Implantation of the blastocyst in the uterus is not only of fundamental importance for the establishment of pregnancy, but it is also a biologic phenomenon of tremendous fascination. Recent progress in implantation research appears to open some ways on how to manipulate this process, particularly during its initial phase.

For heuristic reasons, it seems appropriate to use a narrow definition of implantation, i.e., the specific process that leads to the formation of a specialized, intimate cellular contact between the trophoblast of the embryo (usually in the blastocyst stage) and the endometrium. In case of ectopic implantation, the endometrium may be replaced by other tissues.

Such a narrow definition is, however, not always used, which is unfortunate for the discussion of mechanisms involved. Much of the literature on “anti-implantation agents” is based on experiments in which, after treatment of female animals at any point during the preimplantation phase, uterine swellings (so-called “implantation sites”) are counted at a relatively late stage, i.e., a number of days after initial contact formation between the trophoblast and the endometrium. Certainly, many complex tissue interactions and considerable remodeling processes are required until a mature placenta is established. It is usually impossible, in the described type of experiments, to decide which individual process was influenced by the drug treatment. Systematic morphologic investigations of all successive stages, combined with biochemical studies of compounds suggested to be related to implantation mechanisms, are rarely being carried out. This may be one reason why, although there is no lack of hypotheses on implantation mechanisms, concepts are highly controversial and incongruent, focusing either on regulation by ovarian (or trophoblast-derived) hormones, or on changes of cell surface characteristics, or on uterine motility, or decidualization, or vascular changes in the endometrium, including their regulation by prostaglandins and histamine, for example. According to the specialization of the investigator and depending on the type of
experiment preferred and on the stage chosen for investigation, any of these aspects may appear to be of prominent importance for "implantation."

We would like to concentrate, in our discussion, on the initial phase of implantation, i.e., on mechanisms involved in the formation of the first intimate and specialized contact between trophoblast and uterine epithelium, with subsequent invasion. We will omit a discussion of other important aspects of implantation such as vascular reactions and decidual reaction, the hormonal regulation, the role of histamine and prostaglandins, topics that have been covered in a number of reviews.9, 28, 36, 51, 69, 87, 108

Recent experiments, using predominantly the rabbit and the mouse as models, suggest a central function for certain highly specific proteinases, in the initiation phase of implantation. They should be of interest to the gynecologist since proteinase inhibitors are being proposed and tried as additives to IUDs in order to reduce menstrual blood loss.130, 131, 142 These inhibitors are in part identical with those found highly effective in interfering with implantation initiation, in the animal models.25, 27 It seems necessary, therefore, to consider the possibility that proteinase inhibitors released from IUDs may have additional effects, i.e., interfere with implantation of the blastocyst, also in the human.

EXPERIMENTAL EVIDENCE FOR A SPECIFIC ROLE OF CERTAIN PROTEINASES IN IMPLANTATION INITIATION

It is a very old concept that degradation of maternal tissue components by factors released from the trophoblast may play an important role in implantation. In fact, in 1883, Graf von Spee,126, 127 studying implantation in the guinea pig which is of the interstitial type as in the human, concluded on morphologic grounds that the trophoblast plays an active and cytolytic role, the endometrium showing signs of degeneration and disintegration. Although he apparently thought of an indirect influence by the embryo, suggesting a "biochemical process which is stimulated by the egg," his investigations initiated active search for degradative enzymes released from the trophoblast, particularly proteinases, during the following decades (discussed in Denker25). In fact, proteinases and protease inhibitors were found in both the trophoblast and the endometrium in the human shortly after the turn of the century. However, the simplifying concept that the invading trophoblast forces its way by digesting away components of the maternal tissues was later abandoned for being too mechanistic. It was nearly forgotten after the hormonal regulation of reproductive functions came into the center of interest.

In the light of recent experimental data, a role of hydrolytic enzymes in implantation must be reconsidered, although in a modified concept. Proteinases are certainly involved, but only part of their function, and possibly not even the physiologically most significant one, may be complete hydrolysis of maternal proteins to small peptides and amino acids as caused by cathepsins. Most members of this latter class have an acid pH optimum and are typical lysosomal enzymes. Some of them have been found to be in fact very active at implantation sites: Cathepsin B-type enzymes showing impressive activity in the cat,30 the pig (Denker and Heap, unpublished) and the marsupial, the tammar wallaby;35 a proteinase found in the guinea pig embryo may be related to it.102
Cathepsin D was found to show increased activity in total endometrial homogenates but decreased concentration in the uterine epithelium, at implantation sites of the rat. 93, 144

Particularly interesting are, on the other hand, certain alkaline or neutral proteinases which may act extracellularly and for which experimental data suggest a special role in implantation initiation. According to biochemical studies, some of them may cause hydrolysis of only a few peptide bonds in their physiologic substrates, a process known as limited proteolysis. This interesting process can induce subtle but physiologically very significant changes in the substrate proteins, as demonstrated in a number of other systems: Certain highly specific proteinases are activators of proenzymes, some of them initiating complex chains of biochemical events involving mutual activation of various enzymes (examples: the blood clotting and the complement system) and liberating biologically active peptides (kallikrein-kinin system). When acting on cell surfaces, proteinases can elicit a great variety of cellular responses, e.g., changes in adhesive behavior, cell migration, secretion of proteins (e.g., fibronectin)92 or of other enzymes, and they can cause metabolic changes or mitogenic activation;54 (for additional references, see Denker27) and even trigger cell differentiation.101 Enzymes which show these effects include highly specific proteinases like thrombin, causing limited proteolysis. Even the degradation of extracellular ground substance macromolecules, as part of the process of invasion, seems to involve primarily only partial degradation to high-molecular-weight products.73, 83

In fact, the implantation-associated proteinase which has been studied in most detail so far, blastolemmsme, shows biochemical properties that are in accordance with such a concept, i.e., suggesting that the enzyme action is highly specific, cleaving only a limited number of peptide bonds in its physiologic substrates. The enzyme which can be extracted from the trophoblast of late preimplantation stage rabbit blastocysts exhibits not only a primary specificity for arginyl bonds (P1) but also recognizes the adjacent amino acids in the neighboring positions P2 and P3, so that it can be expected to cleave only some of the arginyl bonds in a protein.31

Concerning the question about the cell biologic mode of action of proteinases in implantation initiation, most available information was obtained in experiments using the rabbit as a model, and some data have been obtained from the mouse.25, 27, 28 The rabbit offers the advantage of having a greatly expanded blastocyst, around 5 mm in diameter at implantation, which facilitates analysis. In addition, due to its copulation-induced ovulation, developmental stages are defined precisely. It has, therefore, been chosen as the standard model in our laboratory, although it does show certain peculiarities mostly connected with its representing the central type of implantation. The problem posed by species differences will be discussed more in detail.

The most interesting proteinase operative in implantation initiation in the rabbit is the trophoblast-dependent "blastolemmsme."21, 26, 25, 27, 31 With use of a gelatin substrate film test, the enzyme is found at the interface between trophoblast and uterine epithelium precisely during the phase of attachment of the abembryonic trophoblast, which is the beginning of implantation in this species. Noticeable blastolemmsme activity is, therefore, demonstrable only for about one day, i.e., between 6 3/4 and 7 3/4 days post coitum (days p.c.).

Two major physiologic functions have been envisaged for blastolemmsme: (1) in the process of dissolution of the so-called blastocyst coverings, and (2) in the attachment of the trophoblast to the uterine epithelium.
There is excellent experimental evidence for a central role of blastolemmase in the first-mentioned process, the dissolution of the blastocyst coverings. These are extracellular coatings composed principally of glycoproteins and equivalent to the zona pellucida, although in our model, the rabbit, they are of a more complex structure than, for example, in the human: when implantation is being initiated, they consist of 3 layers of different origin and composition (for more details, see below). For implantation, they need to be removed in order to bring the trophoblast into contact with the uterine epithelium. Blastolemmase is of central importance in this process, as shown impressively in experiments in which effective but nontoxic proteinase inhibitors (e.g., aprotinin = Trasylol®, antipain, soybean trypsin inhibitor, and others) were administered within the uterus in vivo (Denker25 and unpublished results). The major effects seen were the same with all blastolemmase inhibitors although minor differences were noted.

Due to this treatment, dissolution of the blastocyst coverings does not take place, and they remain interposed between trophoblast and uterine epithelium so that formation of a cellular contact is impossible (Figs. 1A, B; 2). Therefore, blastocysts stay free in the uterine lumen.

An important detail of these experiments is the observation that the blastocysts remain viable for a number of days even though implantation was blocked. We like to put emphasis on this point because, as a rule, other investigations of “anti-implantation” agents have usually not ruled out the possibility that these drugs were perhaps simply causing degeneration of the embryos, which makes it impossible to derive any conclusions on specific cell biologic processes essential for implantation. In the experiments with inhibitors administered within the uterus the blastocysts continue to expand (although at a slightly reduced rate) in spite of their failure to implant. Electron micrographs have not given evidence for any major toxic damage to the trophoblast.25 As a result of continued expansion, most of the blastocysts finally hatch mechanically from their coverings, although a proportion of them remains completely encased (particularly in experiments in which the inhibitor is being infused continuously into the uterine lumen). The fragments of the coverings typically remain sharp-edged, indicating absence of any lytic (blastolemmase) activity. In those blastocysts in which mechanical hatching occurs, the trophoblast can, of course, contact the uterine epithelium, although delayed (1-2 days). Interestingly, not all of the exposed parts of the trophoblast manage to attach under these conditions; the contact which is being formed mostly remains superficial (Fig. 2B) and invasion and erosion of maternal blood vessels are found only in few spots. Although most of the blastocysts in inhibitor-treated uteri are still nicely expanded around 2 1/2 days after the time when implantation has started in the controls (Fig. 3), they are finally being progressively resorbed

In the initial series of these experiments, a single bolus of inhibitor had been injected into the uterine lumen of rabbits at 6 1/2 days post coitum, i.e., half a day before implantation initiation.25 Since the intrauterine inhibitor concentration was found to decline rapidly, a more recent series used continuous infusion into the uterine lumen via Alzet® osmotic minipumps loaded with aprotinin (Trasylol®) and placed within the uterus at 5 1/2 days post coitum when midblastocysts are present.90 Saline-loaded minipumps were used as controls placed in the contralateral uterus. In a sense, the minipumps serve as a model for medicated IUDs. The dimensions of the model used (Alzet® model No. 1701, now replaced by a larger model) easily allow intrauterine in-
Fig. 1. Inhibition of implantation in the rabbit by intrauterine administration of a proteinase inhibitor. Stage shown is 7 1/2 days p.c. A. Control, Araldite section, toluidine blue stain. x 900. Antimesometrial endometrium and part of the abembryonic hemisphere of the blastocyst (above) are seen. A syncytial "trophoblastic knob" (T) has established contact with the endometrium, has invaded it and already reached the subepithelial capillaries. No remnants of blastocyst coverings are present anymore. B. As in A, except that 6 mg of aprotinin (Trasylol®) had been injected into the uterine lumen one day before. Due to inhibition of blastolemmase activity, the extracellular blastocyst coverings (BC) have not been dissolved and remain interposed between the trophoblastic knob (T) and the uterine epithelium.
Fig. 2. Inhibition of implantation in the rabbit by intrauterine administration of a proteinase inhibitor. Stage shown is 8 1/2 days p.c. A. 6 mg of aprotinin (Trasylol®) had been injected into the uterine lumen at 6 1/2 days p.c. Araldite section, toluidine blue stain. x 350. In this segment of the abembryonic part of a blastocyst (above), two syncytial trophoblastic knobs (T) can be seen. Although in the controls they would have invaded the endometrium and a hemochorial contact would have been established by this stage, these knobs are still separated from the uterine epithelium (transformed into a broad symplasma) by the blastocyst coverings (dark line), which have been stretched due to continuing expansion of the blastocyst. B. As in A, another blastocyst from the same animal as shown there. In spite of inhibition of dissolution of the blastocyst coverings, many blastocysts finally manage to hatch mechanically due to their continuing expansion. At the site shown here, no remnants of blastocyst coverings are present anymore. Nevertheless, the trophoblastic knob (T) has not attached to the uterine epithelium and only a small portion of the cytotrophoblast has. Here, a trophoblastic giant cell (GC) has formed. There is no sign of deep invasion.
Fig. 3. The two uteri of a rabbit excised at 9 1/2 days p.c. after 12 mg of aprotinin (Trasylol®) had been injected into the lumen of the left uterus at 6 1/2 days p.c. The right uterus had received the same amount of vehicle fluid as a control. Since the proteinase inhibitor does not cause immediate degeneration of the blastocysts but only interferes with the initiation of their implantation, the two blastocysts at the left side have not degenerated yet but have continued to expand, although at a slower rate than the five conceptuses at the control side (right). (No. of corpora lutea: left side 2, right side 6).

Insertion in the rabbit, and, as the controls reveal, there is only a minor nonspecific mechanical IUD effect (leukocytosis, although variable, unusually remaining slight, and the nonspecific antifertility effect remaining negligible) because this pump occupies only a small part of the length of the uterus, and it distends the uterus only slightly. (Fertility depressing effects of nonmedicated IUDs have been found to be restricted to the endometrial area in contact with the device.) The results of the experiments observed at various stages between 7 1/2 and 11 1/2 days p.c. confirm the main results obtained with single intrauterine injections of the inhibitor: Dissolution of the blastocyst coverings is strongly inhibited whereas the blastocysts survive and continue to expand. Degeneration of the embryoblast is seen more rarely, after continuous infusion of the inhibitor than after a single intrauterine bolus injection. In fact, some embryos were found, in the minipump experiments, beautifully differentiated showing neural tube formation and somites, in spite of the fact that they were still completely encased in their blastocyst coverings, i.e., they were totally nonimplanted and free in the uterine lumen. Such a condition had, to our knowledge, never been seen before in a eutherian mammal except for the horse (Betteridge, personal communication) but was known from marsupials (see, e.g., Renfree).

About 25 percent of the embryos remained completely encased in the coverings,
after continuous intrauterine aprotinin infusion, whereas the others managed to hatch mechanically. It seemed interesting to note whether the trophoblast would be able to attach and to invade into the endometrium, in spite of the presence of the proteinase inhibitor. Some of the exposed trophoblastic knobs of the abembryonic hemisphere were indeed found to invade and to erode maternal blood vessels. Many of them did not, however, even if no remnants of blastocyst coverings were interposed between them and the endometrium any longer. This does not necessarily prove that aprotinin-sensitive proteinases are involved in attachment and invasion; it can, alternatively, be explained using a concept proposed by Böving, i.e., that there is only a small time window for invasion of trophoblastic knobs into the endometrium, the end of the susceptibility of the endometrium being determined by the time when uterine epithelial cells fuse and form a broad symplasma after 7–7 1/2 days p.c. After that time, directional invasion of trophoblastic knobs aiming at subepithelial blood vessels may be impossible (because there is no longer any channeled transport of metabolites). In fact, fusion of trophoblast with the uterine epithelium seems basically to be possible, in the inhibitor-treated uteri, but deep invasion is rare in the abembryonic-antimesometrial region.

Implantation consists of two different phases in the rabbit. The phenomena discussed above are part of the first phase of implantation, which takes place in the abembryonic region of the blastocyst, normally oriented towards the antimesometrial part of the endometrium (Fig. 4). This process results in the formation of a yolk sac placenta, which is essentially an ephemeral organ. About 1 1/2 days after the beginning of abembryonic implantation, fusion of the trophoblast with the uterine epithelium also starts at the embryonic pole of the blastocyst, adjacent to the mesometrial part of the endometrium. This leads to the formation of the definitive chorioallantoic placenta (again hemochorial). After infusion of aprotinin into the uterus with the minipump, the latter process did not seem to be impaired at all in those blastocysts that had managed to hatch mechanically from their coverings, if no remnants of the latter remained interposed. This seems to indicate that adhesion of the trophoblast to the uterine epithelium and invasion may not be dependent on aprotinin-sensitive proteinases like blastolemmase. The physiologic function of the latter then would seem to be restricted to the dissolution of the blastocyst coverings, comparable to a hatching enzyme. However it cannot be excluded at present that higher doses of the inhibitor would perhaps interfere with invasion.

The combined morphologic and biochemical evidence from investigation of normal implantation sites and of inhibitor-treated uteri suggests that all major effects of the mentioned proteinase inhibitors are due to direct inhibition of blastolemmase. However, indirect effects on implantation would be possible if inhibitors (like antipain) interact with proteinases involved in hormone-dependent changes in endometrial cell physiology, as proposed for the mouse and rat. Is there any evidence for a role of a blastolemmase-like enzyme in implantation in other species? Our knowledge of the physiologic way of zona-shedding in other species is, unfortunately, very limited. We do not know anything about the situation in the human, not even the time at which shedding occurs here, because the available specimens of the critical period are rare and have not been preserved in a way appropriate to retain the zona. In the rhesus monkey (which, however, shows a number of differences from the human, such as endometrial plaque reaction, central type of im-
Fig. 4. Schematic sketch of the topographical relationships between blastocyst and uterus at the time of implantation initiation in the rabbit, i.e., 7 days p.c. Cross-section through the uterus. The embryonic disc is oriented towards the mesometrial side of the uterus, the abembryonic pole faces the antimesometrial endometrium. The blastocyst is still completely encased in its extracellular coverings, although the dissolution of the latter is just starting in the abembryonic-antimesometrial region where implantation begins.

implantation, late onset of implantation), empty zonae flushed from the uterus together with zona-free blastocysts seemed to indicate that here the embryos may hatch mechanically, perhaps a day or so before implantation. Nothing is known about any proteinases of the uterine secretion or of the trophoblast which might be involved. In the cat, there is morphologic evidence that the zona pellucida is dissolved under the influence of factors depending on the abembryonic and lateral trophoblast (corresponding to the future girdle-shaped invasion zone?), but the gelatin film failed to identify a specific proteinase possibly responsible, other than the general cathepsin B-like activity of the trophoblast. In the mouse, blastocysts can easily hatch by rupturing the zona, in vitro. However, histologic investigation of implantation sites reveals that the normal process is by lysis. It has been proposed that a uterine secretion-derived enzyme assumed to be identical with a caseinolytic proteinase showing the same stage- and hormone-dependent changes of activity is responsible for this as well as for changing cell surface properties so that attachment of the trophoblast can occur ("implantation-initiating factor," ). No direct experimental evidence is available for the latter
assumption, however, while evidence for a role in zona lysis is good. It is possible that the factor described by Mintz\textsuperscript{21} is identical with a chymotrypsin-like enzyme described recently in mouse uterine flushings\textsuperscript{30, 60} showing the same dependence on an "estrogen surge" (superimposed on progesterone action). (For comparison: rabbit blastolemmase is progesterone-dependent, see Denker\textsuperscript{23}. There is no evidence for a physiologically significant estrogen surge in the rabbit or in the human.) The physiologic function for another estrogen-dependent uterine secretion proteinase described in the rat (and mouse), possibly being more close to elastase, remains to be defined, since it was found capable of dissolving the zona of unfertilized but not of fertilized eggs.\textsuperscript{113, 114}

For a number of other proteinases described in the human and in animals, it remains unclear whether they are at all involved in the process of implantation, because they were studied either in uterine tissues remote from implantation sites, or in trophoblast of stages far beyond those discussed here.\textsuperscript{62, 68, 96, 100, 134, 145}

Investigations of plasminogen activator activity present in mouse trophoblast during the phase of stromal invasion\textsuperscript{128} have received interest because the same enzyme was found to be secreted in considerable amounts by many (although not all) highly invasive tumor cells and was proposed to be of major importance for tumor invasion. Using the macrophage as a model, evidence was presented suggesting that plasminogen activator may be an integral part of the whole proteinase system involved in degradation of connective tissue ground substance.\textsuperscript{139, 140} As discussed earlier,\textsuperscript{25, 27} experimental evidence for a major role in the initiation phase of implantation is lacking; in fact plasminogen activator activity of blastocysts and of endometrial and uterine fluid decreases towards implantation and is low at early implantation sites (see also Denker, unpublished).\textsuperscript{11, 82, 117, 130, 137} The plasminogen activator found in cleavage stage embryos may have been adsorbed at the zona and carried from the ovary; its function is unknown.\textsuperscript{121} ε-Aminocaproic acid, an inhibitor of plasminogen activation that does not affect blastolemmase noticeably, does not interfere with implantation when administered intrauterally in the same way as described for aprotinin for example.\textsuperscript{3, 25} Likewise after oral administration of ε-aminocaproic acid, the implantation rate was not changed significantly and even tended to be slightly increased.\textsuperscript{5} Increased fibrinolytic activity may indeed be an unfavorable condition for implantation because it is found in endometria adjacent to various types of IUDs (apparently not including progesterone-releasing IUDs)\textsuperscript{11, 50, 78, 79} although in the uterine secretion, the activity remains unchanged or is reduced due to the presence of inhibitors.\textsuperscript{11, 130}

The physiologic function of trypsin- and chymotrypsin-like enzymes described as occurring in mouse blastocysts is still unknown.\textsuperscript{17} Proteinase inhibitors including aprotinin have been applied within the uterus also in the mouse using a slow release device,\textsuperscript{16} and an antifertility effect was noted when "implantation sites" were counted at a late postimplantation stage. Unfortunately, no morphologic investigation of peri-implantation stages was performed, so that these investigations do not provide any arguments for our discussion whether the major role of proteinases is in zona lysis, in changing cell surface characteristics for adhesion, or in other physiologic processes. Recently, attachment of the mouse blastocyst to mouse uterine, embryo, or L cell monolayers in vitro was described to be inhibited by soybean trypsin inhibitor (which gives basically the same results as aprotinin in the rabbit in vivo system [Denker, unpublished]), and to be stimulated by trypsin.\textsuperscript{74} In a subsequent series of similar in vitro
experiments using mouse decidual cell monolayers, it was found that soybean trypsin inhibitor interfered particularly with attachment of the blastocyst (indicating the need for a trypsin- or chymotrypsin-like enzyme), while \( p \)-nitrophenyl-\( p' \)-guanidinobenzoate (NPGB) inhibited both attachment and outgrowth (interpreted as indicating a need for plasminogen activator activity in addition to a trypsin-like enzyme). The reaction of experiments with NPGB seems problematic, however, since it was shown to have rather toxic side-effects in the rabbit in vivo system.

In another in vitro model system, digestion of films of extracellular matrix by mouse trophoblast outgrowths is being studied. Early spreading of trophoblast onto the substrate was found to depend upon the presence of plasminogen, but subsequent invasion and matrix digestion were plasminogen-independent. Characterization of the proteinases involved in the latter process has not been achieved thus far. Unfortunately, this fascinating trophoblastic outgrowth system suffers from some of the drawbacks of all two-dimensional culture systems. There is some doubt whether cell polarity is still maintained in the outgrowth, since morphology suggests that the side of the trophoblast that attaches to the substrate may finally represent the basal portion of the cell rather than its apex (if any such polarity can be attributed to it).

It is possible, therefore, that in addition to playing a crucial role in dissolution of blastocyst coverings (zona pellucida), certain proteinases of the trypsin/chymotrypsin family are involved in attachment of the trophoblast to the uterine epithelium and invasion. Since our in vivo systems failed to give clear-cut evidence in favor of this, we prefer to withhold any firm statements until we can perform further experiments, using preferably the in vivo system and applying inhibitors that were first tested in vitro against the various proteinases being isolated from trophoblast, endometrium and uterine secretion.

THE PHYSIOLOGIC REGULATION OF IMPLANTATION INITIATION

We will concentrate here on the cell biologic mechanisms of contact formation and refrain from discussing the hormonal regulation. A word of caution should be adequate, however, in this context: Most investigations on the hormonal regulation have used the rat and mouse as a model, pointing out the importance of a preimplantation rise in estrogen secretion. However there is no evidence for a physiologic function of elevated maternal estrogens in initiation of implantation in most other species (including man), and it may be a special feature of such species that are able to undergo delay of implantation (embryonic diapause). It is possible that the same applies to the interesting factor found in uterine fluid of mice, which inhibits uridine incorporation and which may regulate diapause, although it was claimed that comparable inhibiting factors do exist also in a species without diapause—the pig.

If proteinases play a role in the initiation of implantation, as suggested by the experiments discussed above, proteinase inhibitors can be expected to be involved in regulating this process. Proteinase inhibitors are present in all tissues where proteinases are being produced or serve their physiologic function, as in the pancreas (digestive pro-
teinases and, e.g., pancreatic secretory trypsin inhibitor), mast cells (mast cell proteinases and, in the cattle, aprotinin) or in the blood plasma (blood clotting cascade proteinases and their inhibitors). Proteinase inhibitors have been proposed to mediate, at least in part, the resistance of various tissues to invasion.\textsuperscript{76, 136} It was assumed, therefore, that proteinase inhibitors ought to be present also in the uterus and the trophoblast, and it was possible to prove their existence experimentally (for references see\textsuperscript{25}; for more recent data see\textsuperscript{7, 11, 18, 47, 56, 95, 118, 150}). Although it had been proposed that uteroglobin, the dominant uterine secretion protein of the rabbit in the preimplantation phase, is a proteinases inhibitor,\textsuperscript{5, 65} it was thought that this might be due to a low molecular weight inhibitor co-purifying in the same fraction.\textsuperscript{26} In fact, separation from uteroglobin and characterization as a very specific trypsin inhibitor has recently been achieved.\textsuperscript{56} Plasma proteinase inhibitors shown to be present in human uterine fluid\textsuperscript{11, 130} were found to be able to inhibit rabbit blastolemmase (used as a model as long as corresponding human enzymes are unknown).\textsuperscript{26}

Uterine proteinase inhibitor activities are sensitive to maternal steroid hormone levels.\textsuperscript{4, 11, 47, 56, 95, 130} The low molecular weight trypsin inhibitor of rabbit uterine fluid was found to show a pronounced peak of activity in the preimplantation phase but to decline towards implantation.\textsuperscript{56} Regulation of proteinase inhibitor activity levels in the uterine lumen may be one physiologic way of regulating implantation initiation. Such a concept fits nicely the results of experiments on the interference with implantation initiation by intrauterine administration of exogenous proteinase inhibitors in vivo, as discussed earlier. For the physiologic situation, we would predict that, in addition to regulation of inhibitor levels by maternal steroid hormones, local effects exerted by the blastocyst might also play a role perhaps in a way comparable to phenomena described for endometrial amino acid arylamidase activity:\textsuperscript{25, 135} the blastocyst locally stimulates discharge of arylamidase from surrounding uterine epithelium in the preimplantation phase and causes exhaustion of it at the implantation site.

In preliminary experiments, we did not find any evidence that the low molecular weight inhibitor isolated from rabbit uterine secretion inhibits blastocyst proteinase (as determined with chromogenic substrates), although this had been expected since the enzyme interacts with typical trypsin inhibitors. Therefore, the inhibitor may be directed against the trypsin-like enzyme found in uterine fluid.\textsuperscript{21, 25, 34, 70, 71} The simplistic model that the uterus controls directly, via its inhibitor, the blastocyst proteinase and thereby implantation may not apply.

With regard to the special features of animal models representing the central type of implantation, a word of caution may be appropriate concerning their usefulness when it is intended to extrapolate conclusions to species like the human with interstitial implantation. A typical feature of species with central implantation is to exhibit a considerable degree of blastocyst expansion. As a consequence, a large volume of fluid accumulates in the blastocyst cavity, and surface area increases markedly to permit extensive metabolic exchange. Two consequences can be derived from this. For nutrition of the large blastocyst, considerable amounts of special secretory proteins are being produced by the endometrium, some of them perhaps serving carrier functions as discussed for pig uteroferrin\textsuperscript{111} or rabbit uteroglobin.\textsuperscript{5} On the other hand, effective “buffer” mechanisms are required in order to avoid uncontrolled spreading of biochemical reactions (in the sense of activation of enzymes) or uncontrolled diffusion of metabolites or
mediator molecules in such a large uterine cavity. Many of the proteins present can, therefore, be expected to be (a) typical enzyme inhibitors or (b) molecules with relatively weak binding properties for various compounds. The quantity of such molecules will be much greater in these species than in those with interstitial implantation, and hormone-controlled changes in secretory patterns will be much more pronounced in the former than can be expected to be the case in the human. Those animal models are very useful for studies of the mechanisms of hormone action in general, but when trying to apply the concepts derived we must be very cautious and keep in mind that the secretory phenomena are at least greatly exaggerated, in these animals. It is even possible that certain factors playing an important role in the mentioned species, during the late preimplantation phase, may not be needed at all in the human.

Regulation of secretion of the enzyme (and proenzyme?) itself would, of course, be another way of steering the process of implantation. Production of blastolemmase activity depends on the presence of (abembryonic) trophoblast tissue, as shown in a number of experiments. The enzyme can be extracted from trophoblast of the appropriate stage. However, experiments demonstrating unequivocally that the trophoblast is the site of synthesis of the enzyme molecule are still lacking. Uptake from the uterine secretion cannot be excluded at present, and nothing is known about possible proenzyme activation processes. Proteinases are also present in rabbit uterine secretion and seem to fall into the classes of trypsin-like and chymotrypsin-like enzymes. It may be expected that production of uterine secretion proteinases may be regulated by maternal steroid hormones, but besides the dependence on an estrogen surge in the mouse and rat mentioned above little is known about this at present. There is some evidence that the blastocyst stimulates locally the secretory process or the release of cell surface-bound enzymes of the adjacent uterine epithelium. Trophoblast-derived and uterine secretion-derived proteinases probably act together in a concerted way to cause the dissolution of the blastocyst coverings (and attachment?).

It appears possible that enzymes like blastolemmase may be involved in bringing about the correct orientation of the blastocyst in the uterus at implantation initiation. In the rabbit model, the normal orientation is with the embryonic disc facing the mesometrial endometrium and the abembryonic pole of the blastocyst facing the antimesometrial part of the endometrium (Fig. 4). It was proposed by Böving that a pH gradient between the mesometrial and the antimesometrial part of the uterine epithelial surface and a potential of the abembryonic pole of the blastocyst to develop a rise in pH (due to bicarbonate production, transport and CO₂ release into the maternal circulation) act together in this respect. It was proposed that the result is an increase in adhesiveness of the blastocyst coverings at the abembryonic pole, this pole becoming trapped in the antimesometrial niche of the endometrium, due to increased pH. In fact, measurements with pH microelectrodes in vivo give values of 7.32 and 7.54 for the mesometrial and the antimesometrial surface of the endometrium, respectively, at this stage (Fig. 5). Comparison with the pH profile of blastolemmase activity (Fig. 6) reveals that the higher pH would increase enzyme activity. The blastocyst at first rotates freely in the uterine lumen. After blastolemmase production (which appears to be dependent on the abembryonic trophoblast) has started, a positive feedback mechanism can be expected to come into effect as soon as the abembryonic pole meets the antimesometrial endometrium with the higher pH value. Limited proteolysis of the blastocyst coverings by
Fig. 5. The pH values measured at the endometrial surface in vivo using microelectrodes are markedly different at the mesometrial and the antimesometrial side of the rabbit uterus (7 days p.c., values from 104). The blastolemmanse-dependent dissolution of the blastocyst coverings starts normally in the abembryonic-antimesometrial region.

Fig. 6. The pH profile of rabbit blastocyst proteinase (blastolemmanse) as measured with TosGlyProArg-p-nitroanilide as substrate.31 The difference in pH values measured between the mesometrial and the antimesometrial aspect of the endometrium (Fig. 5) is indicated (hatched area). A pH increase in this range can be expected to cause a marked increase in enzyme activity. This is proposed to be one mechanism involved in bringing about the correct orientation of the blastocyst in the uterus, with its abembryonic pole (higher blastolemmanse activity) facing the antimesometrial endometrium (higher pH value).
blastolemmase will make them soft and adhesive, and may help to anchor the blastocyst in this, the correct, orientation. Manipulation of the uterus during this sensitive phase (as with proteinase inhibitor, or saline, injection; any fibrin deposition in the uterine lumen) was found to increase the incidence of dystopic orientation and implantation in the uterus. 

Thus, the concept of a physiologic role of pH gradients in the uterus for implantation, and the concepts of the function of proteinases like blastolemmase in implantation initiation, can easily be reconciled. The same concepts can be applied, hypothetically, to species in which the lysis of blastocyst coverings (zona pellucida) occurs long before attachment of the trophoblast: Here, changes of adhesiveness of glycoproteins may occur at the cell surfaces rather than at the zona but may obey comparable laws.

**THE ROLE OF FACTORS OTHER THAN PROTEINASES IN THE EARLY PHASES OF IMPLANTATION**

For completeness, we need to bring into the picture additional factors that are probably involved but for which experimental evidence is still very poor. As we have seen in the experiments with intravaginal proteinase inhibitor administration, a role of proteinases in cell contact formation itself remains questionable in spite of effects reported for in vitro systems. Even if proteinases change cell surface properties in such a way that adhesion is promoted, this does not explain the type of interaction involved in the ensuing binding.

Individual uterine luminal proteins seem to be recognized and bound differentially by rat blastocysts but the mechanism is unknown. For many years, attention has been directed at the possibility that carbohydrate side chains of cell surface glycoproteins may be involved in mediating adhesiveness between trophoblast and uterine epithelium. Decrease as well as increase in density of certain carbohydrate groups as well as of negative charge (due to bound sialic acids or sulfate ester groups) have been reported to occur at the cell surface. There are conflicting results about the effects of inhibition of glycoprotein synthesis by tunicamycin, however. It was reported that removal of cell surface-bound sialic acids by neuraminidase [0.2 ml (!)] injected into the uterus, or flushed blastocysts treated in vitro and retransferred] is an effective means of interfering with implantation in mice. Histochemical investigation however failed to show a topographical correlation of neuraminidase activity with the attachment sites in the rabbit, and neuraminidase had no effect on the attachment of mouse blastocysts to glass surfaces in vitro. As far as the type of interaction is concerned, consideration of solely the cell surface charge situation may be thought to be too simplistic. The Roseman hypothesis involving cell surface-bound glycosyl transferases and enzyme-substrate binding, although attractive, thus far lacks experimental support in this system.

Another type of recognition mechanism would involve lectin-like molecules, e.g., present at the surface of one of the two partners and binding to certain carbohydrate side chains of glycoproteins found at the surface of the other one. Binding sites for exogenous lectins, i.e., carbohydrate groups, are being identified at the trophoblast and the uterine epithelial surfaces, some of them showing stage-dependent changes and differential distribution at the embryonic versus the abembryonic pole of the blastocyst.
However, knowledge is still limited and some of the data concerning decrease or increase towards implantation are still controversial. In particular, there is no proof for the presence of lectin-like molecules at these sites, and experimental evidence for an interference with implantation by administration of the appropriate competitive sugars is lacking. Intravaginal injection of the lectin, concanavalin A, was reported to block implantation in the mouse; however, we observed in preliminary experiments performed in the rabbit that this lectin can cause degeneration of the blastocysts so that additional experiments are needed in order to clarify the mode of action (Denker and Nalbach, in preparation). Since several laboratories are working on this complex of questions, we may expect more data to become available during the next few years.

In any case, the initial contact that may still be weak and may be established by the mentioned mechanisms will have to be consolidated during the subsequent phase of firm attachment and invasion during which even intercellular junctions are being formed between trophoblast and uterine epithelium. We are at present testing the hypothesis that cross-linking enzymes may be involved. In fact, there is a considerable polymerizing/cross-linking capacity in the uterus-blastocyst system in our model, the rabbit, as shown impressively by the continuous deposition of extracellular material, the blastocyst coverings, during the preimplantation period (Fig. 7A). These blastocyst coverings are in a sense equivalent to the zona pellucida. In the rabbit, the mucoprotein layer, derived from tubal secretion, is being deposited around the egg, i.e., outside the zona pellucida, during tubal passage; the zona is being dissolved shortly after entering the uterus (i.e., around 3 days before implantation) and is being replaced by a new layer formed at the inside of the persisting mucoprotein layer, the neozona (Fig. 7A). The neozona appears to be formed partly from secretory products provided by the trophoblast, this being a new aspect of the physiology of preimplantation stage trophoblast. However, not only trophoblast-derived factors but also certain components of the uterine secretion appear to be essential. In addition, the mucoprotein layer is also being reinforced by uterine secretion material deposited at the outside of the blastocyst coverings, the so-called gloiolemma.

The continuing deposition of extracellular material around the blastocyst coverings is not unique for the rabbit and not as bizarre as it may appear at first glance. Deposition of an additional layer at the inside of the zona pellucida was shown in the fur seal (so-called subzonal layer) and the horse. All these species have in common that they represent the central type of implantation, i.e., the blastocyst attaining a large size, staying in a topographical sense in the (previous) uterine lumen although the trophoblast may finally erode the endometrium and form a hemochorial (rabbit) or endotheliochorial (carnivores) contact. In many species of this group, no additional layers of blastocyst coverings become morphologically discernible, although the fact that the thickness of what appears to be zona pellucida remains unchanged in spite of considerable expansion of the blastocyst, probably indicating that material is being added (from the inside or the outside) without a stratification becoming apparent (cat, see). On the other hand, there is some indication that the addition of material to the zona may be an even more widespread phenomenon including species in which the blastocyst does not expand considerably, therefore possibly also including the human (for more data see).
The mechanism of deposition of this extracellular material is still unknown. In formation of fertilization membranes, as studied in sea urchins and amphibia, it may involve precipitation of glycoproteins by lectins, perhaps ion-dependent, or cross-linking by enzymes like ovoperoxidase as well as further processing by limited proteolysis. Nothing is known about a comparable role of a peroxidase in the blastocyst-uterus system, although an estrogen-dependent endometrial peroxidase is well known and has been used extensively in studies of hormone action. We have preliminary evidence suggesting that transglutaminase (coagulation factor XIIIa) (forming isopeptide cross-links between glutamic acid and lysine) or a related enzyme may be involved in neozona formation. It has recently been suggested that transglutaminase, which was found to be highly active in the uterus and to show an increase in activity during early pregnancy (although it was not indicated whether this was determined in the endometrium or the myometrium), is involved in masking embryonic antigens at the trophoblast surface by cross-linking them with uteroglobin, the dominant uterine secretion protein during the preimplantation phase in the rabbit (although not found unequivocally in other species). We are testing the alternative hypothesis that a transglutaminase-like enzyme is involved in cross-linking extracellular material as part of the processes of (1) formation of the neozona (and the gloiolemma?) and (2) establishment of firm adhesion of the trophoblast to the uterine epithelium. In fact, the polymerizing potential continues to be present in the rabbit uterus-blastocyst system, during the implantation phase. Normally, all layers of blastocyst coverings are being dissolved under the influence of blastolemmase at implantation initiation (Fig. 7B), uterine secretion enzymes probably contributing to the process so that peptide fragments are finally formed. If blastolemmase is inhibited experimentally, lysis of the coverings is not only prevented but deposition of both neozona and gloiolemma continues, showing the presence of the polymerizing factors.

There is some evidence for a role of transglutaminase in cell adhesion in other systems. Blastolemmase is highly selective to substrates; therefore, after dissolution of blastocyst coverings, (glyco-)proteins may remain at the trophoblast and endometrial surfaces, which are blastolemmase-resistant and which can mediate attachment, at first perhaps by a lectin receptor-like mechanism and then by being cross-linked by an enzyme like transglutaminase. We have immunohistochemical evidence that a fibrinogen-like antigen is present at the uterine epithelial surface (not the trophoblast). We know that fibrin is blastolemmase-resistant and these molecules may, therefore, mediate attachment. After proteinase inhibitor injection into the uterine lumen, fibrin deposits form more often (and cannot be dissolved because of low fibrinolytic activity at implantation sites, or because of direct plasmin inhibition); blastocysts attach preferentially to these clots, often maloriented with respect to the mesometrial-antimesometrial axis.

The molecular mechanisms involved in the phase of implantation that follows adhesion, i.e., epithelial penetration, may again involve the action of certain proteinases, as suggested by investigations done in other systems, showing that proteinases may regulate the degree of cell adhesion. We do not know, however, whether these postulated proteinases, if they exist, are the same as discussed for the initiation phase of implantation or perhaps a different system. In experiments using the so-called trophoblastic outgrowth system in vitro as a model, attachment to the culture dish or to cell monolayers, and outgrowth (the latter being used as a model for invasion) were
PREIMPLANTATION PHASE

A) FORMATION of NEOZONA and GLOIOLEMA

DISSOLUTION of BLASTOCYST COVERINGS

ADHESION
Fig. 7. Simplified diagram to illustrate some major molecular events shown or proposed to be involved in implantation initiation. In the preimplantation phase (A), deposition of extracellular material at the interface between trophoblast and uterine epithelium prevails. This is particularly obvious in the rabbit illustrated here, where the mucoprotein layer formed already in the tubes is being reinforced by addition of two new layers, the neozona and the gloiolemma. It seems to apply also to other species (particularly those representing the central type of implantation) although they usually do not show distinct layers of the blastocyst coverings. For simplicity, it is assumed that only one neozona precursor protein (W) is being secreted by the trophoblast and cross-linked by an enzyme present at the interface, and that another protein (X) is being provided by the endometrium as a precursor for the gloiolemma. However, the number of proteins involved, their origin and the nature and origin of the cross-linking enzyme(s) are still unknown. (Three major glycoproteins are found for example in the zona pellucida of pig and rabbit oocytes.) It must be considered a possibility that lectin-like molecules diffusing against neozona precursor, or gloiolemma precursor, respectively, may bring about the initial precipitation at the inner and outer surface of the mucoprotein layer, the latter being rate-limiting for diffusion. Enzymatic cross-linking may then stabilize the newly formed layer subsequently. However, there is no proof of the presence of lectin-like molecules so far. In the initiation phase of implantation (B), the “barrier” (i.e., the blastocyst coverings) formed or reinforced during the preimplantation phase is being removed by the action of specific proteinases (e.g., blastolemmase). When proteinases are inhibited experimentally, contact formation between trophoblast and uterine epithelium is prevented. In such experiments it becomes obvious, at the same time, that the potential for depositing neozona and gloiolemma material is still present at this phase although in the physiologic situation it is overcome by the degradating action of blastolemmase. Trophoblast and uterine epithelium can come together after removal of the intervening extracellular material of the blastocyst coverings. A similar concept may apply for removal of some components of cell surface coats. Since blastolemmase is a highly specific enzyme, other (glyco-)proteins can be expected to remain undegraded, or only chemically modified due to limited proteolysis, at the cell surfaces. For simplicity, only one such protein (Y) is shown at the surface of the trophoblast and another one (Z) at the uterine epithelial surface. In the adhesion phase (C), contact is formed between the cell surfaces of the trophoblast and the apical end of the uterine epithelium. This may involve, in the beginning, relatively weak bonds (of various chemical types) between cell surface glycoproteins, but the contact gradually becomes more and more firm so that finally the blastocyst cannot be separated from the uterine epithelium without destroying cells. Intercellular junctions including desmosomes are formed between both partners at the end. It is hypothesized that cell surface-bound (glyco-)proteins like those mentioned above (which are blastolemmase-resistant) are being cross-linked in order to reinforce contact, perhaps using again an enzyme like the one involved in deposition of blastocyst coverings material (the latter, however, being blastolemmase-sensitive).
found to be regulated separately. While this system may give useful information on adhesiveness and on some aspects of invasiveness in general, it suffers the drawbacks of two-dimensional systems (discussed in 86), and it may not be a suitable model for the process of epithelial penetration in particular, because cells change many of their properties including the polarity typical for epithelial cells. 40

A useful summary of the various modes of epithelial penetration as seen electron microscopically in different species was given by Schlafke and Enders. 115 The principal modes are (a) penetration by displacement of uterine epithelium from its basement membrane (perhaps a subtype of (c)) (rat, mouse); (b) penetration by fusion of trophoblast and uterine epithelial cells (rabbit); (c) penetration by intrusion of processes of trophoblast between uterine epithelial cells (ferret, others?). It is not known to which type the human belongs since the early phase of epithelial penetration has not been observed here. In a slightly later stage, evidence for fusion between trophoblast and endometrial cells has been found electron microscopically. 77 The paraffin sections of the Carnegie collection, still the best specimens of earliest human implantation stages, also suggest symplasma formation in part of the uterine epithelium adjacent to the invading trophoblast (although it is not known whether this is just a sign of degeneration), and small nuclei of quite similar appearance are seen in the periphery of the earliest syncytiotrophoblast. The human may represent a combination of the intrusion and the fusion type. The same may hold true for the rhesus monkey, although this species shows a number of peculiar features (such as central type of implantation and epithelial plaque reaction), which limit its use as a model for human implantation. 41 (Denker, Enders and Schlafke, in preparation).

In any case, the fact that the trophoblast can interact at all with the apical end of the uterine epithelium (which, as in all epithelia, is primarily nonadhesive) is most astonishing. As seen in microcinematographs of in vitro cultures, other cells tend to withdraw from the advancing trophoblast. 52 In the receptive phase (hormonally controlled 108) uterine epithelial cells in the rat and mouse tend to detach from their basement membrane not only upon contact with the mature trophoblast but even after mechanical irritation (for references see 25, 115). In the rabbit, hemidesmosome-like structures were found to form between, astonishingly, the apical end of the uterine epithelium and remnants of blastocyst coverings when the dissolution of the latter was blocked with aprotinin. 25 Does all this indicate that, perhaps, the uterine epithelium loses or even reverses its functional (apical-basal) polarity, after proper hormonal conditioning, when the trophoblast contacts it? This might be one factor contributing to the introduction of changes in the properties of the apical cell surface of the uterine epithelium, to changes in transport, but also to changes in the lysosomal system, finally leading to cell death. It was indeed found that the rat uterine epithelium undergoes considerable changes in transport phenomena, in the preimplantation phase (also in pseudopregnancy), showing increased endocytotic activity. 52, 103 It was proposed that endocytosis and attachment may be related phenomena in molecular terms, attachment being "frustrated endocytosis or phagocytosis." 109, 124 Does this apply to the attachment between trophoblast and uterine epithelium?

The trophoblast does not seem to be affected by stimuli changing the functional polarity of uterine epithelial cells, in this respect once more resembling tumor cells that are not contact-inhibited. The acquisition of a stable polarity may be a major point in
trophoblast differentiation and maturation as discussed elsewhere.\textsuperscript{29} Formation of a basement membrane underneath the trophoblast cells (in murine rodents attaining considerable thickness as Reichert's membrane, usually thought to control transport to the yolk sac), aided by the primary endoderm, may help in stabilizing this polarity. Basement membranes can imprint polarity on cells, even on certain tumor cells.\textsuperscript{61}

Some information is available on the motility of mouse implantation stage trophoblast in vitro, including observations on actin and myosin.\textsuperscript{52, 125} It has also been proposed to focus attention on its cytoskeletal organization in general.\textsuperscript{122} However, it has rarely been asked specifically why the trophoblast invades the endometrium rather than the blastocyst cavity or the embryonic anlage? Further investigation of factors regulating cell polarity and directional growth may well shed new light on mechanisms of trophoblast invasion.

After the trophoblast has penetrated the uterine epithelium, invasion appears to be halted for a while before the basement membrane is overcome.\textsuperscript{115} The mechanism of basement membrane penetration by the trophoblast is still unknown. Recent studies reveal that certain highly metastatic tumor cells produce collagenases specific for type IV collagen.\textsuperscript{83} Such an enzyme has not yet been shown in the invading trophoblast. Alternatively, loss of functional polarity of uterine epithelial cells, as discussed above, can contribute to the process by reducing deposition of basement membrane material while the degradative activity of normal turnover is unchanged. The trophoblast is not always the first cell to penetrate the basal lamina: in the rat, ectoplasmic processes of stromal cells traverse it earlier.\textsuperscript{44}

The spectrum of molecular events discussed here is still highly hypothetical and is simplified for heuristic purposes. The strongest evidence is available for the role of blastolemmase in the initiation phase of implantation. The other details proposed may help to design appropriate experiments to elucidate the mechanisms of implantation initiation further. We should be aware of the possibility that proteinase inhibitors used as an additive to medicated IUDs may affect implantation in addition to reducing menstrual blood loss. Different proteinase inhibitors may have quite different effects, depending on the particular proteinase attacked. While inhibitors of blastolemmase-type enzymes may interfere with implantation initiation not only in the rabbit but also in other species perhaps including the human, selective plasmin inhibitors may have an implantation-promoting effect if any. Blastolemmase-resistant proteins can be expected to be involved in mediating attachment, and they might be a promising tool in attempts to increase success rates in blastocyst transfer.

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