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Investigations on the Cell Biology of Embryo Implantation

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Implantation of the embryo in the uterus is of fundamental importance for the establisment of pregnancy in Eutherian mammals including man. In addition, this process is of great fascination for the basic scientist, since it includes several phenomena that are little understood and that even appear paradoxical: implantation appears to disobey transplantation laws, since an antigenically different graft (the embryo) is not being rejected, and it appears to disobey certain basic principles of cell biology since e.g. in the initiation phase of implantation contact is being established between otherwise non-adhesive apical cell membranes of two epithelia, the trophoblast and the uterine epithelium (Denker 1986).

We are reporting here on investigations of cell-cell interactions in the initial phase of embryo implantation, a field that is very incompletely understood probably because only very few groups are working in this area. This is in contrast to the hormonal control of implantation and to the hormonal control of endometrial function in general, areas which have been covered by many investigations (for review see Finn 1977, Psychoyos 1976). The application of recent concepts about cell-cell and cell-matrix interaction to implantation physiology appears to enable us to develop some new views about mechanisms involved.

The description of this work will follow the time course of events as outlined in Tab 1. During the preimplantation phase the most relevant feature for our discussion is that the zona pellucida (and its equivalents, the blastocyst coverings) is at first being maintained and even modified and reinforced, in a number of species, and it appears to function as a barrier that prevents premature contacts between embryonic and maternal cells. Initiation of the implantation process requires that these coverings be removed. The respective role of enzymatic lysis or mechanical rupturing in this process varies depending on the species. Data on a crucial role that a proteinase system plays here have been worked out in the rabbit model and will be reported in detail. Shedding of the coverings is followed (more or less directly, dependent on the species) by contact formation between trophoblast and uterine epithelium including firm adhesion and, in invasive types of implantation, by penetration of the trophoblast through the epithelium and, subsequently, by deep invasion into the stroma. We are just starting to develop concepts that may explain these most astonishing processes and may finally allow to gain new experimental and therapeutical access to them. The development of appropriate methodology has played a significant role in the reported research program, including analytical (new histochemical techniques) and experimental approaches (in vivo and in vitro, including a novel organ culture system for implantation studies). These methodological aspects will also be referred to in the appropriate paragraphs.

Preimplantation Phase: Transformation of blastocyst Coverings

Until shortly before implantation, the preimplantation stage embryo remains mobile within the lumen of the genital tract. At least early blastocysts are still encased in the zona pellucida supplemented, in some species, by additional layers of extracellular glycoprotein material. The physiological role of the zona pellucida in these stages is still unclear. While there is good experimental evidence suggesting that the zona pellucida has a morphogenetic role during early cleavage (Lehtonen 1980), holding blastomeres together lightly as long as they have not yet polarized (Johnson et al 1986) nor developed sufficient adhesion to each other through junctions forming during compaction (thus precenting monozygous twinning), there is no reason to see any function like this in blastocyst stages. Interestingly, however, the blastocyst coverings are not lost right after compaction of the embryo but persist during blastocyst stages, for longer or shorter time periods depending on the species.

In species with the central type of implantation the blastocysts attein considerable size. During this expansion new material must have been added to the zona pellucida to prevent overstretching and disrupture. Indeed, the thickness of the "zona" as seen in later preimplantation stages in e.g. the cat suggests that this has taken place although no additional layers of material can be discerned morphologically (Denker et al 1978). On the other hand, the rabbit blastocyst allows to detect changes in the structural composition of these external coatings when a combination of histochemical and electron microscopical methods is being used and when a complete series of states is being investigated. It turns out that a young blastocyst is surrounded by complex *"blastocyst coverings"* consisting of: zona pellucida (formed in the ovary) and mucoprotein layer (deposited on the outer side of the zona pellucida in the oviduct). The zona pellucida is then dissolved within the persisting mucoprotein layer during very early intrauterine life of the blastocysts. Surprisingly, immediately after this, deposition of new material starts at the same place so that a new layer (the "neozona") is formed which replaces the zona pellucida and which is continuously being reinforced during blastocyst expansion until the onset of implantation (Denker, Gerdes 1979). Later on, starting about one day before implantation, even a third layer of material is deposited on the mucoprotein layer, this time on the outside (the gloiolemma, thought to be derived from uterine secretion material, Böving 1963). Thus, the blastocyst coverings which surround rabbit embryos at the onset of implantation are indeed quite complex structures which do not contain any zona pellucida material anymore but, instead, material that is derived from tubal secretion (mucoprotein layer) plus a neozona of unknown origin on the inside and a gloiolemma thought to be derived from uterine secretion, on the outside.

Dissolution of the zona pellucida and formation of the neozona can take place in the typical way only within the uterine milieu. They do not occur in the tubes nor in the usual in vitro culture media (Adams 1973, Fischer et al 1983). The postulated uterine secretion factors do not seem to be dependent on ovarian steroid hormones nor do they seem to be restricted to the uterus (Fischer 1988).

On the other hand there is evidence that neozona formation also depends on factors provided by the trophoblast (Leiser and Denker 1988).

The new aspect derived from these investigations is that the zona pellucida and its equivalents (blastocyst coverings) are being transformed and reinforced during the preimplantation phase, a process that is not only valuable as a marker for normal embryo-maternal interactions during preimplantation development and defects occuring in in-vitro culture but also points to physiological functions of these coverings in these phase which still need to be defined. The phenomenon is by no means restricted to the rabbit. It is also found in other species particularly those representing the central type of implantation and showing considerable blastocyst expansion, e.g. in the horse where a "capsule" that corresponds to the neozona is formed which replaces the zona pellucida and encases the conceptus until some time between day 21 and 28

(Betteridge; see Denker et al 1987a). Transformation of coverings appears indeed to be widespread and is also found in species in which the blastocysts remain small (including the human) (for literature see Denker 1977a, Leiser and Denker 1988). In addition to other functions e.g. immunoprotection (Warner et al 1988) this reinforcement of the coverings may also serve to make sure that premature contact of early trophoblast with uterine epithelium does not occur.

Implantation Phase

The Initial Phase of Implantation: Role of Proteinases in Shedding of Blastocyst Coverings

Before the trophoblast can establish contact with the uterine epithelium at implantation initiation, the blastocyst coverings (zona pellucida and its equivalents described above) that form a barrier between both partners need to be removed. Shedding of these coverings occurs at varying times in various species: it may occur already several days before attachment of the trophoblast (e.g. in ungulates; in certain rodents only in the special situation of delayed implantation) or it may immediately precede implantation initiation (e.g. in our animal model, the rabbit).

This process of removal of blastocyst coverings was shown in the rabbit to involve the action of certain proteinases (endopeptidases). In the center of our studies has been, for a number of years, one of these enzymes which we call blastolemmase.

Blastolemmase: Histochemistry and Biochemistry

Blastolemmase is an endopeptidase which appears to play an indispensable role in dissolution of blastocyst coverings in the rabbit. Indeed, whereas many compounds that can be identified with biochemical methods have been proposed to play some role in implantation since their concentrations show changes correlated with implantation (like various proteins), blastolemmase is the only compound that could, in addition, be demonstrated experimentally to be essential for one of the processes involved in implantation initiation (Denker 1977a).

A special methodology had to be developed for the investigation of this enzyme, i.e. a variant of the histochemical gelatin substrate film test (Denker 1974a, 1977b). With this technique, it was possible to show a clearcut temporal and local correlation with the initiation of implantation in the rabbit (Denker 1971b). Dissolution of blastocyst coverings starts, in this species, in the abembryonic region where syncytial elements of the trophoblast develop, immediately followed by attachment of the "trophoblastic knobs" to the uterine epithelium and by their invasion. Interestingly, blastolemmase activity can be demonstrated with the histochemical test exactly in this region and almost exclusively during the time when these processes are going on, i.e. between 7 and 8 d p.c. It is not found at the embryonic pole, where dissolution of blastocyst coverings and attachment of the trophoblast to the endometrium will start one day later. The histochemical test also reveals that the main proteinase activity is found associated with the blastocyst wall, i.e. the complex of trophoblast and disintegrating blastocyst coverings, the latter being the major source (Kirchner 1972, Denker 1974a). From this observation the interesting question arises whether this enzyme is 1.) produced by the trophoblast alone or in cooperation with the endometrium, as favoured by us, or 2.) secreted by the endometrium, as favoured by Kirchner. A role of the hormonal status of the maternal organism was indeed found in ovariectomy experiments showing clearly a progesterone dependence: when early pregnant animals were ovariectomized at 6 d p.c., the blastolemmase activity did not reach the normal levels and dissolution of the blastocyst coverings did not take place. After replacement therapy with progesterone, both the proteinase activity and the dissolution of the blastocyst coverings as well as subsequent implantation could be normalized (Denker 1972). Of course, a progesterone-dependence does not prove a maternal origin of the enzyme, since the hormone effect could have been an indirect one. Observations on blastocysts implanting in an inverse orientation as well as experiments with models for blastocyst coverings without mature trophoblast argue for

a trophoblast origin of the enzyme or of an activator (Denker 1974b, Denker and Hafez 1975). Additional clues are derived from biochemical determinations (see below).

Biochemically the enzyme appeared to be a new entity that had not been described before. It could not be detected with any of the traditional proteinase assays as available at the time of its discovery so that the earlier investigations were restricted to the histochemical gelatin film test. Proteinase inhibitors then allowed a first tentative classification as a serine proteinase of the trypsin family, being more closely related to trypsin than to chymotrypsin and elastase (Denker 1976). Synthetic oligopeptide substrates which became available later on finally allowed to perform quantitative photometric tests and to proceed with the biochemical characterization. Enzyme preparations extracted from rabbit blastocysts (late preimplantation stage) showed a primary specificity of the active site for arginyl bonds (which are cleaved much better than lysyl bonds, Denker and Fritz 1979). It appears interesting that the enzyme has, in contrast to e.g. trypsin, a very high selectivity: it does not only show the described specificity for the amino acid in position P_1 but also recognizes the neighboring amino acids in positions P_2 and P_3. Hydrophobic amino acids in these positions P_2 and P_3 are preferred. Therefore we can assume that this is an enzyme which physiologically cleaves only a few peptide bonds, i.e. arginyl bonds, and even selectively only few of them, in its natural protein substrates (Denker 1982). This is a characteristic of enzymes which have not a general degradative function (like trypsin) but rather cause more subtle but physiologically important changes in the macromolecular structure of their substrates. The biochemical properties of blastolemmase are comparable to those of proteinases that have a regulatory function, e.g. kallikreins and proteinases of the blood clotting and the complement system.

The described synthetic substrates have also made it possible to perform quantitative photometric assays on endopeptidase activities in uterine flushings, comparing a series of relevant reproductive states (Fig 1). Again an enzyme activity can be detected here which has the same properties as the arginine-specific enzyme of blastocyst extracts described above. The enzyme has been partially purified from uterine flushings of pregnant animals (Tisljar and Denker 1985). We assume that this enzyme is identical with blastolemmase originally described using the gelatin film test, because it shows the same proteinase inhibitor profiles and because it shows identical properties with the predominant enzyme activity that can be extracted from blastocysts (see above). This activity can be demonstrated in rabbit uterine flushings only from 7 d p.c. on and decreases again afterwards, i.e. it is correlated in time with implantation initiation, specifically with dissolution of the blastocyst coverings in the abembryonic hemisphere (Fig 1). The activity is dependent on the presence of blastocysts: it is negligeable in blastocyst-free uteri, e.g. in pseudopregnancy (Plikat and Denker in preparation). We assume, therefore, that the enzyme is released from implanting blastocysts into the uterine milieu. However, this obvious dependence on the presence of blastocysts does again not clarify where the site of synthesis of the enzyme molecule is: synthesis by the blastocyst (trophoblast) appears probable, but synthesis by the endometrium and activation by the blastocyst cannot be excluded on the basis of these data.

Other Proteinases

With a similar type of synthetic oligopeptide substrates another endopeptidase can be shown to be present in rabbit uterine flushings which has a preference for apolar (hydrophobic) amino acids. The profile given in Fig 1 ("proteinase X") was obtained with alanyl-alanyl-alanyl-p-nitroanilide. The enzyme becomes demonstrable in the uterine lumen of the rabbit already some time before initiation of implantation, and, in contrast to the arginine-specific enzyme (blastolemmase), in the pseudopregnant as well as the pregnant state (although the activity rises higher in the presence of blastocysts, Plikat and Denker in preparation). These observations suggest that the enzyme is derived from the endometrium, and the blastocysts may either stimulate extrusion into the uterine lumen or cause activation of the enzyme.

A related and possibly identical enzyme was partially purified from rabbit endometrium by ion exchange chromatography on DEAE-Sephadex A-50 and gel filtration on Sephades G-75 (Tisljar and Denker unpublished). This preparation was free of detectable contaminations by arginyl bonds-splitting activity (blastolemmase). It hydrolyzed the substrates Suc-Ala_2-Pro-Leu-pNA, Suc-Ala_2-Pro-Phe-pNA and Suc-Ala_3-pNA with relative rates of 100, 45 and 41%, respectively. Activities hydrolyzing these three substrates comigrated consistently in these experiments. Unfortunately, this line of research had to be descontinued, and it remains to be determined whether the described activities are due to an elastase-like enzyme, or to a mixture of an elastase-like and a chymotrypsin-like enzyme, or to a metalloproteinase. Preparations from uterine flushings (as used for the stage dependent profiles illustrated in Fig 1) were inhibited by phosphoramidon, a characteristic of metalloproteinases.

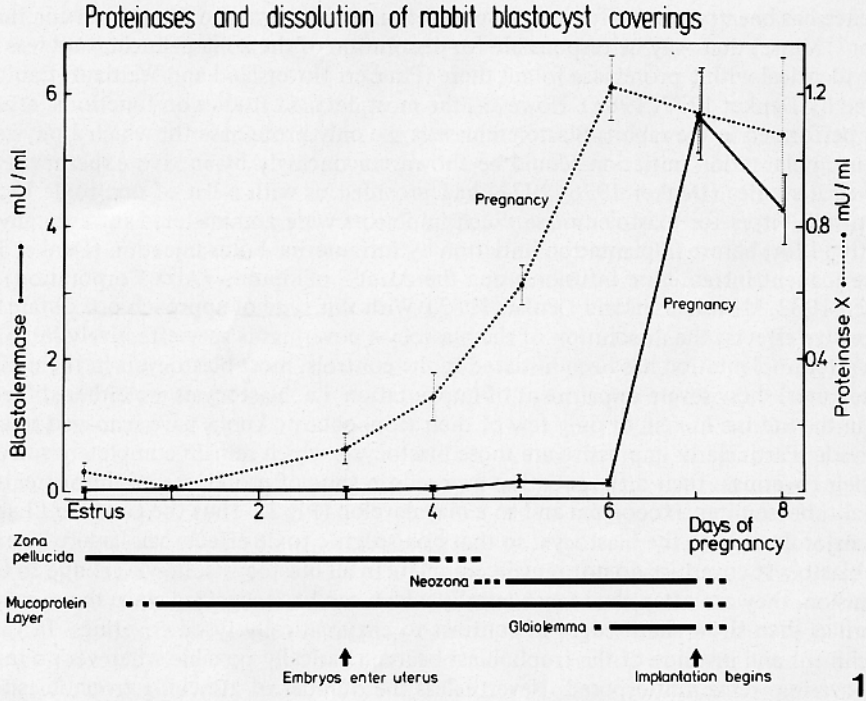
Interestingly, an activation phenomenon could be observed, in the endometrial enzyme preparation, upon addition of a factor(s) that is probably derived from blastocysts, i.e. present in late preimplantation stage uterine flushings containing blastolemmase. Obviously, this process could be of physiological relevance for implantation. The biochemical nature of the factor(s) needs to be identified.

In species other than the rabbit, other trypsin-like, chymotrypsin-like and elastase-like enzymes as well as cathepsins (B, D) have been studied by other groups in blastocysts and endometrium, but data on their biochemical properties and/or possible physiological functions are very limited (for detailed discussion see Denker 1983). A role for the plasminogen activator/plasmin system in embryo implantation has been proposed based on correlation studies in the mouse (Strickland et al 1976). However, besides this correlation experimental evidence for any significant role of the latter system in the initial phase of implantation is lacking, e.g. in vivo inhibitor experiments showing no effects so that we considered it unreasonable to study this proteinase system in detail, in the rabbit (for further discussion, see Denker 1983).

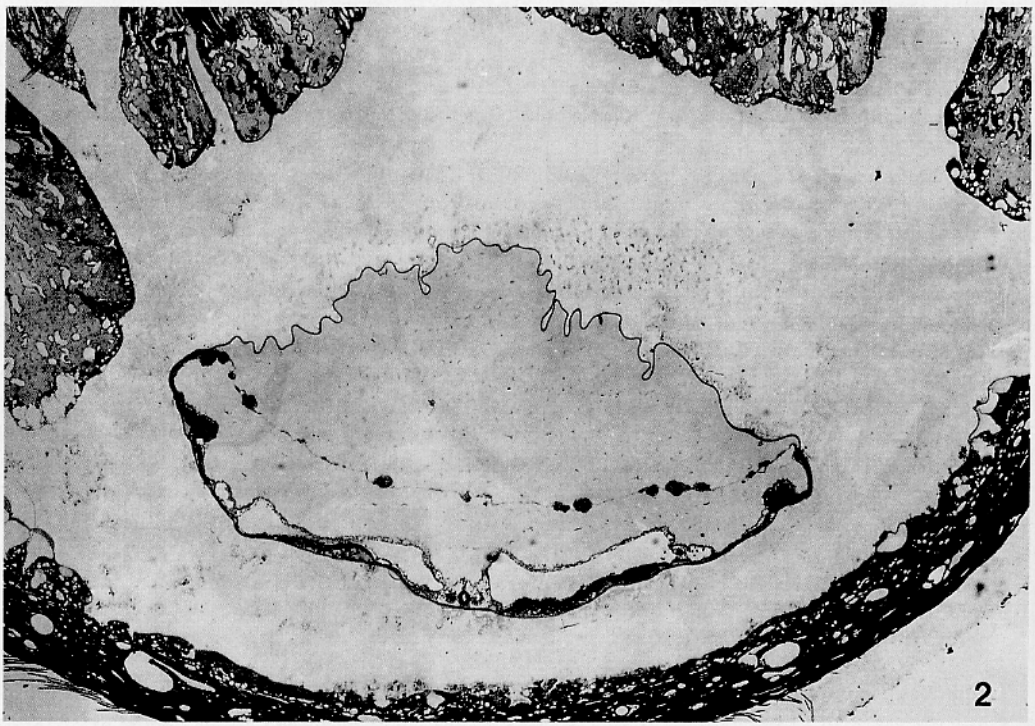
The Physiological Role of Proteinases in the Initial Phase of Implantation

Observations on embryos kept in vitro suggest that in many species blastocysts can hatch mechanically from their coverings (zona pellucida), sharp-edged fragments seeming to suggest that enzymatic lysis is not involved. In vivo, however, the coverings seem to be shed predominantly by mostly locally starting action of enzymes (reviewed by Denker 1983). In the mouse,

Plate 1: Proteinases and embryo implantation in the rabbit. Fig 1: Proteinase activities in rabbit uterine flushings during the preimplantation phase of pregnancy and implantation correlated with transformation of the various layers of the blastocyst coverings and with their dissolution (below). Note the precise correlation between the peak of blastolemmase activity and lysis of the blastocyst coverings at implantation initiation, whereas "proteinase X" activity starts to rise already during the early preimplantation phase. (Blastolemmase activity was determined with 0.3 mMol/l Tosyl-Gly-Pro-Arg-p-nitroanilide as substrate in Tris-HCl buffer ph 9.0, 37° C; "proteinase X" with 1 mMol/l Succinyl-Ala-Ala-Ala-p-nitroanilide in Tris-HCl buffer pH 7.5, 37° C) (Plikat, Denker, unpublished results). **Fig 2:** Inhibition of embryo implantation in vivo by proteinase inhibitors. Aprotinin (Trasylol®) which is a potent blastolemmase inhibitor has been infused intrauterally at a rate of 84 µg/h from 5 1/2 d p.c. on, using the Alzet® osmotic minipump (Meinshausen 1982). The proteinase inhibitor blocks very effectively the lysis of blastocyst coverings and, as a result, implantation. At 9 1/2 d p.c. (i.e. 2 1/2 days after implantation has startet in the controls) this blastocyst lies still completely free in the uterine cavity (The blastocyst has partially collapsed due to fixation and embedding so that it does not fill the uterine lumen anymore). Interstingly, the blastocyst has continued to differentiate thus proving that non-specific toxic side-effects are largely lacking in this experiment: The embryonic anlage proper (facing, atypically, the antimesometrial endometrium, below) shows a neural tube and somites, surrounded symmetrically by exocoelom and the thick syncytiotrophoblast of the embryonic pole; note also well-developed trophoblastic knobs at the abembryonic hemisphere (laterally and above). Embryonic signals have obviously reached the endometrium although attachment is lacking, since placental and paraplacental folds (above) show the same structural characteristics as in normal implantation chambers, and the uterine epithelium is transformed into large symplasms that already start with their typical vacuolar degeneration. Semithin section, toluidine blue, x 23. (after Meinshausen, Denker 1983).


Proteinases and dissolution of rabbit blastocyst coverings
Blastolemmase mU/ml
Proteinase X mU/ml
Pregnancy
Pregnancy
Estrus
Days of pregnancy
Zona pellucida
Neozona
Mucoprotein Layer
Gloiolemma
Embryos enter uterus
Implantation begins

1



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evidence has been presented for a uterine secretion-derived enzyme ("implantation initiating factor", Mintz) that may be responsible for dissolution of the zona pellucida and was assumed to be identical with a proteinase found there (Pinsker, Hoversland and Weitlauf, Dabich, reviewed by Denker 1977, 1983). However, the most detailed studies on functional aspects have been performed in the rabbit. Blastolemmase is the only proteinase for which a physiological role in implantation initiation could be shown convincingly by in vivo experiments. The analytical studies (Denker 1976, 1977) had provided us with a list of non-toxic but highly potent inhibitors for blastolemmase. Such inhibitors were administered intrauterally, either shortly (12 h) before implantation initiation by intrauterine bolus injection (Denker 1977) or by permanent intrauterine infusion using the Alzet® minipump (Alza Corporation) (Meinshausen 1982, Meinshausen and Denker 1983). With this type of approach one obtains very impressive effects: the dissolution of the blastocyst coverings is very effectively inhibited. Two days after implantation has been initiated in the controls, most blastocysts in the inhibitor-treated uteri show severe impairment of implantation, i.e. blastocysts are either still completely free in the uterine lumen, or only few of their trophoblastic knobs have managed to attach and to invade. Particularly impressive are those blastocysts which remain completely surrounded by their coverings: their differentiation proceeds in spite of proteinase inhibitor treatment, and neural tube, somites, exocoelom and so a may develop (Fig 2). Thus the treatment has not led to any major damage to the blastocyst so that non-specific toxic effects can largely be excluded. The blastocyst coverings do not remain complete in all blastocysts, however: due to continuing expansion, they are often shed mechanically which can be recognized since the remnants of the coverings then show sharp edges in contrast to enzymatically lysed coverings. In that case, attachment and invasion of the trophoblast become basically possible wherever no remnants of the coverings remain interposed. Nevertheless the number of attaching trophoblastic knobs remains reduced in the abembryonic hemisphere possibly due to a "missed bus" phenomenon. No inhibition of attachment and invasion of trophoblast could be observed in the second (chorioallantoic) phase, i.e. of trophoblast surrounding the embryonic disc (Meinshausen 1982).

These investigations have shown that blastolemmase plays a central role in one essential process in implantation initiation, i.e. the dissolution of blastocyst coverings, in the rabbit. They may also suggest strongly, that, on the other hand, blastolemmase is not of major importance for the subsequent step, the attachment of the trophoblast to the surface of the uterine epithelium followed by invasion into the endometrium.

On contrast, the physiological role of rabbit "proteinase X"-type enzymes, as found in endometrial homogenates and in the uterine lumen, remains completely unknown so far. The stage-dependent appearance of the enzyme(s) in the uterine lumen and the activation phenomenon mentioned above suggest a role in preimplantation development and/or implantation. A preliminary series of experiments on the effects of the intrauterine administration of appropriate inhibitors in vivo has, again in contrast to blastolemmase, not shown any clearcut effects on implantation. Possibly more potent inhibitors and higher doses than in case of rabbit blastolemmase would be needed since the number of enzyme molecules per uterus and possibilities for compensatory overproduction are probably higher in case of such an endometrium-derived enzyme. A possibility is that "proteinase X" may be involved in dissolution of the zona pellucida inside the mucoprotein layer during early intrauterine life of rabbit blastocysts (see above and Fig 1). Also, it cannot be excluded that the enzyme may play a role in modifying uterine epithelial cell membranes as an element of endometrial "receptivity", i.e. by cleaving off ectodomains of integral membrane proteins (see aminopeptidase studies, below). All these aspects will deserve to be investigated further.

Trophoblast Attachment and Endometrial "Receptivity"

Since the removal of the zona pellucida/blastocyst coverings discussed above occurs at different time points in different species and does not necessarily precede implantation immediately, that

process may not be as crucial for implantation initiation in all species. However, attachment of the trophoblast to the surface of the endometrium is an essential element of implantation in all species (even including the superficial type of implantation as seen in e.g. the pig). We have, therefore, in recent years concentrated our efforts on the cell biological basis of this process.

Lectin Binding Studies

Cell adhesion has been shown to depend on cell surface bound carbohydrate groups in various systems. It has often been proposed that this may also apply to trophoblast adhesion to uterine epithelium in the initial phase of embryo implantation. Our special interest focussed on the stage-specific alterations of rabbit endometrium in the pre- and periimplantation phase of pregnancy and pseudopregnancy assuming that the so-called "receptive phase" is characterized by changed cell surface glycoprotein patterns, resulting in a different carbohydrate composition of the glycocalix. This was studied by the binding of appropriate lectins linked to marker molecules (Nalbach, 1985, Nalbach, Denker 1983, Bükers et al in press).

Seven different FITC-conjugated lectins (WGA, SBA, UEA-I, GSI B4, PNA, RCA-I, DBA) with partially overlapping specificity for different monosaccharide termine were imployed. A general trend was observed towards an increased binding of lectins recognizing galactose (Gal), N-acetylgalactosamine (Gal-NAc) and N-acetylglucosamine (Glc-NAc) at the apical plasma membrane region when time of implantation (7 d p.c.) approached. From then on the placental folds of the implantation chamber (mesometrial part of endometrium) were found to react differently from the (antimesometrial) obplacental and the paraplacental folds. While the reaction on the placental fold decreased rapidly within one day (7–8 d p.c.) the reaction on the other parts of the endometrium increased further. This observation could not be made for the distribution pattern of L-fucose (UEA I).

However, the reactivity within the group of Gal-NAc/Gal recognizing lectins was not, as should be expected, identical. RCA I, GSI B4, PNA and SBA receptor showed the most striking differences between the placental fold and the other parts of the implantation chamber. Binding of PNA, in contrast to the other lectins, decreased towards implantation time at the apical domain of the luminal epithelium and increased at the epithelium in the deep crypts. Since those lectin binding studies were performed with cryostat sections it was not always possible to determine whether the lectin receptor is restricted to the plasma membrane or may also be located in the adjacent cytoplasm.

The general conclusion drawn from our results is that there must be an impressive remodeling of carbohydrate binding sites during the pre- and periimplantation stages in the uterine epithelium of the rabbit. While this is in agreement with data from literature, there are quite important differences in the details as well as in the interpretations derived from them. Other authors (Chavez and Anderson1985, Anderson et al 1986, Chavez in press, Hoffman et al in press) have concluded, largely on the basis of lectin binding studies in situ and using protein fractions, that terminal Gal-containing glycoproteins of the cell surfaces are positively correlated with and play a functional role in endometrial "receptivity" and trophoblast attachment, in spite of the fact that a general trend towards a reduction of the thickness of the glycocalix of the uterine epithelium towards implantation and, in particular, in the implantation chamber was noted. The observations reported here lead to a somewhat different view in particular with respect to attachment of the (embryonic pole) trophoblast during chorioallantoid placentation: At the placental folds, binding of all Gal, Gal NAc and Glc-specific lectins (i.e. including Gal) decreases towards implantation time, but this is where the trophoblast attaches; at the paraplacental folds which are located in immediate vicinity, lectin binding increases, but here attachment of the embryonic pole trophoblast was never seen. Therefore, without negating a possible role of Gal in these processes, these results suggest that induction of Gal-containing glycoconjugates at the cell surface cannot explain the phenomena satisfactorily. Rather they fit into a recently developed concept postulating that, when the uterine epithelium enters the

state of "receptivity", its apical plasma membrane looses part of its apical-type characteristics including the brush border-type glycocalix (Denker 1986, see also below).

Nevertheless, the concept that glycoproteins are involved somehow in implantation remains of interest. These studies are recently being complemented by an investigation of endogenous lectin-like molecules that can be found in rabbit endometrium, uterine secretion and blastocysts (Biermann, Gabius and Denker in preparation). This will hopefully shed light on the validity of the hypothesis that lectin-glycoprotein interactions may be involved in processes like formation of new layers of blastocyst coverings or of trophoblast attachment to the endometrium (Denker 1983).

Development of an In Vitro Model System for Studies on Embryo Implantation

Experimental investigation of the cell biology of trophoblast-endometrial interactions would be much facilitated by the availability of an in vitro model for embryo implantation. We have attempted to develop such a model using rabbit endometrium kept in organ culature, and rabbit blastocysts. The feature that is different in this system as compared to other organ culture models is that endometrial fragments (composed of epithelium and stroma) are not kept stationary at the liquid-air interface but submerged in shaker culture, and that a preculturing is included in which a complete epithelial lining is restored before confrontation with blastocysts is started.

Endometrial fragments of about 1 to 2 mm diameter comprising stroma and epithelium were explanted from pseudopregnant animals 4 to 5 days after injecton of hCG and maintained in organ culture on an orbital shaker at 37° C in a humidified atmosphere with 5% CO_2. Within the first two days the epithelium that was damaged to a limited degree during explantation regenerates and reconstitutes a complete epithelial lining even in the area of the former wound (Denker et al 1984, Hohn et al 1984, Hohn et al in press). The stroma largely keeps its normal architecture. A typical response to steroid hormones in the rabbit uterus during pregnancy is the formation of large symplasms in the epithelium. This morphological transformation

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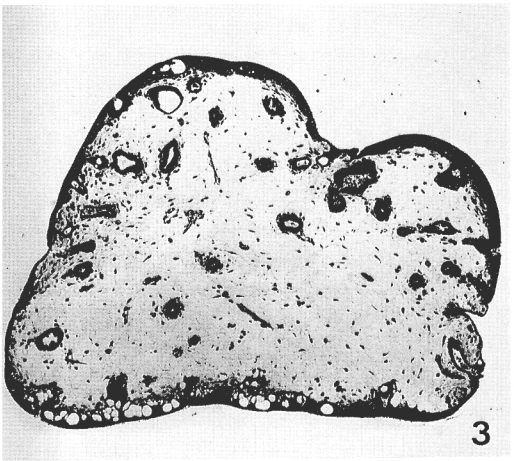
Plate 2: In vitro model for studies on embryo implantation in the rabbit. Fig 3: Endometrial fragment (antimesometrial side) explanted at 5 d of pseudopregnancy and cultured for 2 days in shaker culture with progesterone (32 nMol/l) in MEM with 10% fetal bovine serum. The uterine epithelium has regenerated a complete lining and has, in response to progesterone, transformed into large symplasms. The latter are particularly well developed in this specimen and show already vacuolar degeneration (below) as seen in vivo in the implantation chamber. The stroma appears healthy, no central necrosis. Semithin section, toluidine blue, x 67. **Fig 4:** Scanning electron micrograph of epithelium of an endometrial fragment as in Fig 3. Large symplasms have formed as a response to progesterone (3.2 nMol/l). Cell in the mouth of a crypt and ciliated cells did not fuse. x 1700 (after Hohn et al, in press). **Fig 5:** Embryo co-cultured (without physical contact) with endometrium. Endometrium explanted at 4 1/2 d was first precultured for 2 days (as in Fig 3); then blastocysts explanted 6 1/2 d p.c. were synchronously co-cultured with these fragments for 3 days. The embryonic anlage has attained an advanced state of differentiation: neural folds (above), closing neural tube and somites (middle), anmiotic fold. A plate of thick syncytiotrophoblast surrounds the embryo proper in a horseshoe-shaped configuration. Total preparation, unstained, x 55. **Fig 6:** Not only the embryonic anlage proper develops remarkably well in co-culture with precultured endometrium but also the trophoblast differentiates further. Abembryonic region of a blastocyst as in Fig 5, showing well-developed trophoblastic knobs which partly merge in a (incomplete) ring-like zone surrounding the abembryonic pole. Total preparation, unstained, x 35. **Fig 7:** Attachment of trophoblast to endometrium in confrontation culture. Endometrial fragments were explanted at 4 2/3 d and precultured for 2 days as in Fig 3 and were then combined with a freshly explanted synchronous (6 2/3 d) blastocyst in a mini-chamber securing tight contact, for 2 days. Part of the abembryonic hemisphere of the blastocyst (upper right) can be seen in this section, and part of an endometrial fragment (lower left). Of the 4 trophoblastic knobs, one has attached and fused with the uterine epithelium as proven by TEM. Morphology is largely similar to the in vivo situation. Semithin section, toluidine blue, x 140. **Fig 8:** High magnification of in vitro "implantation" site shown in Fig 7. The fusion product of trophoblast plus uterine epithelium has started to penetrate through the endometrial basal lamina into the stroma. TEM, x 27600.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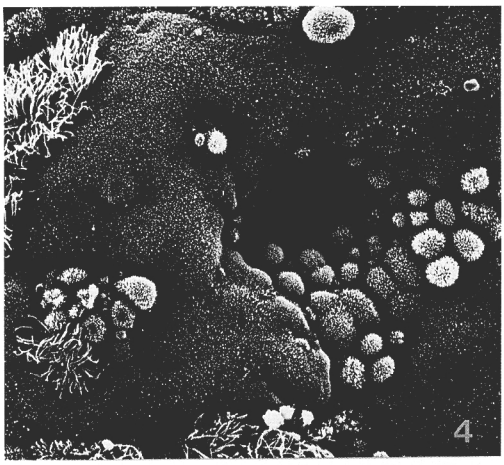
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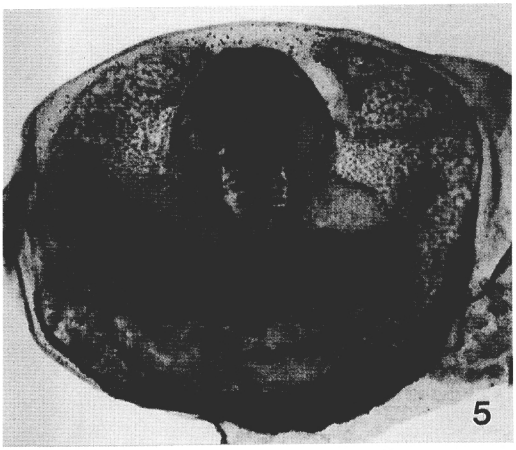
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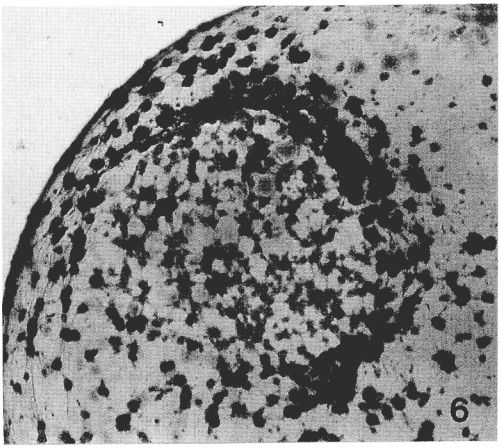
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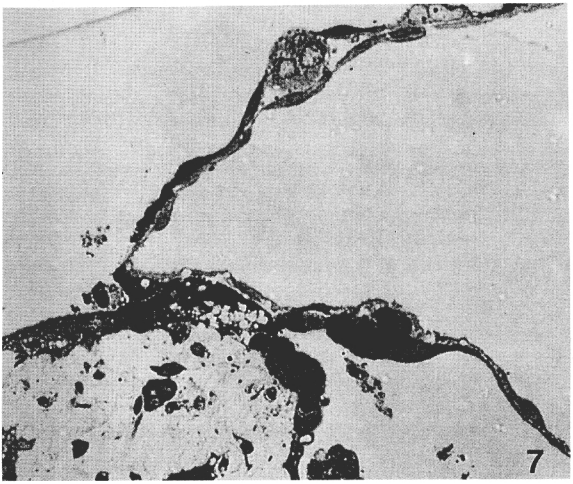
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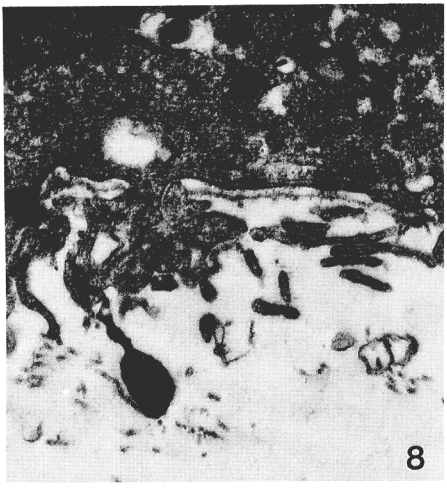
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was obtained when the culture medium was supplemented with progesterone. The extent and the time course of symplasm formation corresponded to the in vivo situation in the implantation chamber (Fig 3, 4).

The development of blastocysts explanted 6 1/2 d p.c. was heavily retarded if they were kept under the same conditions without endometrium, and signs of degeneration were pronounced in the embryonic disc, a well-known in vitro phenomenon. The retardation of development was substantially decreased when blastocysts were transferred after explantation to synchronously developing endometrial fragments that had been precultured for two days and both were kept in co-culture free floating in the medium (Hohn et al, in preparation). The benficial influence of this co-culture system on the embryonic development is indicated by the formation of somites, of neural folds and of the neural tube (Fig 5). Of particular interest for the use of such blastocysts for subsequent studies on implantation in vitro is that syncytiotrophoblast develops well, in this co-culture, in both, the abembryonic hemisphere (trophoblastic knobs, Fig 6) and at the embryonic pole.

Implantation in vitro was obtained only when blastocysts and endometrial fragments were kept constantly in a close contact as they are in the uterus (Fig 7). This was achieved by enclosing them both in dialysis tubings (Hohn, Denker 1985, Hohn et al in press). The blastocyst coverings appeared to be shed in this system either by mechanical disruption or enzymatically. Another important prerequisite for the attachment of endometrial fragments to blastocysts was the continuing expansion of the blastocyst. Evidence for the fusion of trophoblast with symplasms of the uterine epithelium could be demonstrated by electron microscopic examination. As can be seen from the penetration of the uterine basement membrane by processes of the fusion product of trophoblast and uterine epithelium, this in vitro model system does allow to study not only the phases of attachment and fusion but also the incipient invasion into the uterine stroma (Fig 8).

To obtain further information about the relevance of cell surface molecules for the attachment phase of implantation we compared the lectin binding pattern in vivo with the reaction found on the cell membranes of fragments grown in vitro. The fragments from antimesometrial and mesometrial origin were cultured separately. Surprisingly the reactivity for RCA I and SBA on the apical plasma membrane decreased during the first 2–4 days of culture and kept stable afterwards. 5 d explanted fragments showed higher reactivity than 3 d explanted fragments. The binding patterns showed no differences between fragments from mesometrial and antimesometrial origin. The loss of lectin-binding in vitro resembles the behaviour on the placental fold of the implantation chamber where definitive chorioallantoic implantation takes place. It could be argued that this may be the reason why trophoblast attachment was obtained in this in vitro system. However, some details need to be clarified, e.g. only abembryonic trophoblast was found to attach although in vivo this part makes contact normally with the antimesometrial endometrium which has a different lectin binding pattern.

These described interactions between trophoblast and uterine epithelium in our in vitro model appear to show a degree of cell and species specificity (Hohn et al 1985, 1987). Endometrial fragments that were receptive for the attachment of the trophoblast were confronted with spheroids of different tumor cell lines from different origin and tissue type. The attachment of tumor spheroids to endometrial fragments was only possible in areas were the uterine stroma was exposed to direct contact with tumor cells. The intact epithelium, however, allowed only for a loose adherence of the spheroids in a minority of confrontations. Such a "specific receptivity" was not found in in vivo experiments performed in rats and mice where the uterine epithelium at the implantation phase is very labile (Blandau 1949). Here tumor cells transferred into the uterine cavity had been found to invade the uterine stroma very easily via the epithelium during the implantation phase (Wilson, Potts 1970, Short, Yoshinaga 1967). This degree of specificity suggests that the developed in vitro culture system can yeald results that may be less prone to artifacts than those obtained with some of the earlier systems,

Table 1

Preimplantation Phase:	Blastocyst Coverings (Zona pellucida) preserved, transformed, reinforced (Barrier for cell-cell contact)
Implantation Phase:	Shedding of Blastocyst Coverings Lysis (Proteinases!) or Mechanical Hatching
	Epithelial Interactions (Trophoblast and Uterine Epithelium) Attachment Penetration
	Invasion into the Stroma Blood Vessels

and that it may be of value for studies on the cell biology of hormonal responses of the endometrium as well as of trophoblast-endometrial interactions at implantation.

Conclusion

Although our knowledge of basic processes involved in cell-cell interactions at embryo implantation does still remain fragmentary, first contours of a scenario become discernible. Progress has been made in our understanding of most of the individual processes listed, in chronological order, in Table 1, except for the last phases, i.e. invasion sensu strictu and interaction with the vascular system. However, the first phases appear to be most relevant for the regulation of the whole process.

For the preimplantation phase, the traditional view that the zona pellucida is just a vestige from oogenesis and cleavage stages, has to be revised. In many if not most species it undergoes considerable chemical and (in some species) also structural changes and may be replaced by new material, e.g. neozona in the rabbit and capsule in the horse. It appears reasonable to refer to these external coatings of blastocysts as "blastocyst coverings" rather than zona pellucida. The complex structural changes that they undergo point to important physiological functions (morphogenetic, immunological, mechanical) that still have to be defined. Defective transformation of these coverings may serve as a useful indicator for pathological conditions (e.g. in in vitro culture). The mechanisms for the reinforcement of the coverings during preimplantation development need still be elucidate (lectin-glycoprotein precipitation, cross-linked by covalent bonds?).

The central role of blastolemmase in the proteolytic degradation of blastocyst coverings at the initiation phase of implantation was a major finding during earlier years in this series of investigations. Blastolemmase so far remains the only biochemically identified factor for which a role in implantation could be demonstrated experimentally. Leaving aside the hormonal control, inhibition of blastolemmase activity is still the only approach by which one of the processes involved in implantation can be tackled specifically. However, the site of synthesis of blastolemmase (trophoblast, endometrium) and the regulation of its activity still have to be studied. Preliminary data on other proteinases found at implantation sites suggest that we are dealing with a proteinase system that becomes activated at implantation. It appears promising to investigate the interplay between the various components of that system in particular with respect to activation/inactivation phenomena. The limited comparative studies which have been performed show clearly that considerable inter-species differences exist so that with respect to reproductive medicine it appears mandatory to perform studies with human material.

The mechanisms behind the next step after dissolution of blastocyst coverings, the adhesion of the trophoblast to the apical plasma membrane of the uterine epithelium, has long remained enigmatic, but it appears that we are just starting to find definitive clues. While the nature of trophoblast invasiveness still remains as unexplained as invasiveness of malignant tumor cells, we are beginning to get a better understanding of what may be going on in the uterine epithelium when the endometrium enters the "receptive state". Lectin binding studies show that there are very distinct changes in the glycocalix. Some of these changes may be due to de novo expression of cell recognition/adhesion molecules. However, a great number of data compiled recently strongly suggests that the changes in the uterine epithelium are much more fundamental and general (Denker 1986): A partial loss of elements of its apico-basal polarity can be discerned in all parts of the epithelial cell (apical and basolateral membrane domain, cytoskeleton, distribution of organelles, intracellular transport). This concept predicts that changes in the apical plasma membrane in preparation for trophoblast attachment comprise a more generalized loss of apical-type characteristics (which was indeed found, Classen-Linke et al 1987, Winterhager 1985) and that this may be linked to acquisition of basolateral-type characteristics including adhesion molecules. This prediction will lead to look for specific classes of molecules that one may expect to become expressed at the apical plasma membrane of the uterine epithelium in the "receptive phase". It would allow to take a new look at the possible mechanism behind this change since, according to that concept, it must be linked to a change in intracellular sorting of membrane precursors. In analogy to other cell biological systems, this concept allows to design new experimental approaches to the study of endometrial "receptivity" that could ultimately lead to the development of new therapeutical concepts how to influence endometrial "receptivity" by non-hormonal means.

The novel endometrial organ culture model that has been designed in this programme promises to be a valuable tool for studies of molecular mechanisms involved in trophoblast attachment and endometrial "receptivity", in particular since it seems to show a high degree of selectivity for the cell type (trophoblast) as well as for the species. Ongoing experiments suggest that the system is adaptable to the use of human endometrium and human trophoblast cell lines so that it should be of use for future studies of trophoblast-endometrial interactions in the human.

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References

Adams CE (1973) The development of rabbit eggs in the ligated oviduct and their viability after re-transfer to recipient rabbits. J. Embryol 29: 133–144

Anderson TL, Olson GE, Hoffman LH (1986) Stage-specific alterations in the apical membrane glycoproteins of endometrial epithelial cells related to implantation in the rabbit. Biol Reprod 34: 701–720

Blandau RJ (1949) Embryo-endometrial interrelationship in the rat and guinea pig. Anat Rec 104: 331–359

Böving BG (1963) Implantation mechanisms. In: Hartman CG (ed) Conference on Physiological Mechanisms Concerned with Conception. Pergamon Press, Oxford, London, New York, Paris, pp 321–396

Bükers A, Friedrich J, Nalbach BP, Denker H-W (in press) Changes in lectin binding patterns in rabbit endometrium during pseudopregnancy, early pregnancy and implantation. In: Denker H-W, Aplin JD (eds)

Trophoblast Invasion and Endometrial Receptivity: Novel Aspects of the Cell Biology of Embryo Implantation (Trophoblast Res, Vol 4). Plenum Press, New York
Chavez DJ (in press) Possible involvement of D-galactose in the implantation process. In: Denker H-W, Aplin JD (eds) Trophoblast Invasion and Endometrial Receptivity: Novel Aspects of the Cell Biology of Embryo Implantation (Trophoblast Res Vol 4). Plenum Press, New York
Chavez DJ, Anderson TL (1985) The glycocalyx of the mouse uterine luminal epithelium during estrus, early pregnancy, the peri-implantation period, and delayed implantation. I. Acquisition of Ricinus communis I binding sites during pregnancy. Biol Reprod 32: 1135–1142
Classen-Linke I, Denker H-W, Winterhager E (1987) Apical plasma membrane-bound enzymes of rabbit uterine epithelium. Pattern changes during the periimplantation phase. Histochemistry 87: 517–529
Denker H-W (1971a) Substratfilmtest für den Proteasennachweis. Acta histochem Suppl X: 303–305
Denker H-W (1971b) Enzym-Topochemie von Frühentwicklung und Implantation des Kaninchens. III. Proteasen. Histochemie 25: 344–360
Denker H-W (1972) Blastocyst protease and implantation: effect of ovariectomy and progesterone substitution in the rabbit. Acta Endocr 70: 591–602
Denker H-W (1974a) Protease substrate film test. Histochemistry 38: 331–338 and 39: 193
Denker H-W (1974b) Trophoblastic factors involved in lysis of the blastocyst coverings and in implantation in the rabbit: observations on inversely orientated blastocysts. J Embryol exp Morph 32: 739–748
Denker H-W (1976) Interaction of proteinase inhibitors with blastocyst proteinases involved in implantation. In: Peeters H (ed) Protides of the Biological Fluids – 23rd Colloquium. Pergamon Press, Oxford, New York, pp 63–68
Denker H-W (1977a) Implantation: the role of proteinases, and blockage of implantation by proteinase inhibitors. Springer-Verlag, Berlin, Heidelberg, New York (= Adv Anat Embryol Cell Biol 53, Fasc 5)
Denker H-W (1977b) Zur Spezifität und Empfindlichkeit des Proteasen-Substratfilmtests auf Gelatinebasis. Acta histochem, Suppl XVIII: 153–158
Denker H-W (1982) Proteases of the blastocyst and of the uterus. In: Beier HM, Karlson P (eds) Proteins and Steroids in Early Pregnancy. Springer-Verlag, Berlin, Heidelberg, New York, pp 183–208
Denker H-W (1983) Basic aspects of ovoimplantation. In: Wynn RM (ed) Obstetrics and Gynecology Annual Vol 12. Appleton-Century-Crofts, Norwalk, Connecticut, pp 15–42
Denker H-W (1986) Epithel-Epithel-Interaktionen bei der Embryo-Implantation: Ansätze zur Lösung eines zellbiologischen Paradoxons. Verh Anat Ges 80 (Prag 1985), Anat Anz Suppl 160: 93–114
Denker H-W, Betteridge KJ, Sirois J (1987a) Shedding of the 'capsule' and proteinase activity in the horse embryo. J Reprod Fert, Suppl. 35: 703
Denker H-W, Busch LC, Kühnel W (1984) Endometrial organ culture: development of an in-vitro model for embryo implantation. Anat Anz, Jena 156: 142
Denker H-W, Eng LA, Hamner CE (1978) Studies on the early development and implantation in the cat. II. Implantation: proteinases. Anat Embryol 154: 39–54
Denker H-W, Fritz H (1979) Enzymic characterization of rabbit blastocyst proteinase with synthetic substrates of trypsin-like enzymes. Hoppe-Seyler's Z Physiol Chem 360: 107–113
Denker H-W, Gerdes H-J (1979) The dynamic structure of rabbit blastocyst coverings. I. Transformation during regular preimplantation development. Anat Embryol 157: 15–34
Denker H-W, Hafez ESE (1975) Proteases and implantation in the rabbit: role of trophoblast vs. uterine secretion. Cytobiologie 11: 101–109
Denker H-W, Hohn H-P, Winterhager E (1987b) Dreidimensionale Aggregate von Chorionkarzinomzellen als ein In-vitro-Modell für den invasiven Trophoblasten. Verh Anat Ges 81, Anat Anz, Suppl 162: 499–500
Finn CA (1977) The implantation reaction. In: Wynn RM (ed) Biology of the uterus. Plenum Press, New York, pp 245–308
Fischer B (1988) Charakterisierung entwicklungsspezifischer Strukturen und Lebensäußerungen von Präimplantationsembryonen in vitro und in vivo. Habilitationsschrift, Medizinische Fakultät, RWTH Aachen
Fischer B, Denker H-W, Beier HM, Gerdes H-J (1983) Der Umbau der extrazellulären Blastozystenhüllen als ein empfindlicher Indikator für unphysiologische Milieubedingungen in der In-vitro-Kultur. Verh Anat Ges 77, Anat Anz, Suppl 154: 427–429
Hoffman LH, Winfrey VP, Anderson TL, Olson GE (in press) Uterine receptivity to implantation in the rabbit: evidence for a 42 kDa glycoprotein as a marker of receptivity. In: Denker H-W, Aplin JD (eds) Trophoblast Invasion and Endometrial Receptivity: Novel Aspects of the Cell Biology of Embryo Implantation (Trophoblast Res Vol 4). Plenum Press, New York
Hohn H-P, Busch LC, Denker H-W (1984) Endometrium in organ culture: a model host tissue for studies of trophoblast invasion. Eur J Cell Biol 33, Suppl 5: 17
Hohn H-P, Denker H-W (1985) Attachment and invasion of rabbit blastocysts confronted with endometrium in organ culture. Eur J Cell Biol 36, Suppl 7: 28
Hohn H-P, Denker H-W (in press) A three-dimensional organ culture model for the study of implantation of rabbit blastocusts in vitro. In: Denker H-W, Aplin JD (eds) Trophoblast Invasion and Endometrial Re-

ceptivity: Novel Aspects of the Cell Biology of Embryo Implantation (Trophoblast Res Vol 4). Plenum Press, New York
Hohn H-P, Donner A, Denker H-W (1985) Evaluation of an in vitro model for embryo implantation: Selective receptivity of rabbit endometrium for trophoblast attachment. Eur J Cell Biol 39, Suppl 12: 18
Hohn H-P, Donner A, Denker H-W (1987) Specific receptivity of rabbit endometrium for attachment of trophoblast but not of tumor cells in vitro. J Cell Biol 105: 306a
Hohn H-P, Winterhager E, Busch LC, Mareel MM, Denker H-W (in press) Rabbit endometrium in organ culture: Morphological evidence for progestational differentiation in vitro. Cell Tissue Res.
Johnson MH, Chisholm JC, Fleming TP, Houliston E (1986) A role for cytoplasmic determinants in the development of the mouse early embryo? J Embryol exp Morph 97, Suppl: 97–121
Kirchner C (1972) Uterine protease activities and lysis of the blastocyst covering in the rabbit. J Embryol exp Morph 28: 177–183
Lehtonen E (1980) Changes in cell dimensions and intercellular contacts during cleavage-stage cell cycles in mouse embryonic cells. J Embryol exp Morph 58: 231–249
Leiser R, Denker H-W (1988) The dynamic structure of rabbit blastocyst coverings. II. Ultrastructural evidence for a role of the trophoblast in neozona formation. Anat Embryol 179: 129–134
Meinshausen E (1982) Beeinflussung der Embryo-Implantation durch fortlaufende intrauterine Verabreichung eines Proteinaseinhibitors. Experimentelle Untersuchungen am Kaninchen. Dissertation, Medizinische Fakultät, RWTH Aachen
Meinshausen E, Denker H-W (1983) Entwicklungsleistungen von Kaninchenembryonen trotz Hemmung der Anheftung in utero. Verh Anat Ges 77: 421–423
Nalbach BP (1985) Lektinbindungsmuster in Uterus und Blastozyste des Kaninchens während der Präimplantationsphase und der frühen Implantation. Histochemie und Methodenkritik. Dissertation, Medizinische Fakultät, RWTH Aachen
Nalbach BP, Denker H-W (1983) Stage-dependent changes in lectin binding patterns in rabbit uterus and blastocyst during the preimplantation period and implantation. Eur J Cell Biol, Suppl 4: 13
Psychoyos A (1976) Hormonal control pf uterine receptivity for nidation. J Reprod Fertil, Suppl 25: 17–28
Short RV, Yoshinaga K (1967) Hormonal influences on tumour growth in the uterus of the rat. J Reprod Fert 14: 287–293
Strickland S, Reich E, Sherman MI (1976) Plaminogen activator in early embryogenesis: Enzyme production by trophoblast and parietal endoderm. Cell 9: 231–240
Tisljar U, Denker H-W (1985) Partial purification and characterization of a trypsin-like proteinase from rabbit preimplantation uterine fluid. Biol Chem Hoppe-Seyler 366: 1053–1055
Warner CM, Brownell MS, Ewoldsen MA (1988) Why aren't embryos immunologically rejected by their mothers? Biol Reprod 38: 17–29
Wilson IB, Potts DM (1970) Melanoma invasion in the mouse uterus. J Reprod Fert 22: 429–434
Winterhager E (1985) Dynamik der Zellmembran: Modellstudien während der Implantationsreaktion beim Kaninchen. Habilitationsschrift, Medizinische Fakultät, RWTH Aachen.

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