bent G. Böving, whose work stimulated me to study the physiology of implantation, in honor of his 60th birthday.
effectively prevent the attachment of the blastocyst in the uterus [19] (see also below). Furthermore, there is evidence that the physiological regulation of initiation of implantation as governed by maternal hormones may at least in part be mediated by changes in protease and/or protease inhibitor activity of the uterine tissues and the trophoblast.

_Trophoblast-Dependent Blastocyst Proteinase (Blastoemmase): Occurrence and Physiological Function_

A peculiar gelatin-dissolving proteinase is found in the trophoblast and at the surface of implanting rabbit blastocysts. In fact, this enzyme seems to be of great importance for initiation of implantation, as was already assumed at the time of its first description [11] and as could be substantiated by a number of investigations during the following years [for review, cf. 19]. In particular it seems to be involved in dissolution of the extracellular blastocyst coverings and it is therefore named 'blastoemmase'.

When the highly sensitive gelatin substrate film test is applied which was designed and optimized for this enzyme [12, 16], blastoemmase activity is nondetectable before 6 days post coitum (p. c.) (sometimes traces are found at 5–6 days p. c.), but rises abruptly at 6½ days p. c., i.e. few hours before the dissolution of the blastocyst coverings begins. At 7 to 7½ days p. c. when the lysis of the coverings is under way and the trophoblast attaches to the uterine epithelium [7, 13], the proteinase activity is restricted to the area in which these processes are going on, i.e. the abembryonic hemisphere of the blastocyst (fig. 1). In contrast, the embryonic pole where the blastocyst coverings remain still intact and where no attachment takes place in this phase does not show any activity worth of mentioning. After completion of the abembryonic dissolution of the coverings and attachment of the trophoblast, the enzyme activity disappears abruptly so that only minor remnants of activity can be traced irregularly in some places between trophoblast and uterine epithelium from 8 days p. c. on.

Some controversy has developed on the question whether this proteinase derives from the trophoblast as suggested by the described observation, or from the uterine secretion as proposed by Kirchner [28, 29]. There is much evidence, however, that this proteinase at least depends in some way on the abembryonic trophoblast: the maximum of enzyme activity and the beginning of the dissolution of the blastocyst coverings are
Fig. 1. Inhibition of blastolemmsae activity and of implantation by administration of proteinase inhibitors in vivo in the rabbit. Inhibitors were injected into the uterine lumen at $6^{1/2}$ days p.c. [for details, cf. 19], and animals were sacrificed at $7^{1/2}$ days p.c. Cryostat sections through blastocyst sites, gelatin substrate film test for demonstration of proteinase activity, incubation time 105 min. a After administration of 6 mg of ε-aminocaproic acid (which does not inhibit blastolemmsae), a completely normal situation is found which corresponds perfectly to the controls. Three trophoblastic knobs are seen in this segment of the abembryonic-antimesometrial region. Blastolemmsae activity (bright lysis zones between trophoblast and uterine epithelium) is normal, the blastocyst coverings have been dissolved, and the trophoblastic knobs are establishing contact with the uterine epithelium. ×130. b After administration of 6 mg of aprotinin (Trasylol), blastolemmsae activity is completely inhibited, the blastocyst coverings (dark band-like structure) are still intact and the trophoblastic knobs (two visible) have not been able to attach to the uterine epithelium. ×210.
always observed at the abembryonic pole of the blastocyst, even if it is abnormally oriented in the uterus, i.e. facing the mesometrial instead of the antimesometrial endometrium (maloriented blastocysts) [17, 19, 20]. Models of blastocyst coverings without trophoblast do not gain any comparable proteinase activity and are not dissolved in the 7- to 8-day uterus [25]. It became very clear from these investigations that the trophoblast has to provide a factor (or factors) which is (are) essential for the development of this proteinase activity. So far it is not completely clear, however, whether this factor is an activator for a uterus-derived proteinase or whether it is the gelatinolytic enzyme (or proenzyme) itself. The latter assumption is probably correct because it was possible to demonstrate very high gelatinolytic proteinase activity of the same electrophoretic mobility in both the trophoblast and the disintegrating blastocyst coverings of the rabbit, while uterine secretion proteinase appears to be a different entity [26]. Recently it became possible to study proteinase activity in trophoblast homogenates using novel chromogenic tri- or tetrapeptide substrates and to define the biochemical properties of the trophoblastic enzyme(s) more precisely [23] (see also below).

The main physiological function of blastolemmanse seems to be connected with the dissolution of the blastocyst coverings. One may be tempted, therefore, to compare it with hatching enzymes of lower animals [19]. According to its biochemical properties (particularly its substrate specificity) which will be discussed below, it is probably not responsible for complete digestion of the coverings but for softening them due to hydrolysis of just a few peptide bonds. Such a limited hydrolysis might also change cell surface properties and elicit certain cellular responses (see Introduction) forming part of the attachment process.

The role in the dissolution of the blastocyst coverings is most clearly demonstrated by in vivo inhibition experiments [19, 20]. Specific proteinase inhibitors which had previously been shown to inhibit blastolemmanse strongly in vitro [18], i.e. aprotinin (Trasylol), antipain, p-nitrophenyl-p′-guanidinobenzoate, boar seminal plasma trypsin-αcrsin inhibitor, were administered into the uterus of rabbits half a day before implantation (i.e. at 6½ days p.c.). Detailed morphological and biochemical studies showed that after this treatment, blastolemmanse activity is nondetectable or greatly reduced, and the dissolution of the blastocyst coverings is very effectively inhibited (fig. 1–4). Since the coverings remain as a barrier between the trophoblast and the uterine epithelium, the formation of a cellular contact is not possible. Instead, the blastocyst re-
Fig. 2. Inhibition of dissolution of the blastocyst coverings and of implantation in the rabbit by intrauterine administration of 6 mg of aprotinin at 6½ days p. c. Electron micrograph. ×16,150. Stage shown is 8½ days p. c. The blastocyst coverings (BC) are still interposed between trophoblast (T) and uterine epithelium (U) thus preventing formation of a cellular contact. Hemidesmosome-like structures are found at the surface of the uterine epithelial symplasma facing the nondissolved blastocyst coverings.
Fig. 3. Inhibition of blastocyst implantation in the rabbit by intrauterine administration of 6 mg of aprotinin at 6½ days p. c. Cryostat section through blastocyst site, 9½ days p. c., gelatin substrate film test for demonstration of proteinase activity, incubation time 105 min. ×9. Even 2½ days after implantation has started in the control the blastocyst lies still free in the inhibitor-treated uterine lumen. The nondissolved blastocyst coverings are mechanically ruptured due to continued expansion of the blastocyst (remnants seen at lower right side). No proteinase activity except for scattered endometrial stroma cells. Protein-rich uterine fluid surrounds the blastocyst, whereas the blastocyst fluid contains very little protein precipitates.

mains free in the uterine lumen. Expansion continues, although at a reduced rate. Due to this continued expansion, the blastocyst coverings are finally ruptured mechanically instead of being dissolved enzymatically. This process bears some resemblance to the hatching from the zona pellucida as seen in the mouse under certain hormonal conditions and in vitro. Due to this rupturing, the trophoblast can come into contact with the uterine epithelium in some places. Nevertheless, interestingly, attachment and fusion occurs only focally, i.e. many trophoblastic knobs remain unattached. This may be due to the fact that the trophoblast is now 'out of
Fig. 4. Inhibition of blastocyst implantation in the rabbit by intrauterine administration of 0.6 mg of p-nitrophenyl-p'-guanidinobenzoate (NPGB) at 6 1/2 days p.c. Cryostat section through blastocyst site, 7 1/2 days p.c., gelatin substrate film test for demonstration of proteinase activity, incubation time 105 min. × 60. The non-dissolved blastocyst coverings are clearly seen as a darkly stained band. No proteinase activity except for two endometrial stroma cells. In contrast to aprotinin (TrasyloL), the synthetic inhibitor NPGB shows toxic effects, causing degeneration of blastocyst tissues. Other blastocysts were found to survive NPGB action and to be able to recover after decline of intrauterine inhibitor activity, and to implant normally [19].
phase' with the uterine epithelium which has in the meantime been transformed into a broad symplasma.

In the described experiments, the intrauterine inhibitor concentration had already dropped down considerably at the time at which this delayed focal attachment was observed, due to the fact that only one injection of inhibitor was applied. We are at present investigating whether trophoblast attachment can be completely prevented when the inhibitor concentration is kept high. This would indicate that proteinase action is necessary not only for the dissolution of the blastocyst coverings but also directly involved in the formation of a cellular contact.

Certain differences in the efficacy of the various inhibitors employed were observed and could in part be correlated with differences in elimination rates [19]. The most effective inhibitor in the tested series was aprotnin (Trasylol). On the other hand, ε-aminocaproic acid, an inhibitor of plasminogen activation which does not alter blastolemase activity in vitro, was administered as a control. It was found to be without any effect on the dissolution of the blastocyst coverings and on attachment of the trophoblast even when administered in 100 times higher molar doses than those proven to be effective for aprotnin. This suggests that plasminogen activators and plasmin probably do not play a major role in the initiation phase of implantation. This does not exclude that this system might be involved in the later process of stromal penetration.

In fact, plasminogen activator activity was described to increase in mouse trophoblast under in vitro culture conditions at a phase equivalent to implantation, and the maximum was found at the stage equivalent to the phase shortly after epithelial penetration [42]. However, using fresh, noncultured material from the rat, Liedholm and Åstedt [31] observed a decrease of plasminogen activator activity towards implantation in both the embryos and the endometrium. Likewise, no particularly high plasminogen activator activity was detected in rabbit implantation sites, using a comparable technique [Denker, unpublished].

Apart from the rabbit, proteinase inhibitors have been administered into the uterus during pregnancy also in the mouse, and an antifertility effect was noted [9]. Although no analysis of implantation and early post-implantation stages was carried out in this case, the authors assumed that it was the implantation process which was disturbed also in this species. Recently, it was reported that the attachment of mouse blastocysts to cell monolayers in vitro may involve the action of proteinases because addition of soybean trypsin inhibitor inhibited and trypsin stimulated this process [30].
<table>
<thead>
<tr>
<th>Substrates</th>
<th>Gelatin; certain obligopeptides with arginyln bonds (no measurable hydrolysis of: low molecular weight trypsin substrates like BANA etc., or comparable chymotrypsin or elastase substrates, or casein or fibrin)</th>
</tr>
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<tbody>
<tr>
<td>Inhibitors</td>
<td>Aprotinin (Trasylol®), SBTI, antipain, leupeptin, pancreatic secretory trypsin inhibitor (Kazal), α1-antitrypsin, p-nitrophenyl-p’-guanidinobenzoate, etc. (not inhibited by EDTA, iodoacetamide or ε-aminocaproic acid)</td>
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<tr>
<td>pH optimum</td>
<td>pH 8.0–8.5 (substrate: gelatin)                                                                                           pH 8.5 (substrate: Tosyl-Gly-Pro-Arg-p-nitroanilide)</td>
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<td>Active center</td>
<td>related to that of trypsin, but more restricted substrate specificity</td>
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**Biochemical Properties**

Until very recently, investigations of rabbit blastocyst proteinases were generally based on gelatin substrate film tests, particularly on one version which had been specifically designed and optimized for this purpose [12, 16]. Although previously the lack of a kinetic assay was a severe obstacle for detailed biochemical investigations, many interesting data could, nevertheless, already be collected. Experiments on the inhibition of rabbit **trophoblast-dependent blastocyst proteinase (blastolemmase)** by specific proteinase inhibitors *in vitro* [18] revealed that the *active center* of this enzyme is closely related to that of trypsin (table I). However, its *substrate specificity* is much more restricted: the classical chromogenic low molecular weight trypsin substrates like benzoyl-arginine-β-naphthylamide or benzoyl-arginine ethyl ester are not hydrolyzed at any measurable rates. The pH optimum lies in the alkaline range (around pH 8.0–8.5 with the gelatin substrate film test [cf. 19]).

Recently, these findings could be substantiated and more accurate data were obtained using quantitative biochemical assays based on the hydrolysis of novel tri- and tetrapeptide-p-nitroanilide substrates [23]. These investigations provided evidence that the proteinase(s) present in rabbit trophoblast homogenates is (are) highly specific insofar as in addition to a specificity for arginyln bonds (P1), the amino acids in the neighboring positions P2 and P3 are also recognized. In fact, substrate specificity
is quite comparable to that of highly specific enzymes like thrombin and the kallikreins. This suggests that trophoblast proteinase(s) may hydrolyse only few peptide bonds in the physiological substrates, as typical for enzymes which serve a more regulative than a digestive function. Although in addition to blastolemmase, at least one other highly specific endopeptidase is present in rabbit trophoblast extracts, there is evidence that predominantly blastolemmase was measured in these assays [21]. Thus is appears justified to assume that the physiological role of this enzyme is not to cause complete digestion of the blastocyst coverings but to soften them. It remains to be investigated whether these trophoblast-derived proteinases might also be involved in other, more regulative functions like introducing changes in cell adhesion and in invasive potential, proenzyme activation or liberation of biologically active peptides.

In rabbit implantation stage uterine secretion, a trypsin-like enzyme is found which appears to resemble trypsin more closely than blastolemmase: it is highly basic [19, 28] and shows detectable benzoyl-arginine-\(\beta\)-naphthylamide-splitting activity [26]. It is, like the trophoblast-dependent enzyme, being inhibited by the specific high molecular weight trypsin inhibitors. Some first evidence was found that trypsin-inhibiting activity found in the uteroglobin fraction [4] is directed also against this enzyme [26]. Problems of purity of this fraction are discussed elsewhere [21]. Furthermore, blood plasma inhibitors (like \(a_1\)-antitrypsin, \(a_1\)-antichymotrypsin, inter-\(a\)-trypsin inhibitor, \(a_2\)-macroglobulin) were shown to be present in human endometrium and uterine secretion [41, 43]. Some of them were found to be able to interact with rabbit blastocyst proteinase (blastolemmase) [21]. It appears, therefore, reasonable to assume that, in vivo, the activity of blastocyst-derived and of uterine secretion proteinases is strictly controlled by such inhibitors. Inhibitor activity in the uterine secretion, in turn, was shown to be hormonally regulated [3].

Minor activities of chymotrypsin-like enzymes have also been detected in rabbit uterine secretion and blastocysts [26] but have not been studied in detail so far.

As far as other species are concerned, some comparable studies have only been performed in the mouse. In mouse uterine secretion, a zona pellucida – dissolving activity was reported to increase before implantation ('implantation initiating factor' [32]) and was attributed to a casein-splitting endopeptidase showing parallel changes in activity [38]. Several trypsin-like (BANA splitting) and chymotrypsin-like (GPNA splitting) as well as casein and hemoglobin hydrolyzing enzymes have been detected
biochemically in blastocysts and uterine flushings of this species [1, 9]. Since only insignificant endopeptidase activity could be demonstrated in implanting mouse blastocysts using histochemical substrate film tests [5; Denker, unpublished] it was assumed that uterine secretion proteinase activity is physiologically more significant in this species [32]. Species differences were also emphasized by Blandau [6] who found gelatinolytic activity in late preimplantation blastocysts of the guinea pig (which show more invasive properties) but not in those of the rat (where the trophoblast seems to be less invasive while the uterine epithelium degenerates more readily). The physiological function of the biochemically detected proteases of mouse blastocysts remains obscure in this context in spite of observations on an increase in activity of some of these enzymes towards implantation [1, 9].

Only histochemical investigations have been performed so far in the guinea pig and the cat. In the cat, considerable gelatinolytic activity is found in late preimplantation trophoblast and increases towards implantation. It is particularly high in the girdle-shaped invasion zone at 14 days p. c. where it is located in the trophoblast, between it and the uterine epithelium and in the widened endometrial crypts [22]. Experiments on the pH dependence and on inhibition by various inhibitors revealed that this activity is predominantly due to an enzyme which is totally different from rabbit blastolemmase, namely to an SH-dependent cathepsin with an acid pH optimum. It is in many respects similar to cathepsin B, but since no BANA-splitting activity could be demonstrated so far it may also be related to cathepsin L or the 'collagenolytic cathepsin' [2], which is possibly also true for rabbit endodermal cathepsin [19]. In the guinea pig, only limited experiments on the pH dependence and on inhibition by various inhibitors have been performed on postimplantation stages [34 to 37]. The obtained results suggest that here, too, the predominant proteolytic activity is due to a cathepsin which might be of the same type. According to the acid pH optimum, an intracellular (intralysosomal) site of action has to be considered for cathepsins, thus differing from the assumed site of action of rabbit blastolemmase (i.e. at the interface between trophoblast and endometrium where an alkaline pH is measured). In both the cat and the guinea pig, however, available data do not exclude the presence of, in addition, a trypsin-like enzyme comparable to blastolemmase particularly in preattachment and attachment stages where it might be involved in the dissolution of the zona pellucida [19]. This point will merit further investigation.
Conclusion

The inhibition of implantation by administration of specific proteinase inhibitors may be considered a new concept possibly applicable for fertility regulation. More extended studies of this aspect including various synthetic low molecular weight inhibitors are in progress in our laboratory. Possible side effects, as increased incidence of dystopic implantation, and ethical aspects should, however, be taken into consideration [19]. It should, furthermore, be kept in mind that whenever proteinase inhibitors are being administered into the uterus as in attempts to prevent increased menstrual blood loss associated with IUD use [44, 46], implantation of blastocysts may be disturbed at the same time.

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