CHANGES OF INTERMEDIATE FILAMENT PROTEIN LOCALIZATION IN ENDOMETRIAL CELLS DURING EARLY PREGNANCY OF RABBITS

Axel Hochfeld, Henning M. Beier, and Hans-Werner Denker

Institut für Anatomie
Medizinische Fakultät der RWTH Aachen
Melatener Strasse 211
D-5100 Aachen, Federal Republic of Germany

1Lehrstuhl für Anatomie und Reproduktionsbiologie
Medizinische Fakultät der RWTH Aachen

2Lehr- und Forschungsgebiet Anatomie und Reproduktionsbiologie
Medizinische Fakultät der RWTH Aachen

INTRODUCTION

Implantation is initiated by an interaction of trophoblast with the uterine epithelium via the apical cell poles of both partners. Aspects of changes that must take place in the glyocalyx and in the plasma membrane, to allow this process to be initiated are discussed in other contributions to this volume. There appears to be good reason, however, to bring the cytoskeleton into the picture, since findings obtained in other systems give strong evidence for interactions between it and the cell membranes (Tachi et al., 1970; Jones and Goldman, 1985; Perides et al., 1986a,b; Traub et al., 1987; Lazarides, 1980, for review see: Cowin et al., 1985; Geiger et al., 1985). There are only a few reports on the occurrence of intermediate filaments (IF) in the uterine epithelium. Franke et al. (1986) have studied IF in the proliferative phase of the human endometrial epithelium. Khong et al. (1986) concentrated on the expression of IF in the placenta, amniochorion, and placental bed in humans and Dabbs et al. (1986) used IF as diagnostic tools for histologic differentiation of uterine adenocarcinomas. Viale et al. (1988) described coexpression of vimentin and keratin in endometrial glands.

At present there is no literature concerning the distribution of IF in the uterine epithelium in the phase when attachment is being initiated between it and the trophoblast. In this study, regional distribution and cellular expression of vimentin as a marker for mesenchymal cells, and cytokeratins for epithelial cells, has been investigated during this phase. Also there was interest in the localization characteristics of these two intermediate filament proteins in the corresponding phases of pseudopregnancy. Specifically, the following questions were asked: Are there differences in the distribution between pregnancy and pseudopregnancy? Does the blastocyst influence the maternal cytoskeleton of the uterine epithelium?

3 Present address: Institut für Anatomie, Lehrstuhl für Anatomie und Entwicklungsbio logie, Universitätsklinikum, Hufelandstrasse 55, D-4300 Essen, Federal Republic of Germany
MATERIALS AND METHODS

Rabbits were caged as described by Fischer and Meuser-Odenkirchen (1988). Does were mated to two fertile bucks. Pseudopregnancy was induced by intravenous injection of 75 I.U. of human chorionic gonadotropin (hCG) (Prolan®, Bayer AG, Leverkusen, FRG). The day of mating and of hCG injection was designated day 0 of pregnancy/pseudopregnancy. Two rabbits each were killed by stunning and exsanguination at 3, 4, 5, 6, 7, 8, and 9 days post coitum (p.c./post hCG (p.hCG). The uteri were quickly removed, cut into pieces, frozen unfixed in liquid nitrogen and stored at -25°C to -30°C in air tight plastic bags until use.

Uterine ligation was performed under thiobarbital anesthesia (Thiogenal®, Merck, 40 mg/kg body weight given i.v. plus 20 - 40 mg/kg body weight i.p. during surgery) after perphenazine premedication (Decentan®, Merck, Darmstadt, FRG, 5 mg/rabbit i.m.). Twenty-four hours after mating, one of the uteri was ligated near the uterotubal junction to prevent passage of embryos from the Fallopian Tube into the uterine lumen. Animals were killed 8 d p.c. The uteri were handled as described.

Sections (10 μm) were taken in longitudinal or transverse orientation, on a cryostat (Dittes Duspiva, Heidelberg, FRG) at -25°C, mounted on gelatin coated glass slides, and stored in phosphate buffered saline (PBS: Ca++ and Mg++ free, Seromed, Berlin, FRG) for about 10 minutes until use.

Immunofluorescence Staining

Unfixed uterine sections were incubated with: rabbit serum diluted 1:20 in PBS with 1.5% bovine serum albumin (BSA, Sigma No. A 7906, Deisenhofen, FRG), followed by first antibody: 1:40 diluted anti-keratin (KL4, Dianova, Hamburg, FRG, reacting with polypeptides in the molecular range of 50 - 67 kilodaltons) or 1:40 diluted anti-vimentin (Dakopatts, Glostrup, Denmark, reacting with the 57 kilodaltons protein) 2 hours at room temperature. Controls were incubated with mouse serum (1:100) instead. After washing with 4 changes of PBS for 10 minutes each, 1:40 diluted FITC-conjugated rabbit anti-mouse IgG (F 232, Dakopatts, Glostrup) was applied for 1 hour in the dark at room temperature. Sections were again washed in 4 changes of PBS. The sections were then mounted in glycerol:PBS (9:1) and examined under a Zeiss photomicroscope II with an UV-epiilluminator and a filter set for FITC. Samples were photographed immediately after fluorescence-reaction on Kodak Tri X pan 400 film with standardized illumination and identical exposure time.

RESULTS

Distribution Of Vimentin

Myometrial smooth muscle cells and blood vessels were recognized by the vimentin antibody in all sections of uteri, regardless of the state of pregnancy and pseudopregnancy. The stromal cells were also positively stained with anti-vimentin in all cryostat sections. In pregnancy the cells of the endometrial epithelium showed stage-dependent alterations.
# Table 1

Intracellular Distribution Of Vimentin In Endometrial Epithelial Cells

<table>
<thead>
<tr>
<th>Day</th>
<th>Pseudopregnancy</th>
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<th>Pregnancy</th>
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<td>Luminal Epithelial Cells</td>
<td>Deepest Parts Of The Crypts</td>
<td>Interblastocyst Segments</td>
<td>Luminal Epithelial Cells</td>
<td>Deepest Parts Of The Crypts</td>
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Figure 1. Vimentin immunoreactivity. (a) Implantation chamber 7 days p.c. Epithelium (E) adjacent to the blastocyst shows an intense vimentin reaction in the apical cytoplasm of the luminal cells (arrows). Stromal components were also significantly stained (S). There is no reaction of the blastocyst coverings (arrowhead). (b) Phase contrast, X370.

At 3 days p.c. to 5 days p.c., there was only a weak vimentin reaction in the luminal epithelium, restricted to the basolateral regions of the cells. There was no reaction in the deepest part of the crypts (Table 1).

A difference from these early stages first became detectable at 6 days p.c. The epithelium surrounding the blastocyst in the implantation chamber was more intensely stained, but always mainly restricted to the basolateral regions of the epithelial cells.

At 7 days p.c. the anti-vimentin reaction in the implantation chamber became much more pronounced. The luminal epithelium was significantly more intensely stained in particular at the antimesometrial side. This anti-vimentin reaction was no longer restricted to the basolateral regions of the cells but rather vimentin-positive structures in the apical cytoplasm of the luminal cells were noted (Figure 1). In the deepest parts of the crypts, near the myometrium, still no positive vimentin reaction was visible (Table 1).
At 8 days p.c. the epithelial cells in the implantation chamber were strongly stained for vimentin in the apical cytoplasm. A moderate apical reaction could also be detected in the middle parts of the crypts. Again there was no reaction in the deepest parts of the crypts (Table 1).

Nine days p.c. the fluorescence reached a maximum in the epithelial cells surrounding the blastocyst. A massive band of fluorescence could be detected in the apical and basal cytoplasm of the luminal epithelium, clearly marked off from the uterine stromal cells (Figure 3). Decidual cells located near small blood vessels were positively stained with anti-vimentin.

In all states of pseudopregnancy from day 3 to day 9 p. hCG, only a delicate vimentin reaction in the luminal and middle parts of the endometrial epithelium was noted, corresponding with the observations in pregnancy from day 3 p.c. to day 5 p.c. As in pregnancy there was never any positive reaction in the deepest parts of the crypts (Figures 5 and 6).
Vimentin immunoreactivity. (a) Implantation chamber 9 days p.c.. Reaction reached a maximum in the apical and basolateral cytoplasm of the epithelial cells surrounding the blastocyst. There is a delicate immunofluorescence in mesoderm (arrowheads). Arrows = trophoblast; BC = blastocyst cavity; L = uterine lumen; S = stroma. (b) Phase contrast, X370.

Vimentin distribution in the interblastocyst segments of pregnant uteri was from day 6 p.c. to day 9 p.c. quite similar to the results in all stages of pseudopregnancy (Figures 2 and 4). After tubal ligation, the epithelium of the ligated side (no blastocyst present) showed a vimentin distribution which was identical with that of interblastocyst segments or of pseudopregnancy. At the contralateral side serving as a control (blastocysts present), the typical distribution pattern of vimentin with increased apical reaction of the epithelium of the implantation chamber was seen.

In the blastocyst there was no distinct vimentin reaction at 6 days p.c. and 7 days p.c., either in fragments of blastocyst coverings or in the trophoblast cells or endoderm. At 8 days p.c. and 9 days p.c. a delicate immunofluorescence was detected in extraembryonic cells other than trophoblast, probably extraembryonic mesoderm. Vimentin was not detected in trophoblast cells (Figure 3).

Distribution Of Cytokeratin

In pregnancy and pseudopregnancy an intense reaction for cytokeratin was noted in the endometrial epithelium, with a higher concentration in the subapical region. There were no significant differences in staining patterns between the various stages; also the deepest parts of the crypts showed the same cytokeratin reaction (Figures 7 and 8; Table 2). Other parts of the uterus including stromal cells or myometrium did not react with the cytokeratin antibody, except for the peritoneal epithelium.
Figure 4. Vimentin immunoreactivity. (a) Interblastocyst segments 9 days p.c.. There is again only a weak reaction in the basolateral regions of the epithelial cells (arrows) and a positive staining of the stromal components (S). (b) Phase contrast, X370.

Distribution Of Cytokeratin In The Blastocyst

In the blastocyst from 6 days p.c. on a faint cytokeratin reaction was visible only in trophoblast cells. Fragments of the zona pellucida showed no staining (Figure 8).

DISCUSSION

According to earlier investigations, the physiological and histological transformation of the rabbit endometrium seems to be very similar in pregnancy and pseudopregnancy (Beier and Kühnel, 1973; Busch, 1982; Winterhager, 1985) except for the implantation chamber (from 6 1/2 - 7 days p.c. on). Biochemical investigations, however, have revealed time-dependent quantitative differences in protein patterns between pregnancy and pseudopregnancy. These differences are obviously due to the presence or absence, respectively, of blastocysts (Beier et al., 1974, 1979).

From embryo transfer experiments it appears that the pseudopregnant uterus acquires characteristics of a pregnant uterus when blastocysts are surgically transferred at any stage between 3 1/2 and 6 days p.c. into such pseudopregnant milieu (Fischer, 1988). It is, however, not yet clear whether success of embryo transfer is only a matter of "appropriateness" of the pseudopregnant endometrium or whether this condition is triggered by the transferred blastocysts.
Table 2

Intracellular Distribution Of Cytokeratin In Endometrial Epithelial Cells

<table>
<thead>
<tr>
<th>Day</th>
<th>Pseudopregnancy</th>
<th>Pregnancy</th>
<th>Interblastocyst Segments</th>
<th>Implantation Chamber</th>
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<td>Apical Region</td>
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The possibility that embryonic signals trigger reactions of the endometrium has been discussed by Beier (1968), when protein patterns of rabbit uterine secretions were analyzed and compared. These investigations demonstrated that normal pregnancy patterns differed clearly from pseudopregnant patterns. Those differences suggested that embryonic signals act as conditioning factors for an appropriate preparation of the intrauterine protein milieu for preimplantation development and implantation (Beier, 1968).

The earliest differences between pseudopregnancy and pregnancy in the rabbit model have been found in the immediate vicinity of blastocysts from 6 d to 6 d 16 h p.c. when membrane-bound enzymes or gap junction proteins were investigated (van Hoorn and Denker, 1975; Winterhager et al., 1988). The results documented in the present communication for vimentin are quite consistent with these findings. During the preimplantation phase the histochemical findings are quite similar for pseudopregnancy and for pregnancy until 5 d p.c./p. hCG. The reaction is weak but clearly positive in the basolateral region of all epithelial cells. In both physiological states, the reaction remains negative in the deepest parts of crypt epithelia. This shows that the uterine epithelium consists of cell populations that clearly differ in their content of vimentin intermediate filaments, while appearing similar with respect to cytokeratin content.
Figure 6. Vimentin immunoreactivity. Overview over the implantation chamber, 8 days p.c. This photograph is from the non-ligated uterus. There is abundant vimentin in the apical regions of the luminal epithelium, but no reaction in the deepest parts of the crypts (arrowheads). (L = uterine lumen; E = luminal epithelial cells). X92.

There are other reports consistent with the finding that the uterine epithelium of the rabbit represents a combination of different populations of cells. Conti et al. (1984) have described a differential reaction of various cell types of the rabbit uterine epithelium to stimulation by ovarian steroid hormones. A differential response is also suggested to play a role for the outcome of in vitro culture experiments (Gerschenson et al., 1979; Mulholland et al., 1988). Histochemical investigations of in vivo material have revealed that deep parts of crypts react in a different manner as compared with the middle and upper parts of crypts and the surface epithelium of the uterus (phosphorylase: Denker, 1971;

In contrast to vimentin, cytokeratin immunoreactivity does not show any differences between the uterine luminal (surface) epithelium and any parts of the endometrial crypts including the deepest parts which are all likewise positive. In all investigated stages, cytokeratin immunoreactivity is dominant in the apical part of the cytoplasm of uterine epithelial cells. Cytokeratin filaments are here obviously attached to desmosomes (Franke et al., 1986; Lazarides, 1980; Gounon et al., 1987).

On the basis of these findings it appears that a combination of cytochemical tests for vimentin and cytokeratins is very useful for the identification of various populations of cells within the uterine epithelium of the rabbit. Interestingly, the lack of vimentin immunoreactivity seen in the deepest parts of cryptal epithelium remained constant throughout all investigated stages. This could be useful for the identification, in in vitro culture, of various endometrial cells since exclusively vimentin positivity is found in stroma cells; epithelial cells of middle and upper parts of crypts and of surface epithelium are positive for both vimentin and cytokeratin, whereas epithelial cells of deep crypts are negative for vimentin but positive for cytokeratin. Whether these characteristics remain stable in culture will have to be investigated further.

Figure 7. Cytokeratin immunoreactivity, 6 days p.c. Intense reaction only in the uterine epithelium including the deepest parts of the crypts.(BC = blastocyst cavity; S = stroma). X92
Figure 8. Cytokeratin immunoreactivity. (a) Interblastocyst segments, 6 days p.c.. In pregnancy and pseudopregnancy (not shown) a high cytokeratin reaction is noted in the subapical cytoplasm of the epithelial cells. (L = uterine lumen; S = stroma; E = epithelium). (b) Phase contrast, X370.

Changes of immunoreactivity of intermediate filament proteins in the rabbit uterus during early pregnancy and pseudopregnancy have not been described before. The presence of intermediate filaments in the uterine epithelium has been reported for a number of species, but data on any influence that the blastocyst might exert have been lacking (Franke et al., 1986; Khong et al., 1986). Franke et al. reported a case of well differentiated adenocarcinoma in the human where vimentin was found in both an apical and basal location within the cells. Glasser et al. (1986, 1987) reported an increase of intermediate filaments, namely vimentin and desmin, in rat uterine stromal cells undergoing decidualization in vivo and in vitro. They demonstrated that vimentin is common in all uterine stromal cells but increased during in vivo and in vitro decidualization. The increase in vimentin concentration per cell was found to be proportional to the increase in total cell protein. Desmin was selectively expressed in decidualizing stroma and may serve as a marker for it.
In the implantation chamber of the rabbit, i.e., in the immediate vicinity of the blastocyst, uterine epithelial vimentin is observed to show a change in the intensity of expression as well as in intracellular distribution from 6 days p.c. on, i.e., before attachment of the trophoblast to the uterine epithelium which occurs at 7 days p.c. Vimentin immunoreactivity increases in the basal parts of the epithelial cytoplasm, but, interestingly, also starts to become positive at the apical cell pole. At the end of the observation period, i.e., 9 days p.c., vimentin is abundant both apically and basally. The apical reaction is most impressive in the surface epithelium adjacent to the blastocyst but reaches also down into the middle parts of crypts. The deepest parts of crypts, however, remain negative even in these regions of the implantation chamber.

The described changes of vimentin reactivity in middle and upper parts of crypts and in the surface epithelium are not seen in inter-blastocyst segments of the uterus. This points to signals that are locally produced by the blastocyst and influence the state of the adjacent uterine epithelium.

Tubal ligation experiments were performed in order to demonstrate the importance of such locally acting signals from the blastocyst more clearly, within the same animal. The increase in vimentin reactivity, and in particular, the apical localization of vimentin was seen only in the implantation chambers of the uterus of the non-ligated side, whereas the blastocyst-free uterus at the ligated side showed the same picture as in pseudopregnancy. Thus the changes in vimentin expression and intracellular distribution depend clearly on the presence of blastocysts. The distribution of cytokeratins does not appear to change in the presence of an embryo.

Signals that the blastocyst might emit just before attaching to the uterine epithelium initiating implantation have often been postulated but have not been identified chemically (cf. Beier, 1984; Kennedy, 1983). Dickmann et al. (1976) have postulated that such signals are identical with steroid hormones produced by the blastocyst. However, although the blastocyst of various species has been shown to possess steroid metabolizing enzymes, actual steroid production by the blastocyst has been shown unequivocally only in the pig (Perry et al., 1973; Heap et al., 1979).

Alternatively, it is known that prostaglandins can exert various effects on the endometrium. It is very probable that cAMP acts as a second messenger for various reactions on the endometrium (Kasamo et al., 1986; Dey et al., 1980). Lazarides (1980) and Inagaki (1987) discussed that cAMP-dependent kinases may be involved in the regulation of cytoskeletal structures by affecting the phosphorylation of intermediate filament proteins, especially vimentin.

As far as the general function of intermediate filaments in uterine epithelial cells is concerned, their role for maintaining the mechanical stability of cells should be considered. Vimentin immunoreactivity was maximal in the large symplasms of uterine epithelium formed in the rabbit implantation chamber. After the lateral membranes have been lost due to cell fusion, intermediate filaments probably have important functions in stabilizing the multinuclear cytoplasmic masses formed in this way. Interestingly, the smaller symplasms formed during pseudopregnancy (Winterhager, 1985; Busch, 1982) do not show any change in
vimentin expression and its intracellular distribution, supporting the view that a
different type of symplasm is found in the implantation chamber.

An interesting question appears to be to what extent the changes in the
intermediate filament proteins described here may be related to membrane
changes taking place in uterine epithelial cells (discussed in other contributions to
this volume, cf. Classen-Linke et al., 1990; Winterhager and Denker, 1990;
Denker, 1986; Lampelo et al., 1985). According to Traub et al. (1987) neutral lipids
which are part of all cell membranes may be associated with vimentin filaments.
In this way vimentin filaments could influence the fluidity of membranes and the
lateral diffusion of integral membrane proteins. The polarity of epithelial cell
membranes could easily be influenced. Thus, the current results on changes in the
intracellular distribution of vimentin in the uterine epithelium in the
implantation chamber should be of relevance for the hypothesis that fundamental
changes in the composition of the apical plasma membrane of the uterine
epithelium are a prerequisite for trophoblast attachment, and that this depends on a
general change of apico-basal polarity characteristics of uterine epithelial cells
during this "receptive" phase (Denker, 1986).

SUMMARY

The localization of intermediate filament proteins was analyzed
immunohistochemically in the uterus and blastocyst during early pregnancy of the
rabbit. Monoclonal antibodies to vimentin (reacting with the 57 kilodalton protein)
and cytokeratin (reacting with polypeptides in the molecular range of 50 - 67
kilodaltons) were used. Uteri were investigated from 3 to 9 days post coitum (days
p.c.). These uteri were compared with specimens obtained in hCG-induced
pseudopregnancy (p. hCG) to demonstrate changes of intermediate filament
localization in endometrial cells which might be induced locally by blastocyst-
derived signals.

In all stages of pseudopregnancy from day 3 to day 9 p.hCG we noted a
delicate vimentin reaction in the basolateral compartments of uterine epithelial
cells which were situated mainly in the upper and luminal parts of the endometrial
crypts. An identical distribution was found in pregnancy at 3 to 5 days p.c. In the
deepest parts of the crypts no positive vimentin reaction was found at any stage of
pregnancy or pseudopregnancy.

However, at 7 days p.c. the anti-vimentin reaction in the epithelium
surrounding the blastocyst in the implantation chamber became much more
pronounced. This immunofluorescence reaction was no longer restricted to the
basolateral regions of the cells: vimentin-positive structures in the apical
cytoplasm of luminal cells, which reached a maximum at 9 days p.c., were also
noted.

An intensely positive reaction of cytokeratin was seen in all uterine
epithelial cells. There were no significant differences in staining patterns among
any stages of pregnancy or pseudopregnancy.
In the blastocyst, a faint cytokeratin reaction in trophoblast cells was recognized. Vimentin reacted positively in cells which likely represented the mesoderm.

The current results suggest the following: (1) The uterine epithelium of the adult rabbit (except for the deepest parts of endometrial crypts) coexpresses both types of intermediate filament proteins, cytokeratin, and vimentin; (2) the characteristic intracellular distribution as well as the total amount of vimentin change considerably in the uterine epithelium of the implantation chamber; (3) these changes are assumed to be the result of signals released from the implanting blastocyst; (4) vimentin seems to react to these postulated signals, whereas in contrast the class of cytokeratins investigated here does not show any change of intracellular distribution.

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Edited by

Hans-Werner Denker

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and

John D. Aplin

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