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Endometrial Receptivity: Selective Adhesion Competence of Rabbit Uterine Epithelium for Trophoblast but Not for Various Tumor Cells

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Key Words
Uterine epithelium · Cell-cell adhesion in vitro · Trophoblast · Tumor cells · Embryo implantation

Abstract
A modification of an established in vitro model for embryo implantation was used to probe the receptive uterine epithelium for any specificity of interaction with various invasive cells other than trophoblast. Endometrial explants consisting of stroma and epithelium taken from pseudopregnant rabbits were cultured in the presence of progesterone in order to regenerate a complete epithelial lining while maintaining the receptive state. Such precultured fragments were brought into contact with multicellular spheroids of different invasive tumor cell lines from different species. In contrast to the trophoblast of the rabbit blastocyst (previous publication), none of the tumor cell lines was able to adhere to intact epithelium of endometrial fragments nor to penetrate it. The uterine epithelium was also an insurmountable barrier for tumor cell spheroids confronted with the epithelium of fresh complex explants consisting of endometrium and myometrium or for spheroids introduced into the uterine lumen of pregnant/pseudopregnant rabbits at the periimplantation phase. However, all tumor cells were able to adhere to and mostly also to invade into the endometrial stroma when it was exposed artificially, i.e. when the epithelium was removed. These results suggest that the receptivity of rabbit uterine epithelium shows a remarkable selectivity with respect to cell type (trophoblast) and species (rabbit, not human, mouse, or rat).

Abbreviations used in this paper

hCG	human chorionic gonadotropin
p.c.	post coitum
p.i. hCG	post injectionem hCG = after injection of hCG
FBS	fetal bovine serum

Introduction

Implantation of the mammalian embryo is initiated by adhesion of the trophoblast to the uterine epithelium. This adhesion does not only serve to anchor the conceptus at its site of subsequent placentation but also initiates a cascade of cellular interactions that appears to be an essential element of implantation initiation in all species: in epithelio-chorial placentation (e.g. pigs) it is maintained throughout pregnancy, while in invasive types of placentation (e.g. hemochorial as in the human) it is fol-

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lowed by a cascade of secondary events leading to penetration of the epithelium by the trophoblast cells and their invasion into the uterine stroma. These phenomena of adhesion and invasion appear to be possible only when the differentiation processes in trophoblast as well as in the uterine epithelium are delicately synchronized: trophoblast cells have to develop adhesive and invasive properties at the timepoint when the uterine epithelium becomes permissive for trophoblast adhesion and penetration. Endometrial permissiveness ('receptivity') appears indeed to be tightly limited temporally under the control of progesterone/estrogen ratios [Psychoyos, 1976; de Ziegler et al., 1998] plus locally acting blastocyst-derived signals the nature of which has recently been discussed vividly [Rice and Chard, 1998; Sharkey, 1998; Simon et al., 1998].

Under cell biological aspects, the adhesion of trophoblast to uterine epithelium appears to be a very unusual phenomenon and poses a biological paradox [Denker, 1993] because the apical surfaces of epithelial cells are not normally adhesive for other cell types [Easty and Easty, 1974; de Ridder et al., 1975, 1977]. Data have been presented suggesting that the uterine epithelium has the unusual ability to downregulate part of its apico-basal polarity in order to develop receptivity [Denker, 1994]. So far, however, it has not been established clearly to what degree any specificity can be detected in this interaction with respect to the cell types that are able to adhere. The invasive behavior of trophoblast cells has been compared to that of tumor cells for a long time [Denker, 1980; Yagel et al., 1988]. Indeed, Kirby has shown that trophoblast cells transplanted into extrauterine tissues like kidney or testes exhibit destructive invasiveness [Kirby, 1965, 1967, 1970]. In turn, tumor cells have been successfully introduced into the rodent uterus as a tumor tissue analogue for the blastocyst [Wilson, 1963; Short and Yoshinaga, 1967; Lions, 1970; Wilson and Potts, 1970]. In these studies, invasion of tumor cells into the uterine wall was observed only in receptive endometrium, i.e. during pseudopregnancy or under appropriate stimulation with progesterone and estradiol. Some authors, however, were not able to confirm this hormone dependency (see 'Discussion'). On the other hand, the rodent model appears to be quite problematical with respect to any studies involving possible irritations of the uterine epithelium: the latter is programmed at receptivity to undergo apoptosis in the implantation chamber and even around foreign bodies like beads or oil droplets [Abrahamsohn and Zorn, 1993], and thus is known to slough off easily after mechanical irritation as would occur during transplantation of tumor tissue fragments or injection of tumor cell suspensions. Therefore, it remains questionable whether in rodents the uterine epithelium forms an effective barrier that the invading trophoblast has to interact with as might be the case in other species where the epithelium is less prone to undergoing degeneration [for discussion, see Friedrich, 1991].

The present communication reports on experiments performed with rabbit endometrium, using established in vitro and in vivo procedures, and focuses on the interaction of the uterine epithelium with tumor cell lines derived from different origins regarding organ and species. The in vitro model had previously been used successfully for the study of the interaction of the trophoblast of rabbit blastocysts with uterine epithelium of explanted endometrial fragments [Hohn et al., 1989; Hohn and Denker, 1990]. In a modification of this model, human choriocarcinoma cells (i.e. malignant trophoblast cells) have been shown to be able to adhere to and invade the epithelial lining of human endometrial explants in vitro [Grümmer et al., 1994]. The present investigation has provided data suggesting that there is a remarkable degree of cell type specificity in the adhesive interaction of invasive cells with rabbit uterine epithelium, but not with stroma, pointing to specific recognition phenomena for which no evidence had been shown in previous reports.

Materials and Methods

Cell Lines and Cell Culture

Most of the cell lines used in these experiments were kindly provided by Dr. M. Mareel (Gent, Belgium): MO_4 cells are Kirsten-MSV transformed murine embryonic fibrosarcoma cells [Billiau et al., 1973]. NBT II cells have been derived from a rat urinary bladder tumor induced with N-butyl-N-(4-hydroxybutyl)-nitrosamine [Toyoshima et al., 1971]. MCF-7 cells go back to a human mammary carcinoma dissected from a pleural effusion [Soule et al., 1973]. 12R1 C-RK cells are described as a rat kidney tumor transformed by adenovirus type 12 [Jochemsen et al., 1982]. LLC-H61 cells represent a human lung carcinoma [van Lamsweerde et al., 1983].

The rabbit carcinoma cell line V2 was obtained from Dr. B. Jockusch (Bielefeld, Germany) [Graf et al., 1981] while Jeg-3 cells, a human choriocarcinoma cell line, were purchased from the American Type Culture Collection, ATCC-No. HTB 36 [Kohler and Bridson, 1971].

All cells were routinely maintained at 37°C in humidified air containing 5% CO_2. MO_4, NBT II, and MCF-7 cells were cultured in MEM-Rega-1 medium (Gibco, Eggenstein, Germany) while 12R1 C-RK, LLC-H61, and V2 cells were kept in MEM Dulbecco (Boehringer, Mannheim, Germany). MEM (Gibco) was used for Jeg-3 cells. All media were supplemented with 10% fetal bovine serum (FBS, Gibco), 3.4 m*M* *L*-glutamine (Gibco), and penicillin (100 IU/ml)/streptomycin (100 μg/ml) (Boehringer).

Multicellular spheroids were generated by suspending cells from monolayer cultures after treatment with 0.05% trypsin/0.02 EDTA (Gibco). 6×10^5 cells in 6 ml medium were aliquoted into 25-ml Erlenmeyer flasks and cultured for different periods (2–4 days depending on the proliferation rate of the respective cell line) in humid atmosphere containing 5% CO_2 in air at 37°C on a gyratory shaker set at 70 rpm as described previously for choriocarcinoma cells [Grümmer et al., 1990, 1994].

Maintenance and Preparation of Rabbits

Mixed breed rabbits were kept in single cages in air-conditioned quarters under a light-dark cycle of 12/12 h. They were fed a standard pellet food ad libitum. Pseudopregnancy was induced by a single i.v. injection of 75 IU hCG (Prolan®, Bayer, Leverkusen, Germany). Pregnant rabbits were obtained by mating does with two fertile males. At the desired stage, animals were sacrificed by stunning and exsanguination. Animal experiments had been approved by the local authorities (Regierungspräsident).

Preparation and Culture of Endometrial Fragments

The generation and pre-culture of endometrial fragments has been described previously [Hohn et al., 1989]. In brief, uteri were excised from pseudopregnant rabbits and opened longitudinally under aseptic conditions. Endometrial tissue containing stroma and epithelium was taken from mesometrial and antimesometrial regions and subdivided into fragments of about 1 mm in diameter. Such fragments (20–25 in 10 ml medium in 25-ml Erlenmeyer flasks) were cultured in Eagle's MEM containing 10% FBS, penicillin/streptomycin (100 IU/ml, 100 µg/ml) as well as 3.4 m*M L*-glutamine under the same conditions (gyratory shaker) as were the tumor cell spheroids except that the shaker was set at 110 rpm and that the incubator temperature was at 38.8°C. During this culture period, the uterine epithelium grows around the fragment to cover the exposed stroma [cf. Hohn et al., 1989]. The media were supplemented with 10 ng/ml progesterone (Merck, Darmstadt, Germany); under these conditions, the receptive state has been shown to be maintained in vitro (as judged by the formation of symplasms in the original epithelium) [Hohn and Denker, 1990]. The culture media were changed daily.

Confrontation of Endometrial Fragments with Tumor Cell Spheroids in vitro

Endometrial fragments were routinely explanted at 4 days and 16 h after the injection of hCG (4 d 16 h p.i. hCG) and were precultured for additional 2 days in order to start the experiments at 6 d 16 h p.i. hCG which corresponds to the onset of antimesometrial implantation in vivo in the rabbit.

In order to probe various types of tumor cell spheroids for their ability to adhere to and invade the endometrium, precultured endometrial fragments, completely covered with epithelium, were brought in contact with the spheroids and kept in confrontation culture as reported previously (c.f. fig. 11) [Grümmer et al., 1994]. In brief, endometrial fragments were transferred onto the surface of a semisolid agar medium (2.5% w/v in 50% MEM) together with several tumor cell spheroids (4–8 per fragment). After 4 h of stationary incubation at 37°C at the air/liquid interface (using the surface tension of the water to ensure close contact) the slightly adhering tissues were carefully lifted off the agar surface by overlaying them with media and were transferred into the shaking culture system described above. In the following text the term 'confrontation' will be used for this experimental procedure of induced contact.

In another series of experiments that was performed only with MO_4 cell spheroids endometrial fragments were explanted at 4 d 2 h p.i. hCG and were precultured for 48, 52, 56, etc. up to 96 hours; i.e. confrontation with MO_4 spheroids started at either 6 d 2 h, 6 d 6 h, 6 d 12 h, etc. up to 8 d p.i. hCG. Again, after 4 h of stationary confrontation the confronted tissues were transferred into shaker culture.

While in the above-mentioned experiments tumor cell spheroids were confronted with intact epithelium of endometrial fragments, confrontation was performed with exposed endometrial stroma (c.f. fig. 11) in two different settings: For confrontation with either of the tumor cell lines, endometrial fragments explanted at 6 d 16 h p.i. hCG were used immediately after explantation from the uterus, i.e. without preculturing the endometrium so that the uterine stroma remained exposed at the cut edges of the fragments. After 4 h of confrontation on agar the confronted tissues were kept in shaker culture for 24, 48, or 96 h, respectively. In an additional experiment, for confrontation with Jeg-3 cells, endometrial explants were explanted at 4 d 16 h p.i. hCG and precultured. After 2 days of preculturing the fragments were cut in half and the cut edge was confronted with Jeg-3-cell spheroids.

In experiments on fragments with a completely regenerated epithelial lining a total of about 50 endometrial fragments (originating from at least 2 animals) were used for each condition. In experiments involving exposed stroma 30–40 fragments were examined for each condition.

Experiments in vivo

MO_4 cells were transferred into the uterine lumen of one pregnant and two pseudopregnant rabbits at 6 d 16 h post coitum (p.c.)/p.i. hCG during laparotomy under neurolept-thiobarbiturate anesthesia (Decentan®, Merck; Thiogenal®, Merck). Suspensions of tumor cell spheroids were pipetted with a Pasteur pipette into the uterine lumen through several small incisions made with fine scissors. Approximately 200 spheroids were injected together with 0.2 ml culture medium through each incision before the incision was closed with a one-stitch suture. In the pregnant animal injections were made 1 cm proximally as well as distally of blastocysts. In pseudopregnant rabbits one incision was located in the tubal third of the right horn with a ligature 3 cm towards the vaginal end so that the vaginal third could serve as a control. In the left horn, three spheroid suspensions were injected through three incisions distributed homogeneously over the whole length with ligatures located 1 cm towards the vaginal end for each injection. The animals were sacrificed after 48 h for the pregnant rabbit or 32 h for the pseudopregnant one, respectively. The injected regions were removed from the uteri, embedded, and analyzed in serial sections.

Morphology

For light microscopy, tissues were fixed in Bouin Hollande, embedded in paraffin, serially sectioned, stained with hematoxylin and eosin, and examined with an Axiophot (Zeiss, Oberkochen, Germany). For electron microscopy, tissues were fixed in glutaraldehyde (2.5% in 0.1 *M* cacodylate buffer, pH 7.4), postfixed with 2% osmium tetroxide, contrasted with 1% uranyl acetate, and embedded in a mixture of Epon and Araldite. Sections were contrasted with uranyl acetate and lead citrate and studied with a Zeiss EM 10 or a Phillips EM 200 electron microscope.

Results

Confrontation with Epithelium of Precultured Endometrium at 6 d 16 h p.i. hCG in vitro

As a standard experiment performed for all tumor cell lines used in this study, tumor cell spheroids were brought into tight contact with endometrial fragments explanted from pseudopregnant rabbits at 4 d 16 h p.i. hCG and precultured for 2 days in the presence of progesterone. The histological state of these fragments corresponded well to that described previously [Hohn et al., 1989], i.e. after preculturing larger areas of necrotic or apoptotic cells were not observed, and flat (squamous) to cubic epithelium had regenerated covering cut surfaces. When after 4 h the phase of stationary confrontation between spheroid and endometrial fragments on agar was terminated almost all (i.e. about 90–95%) tumor cell spheroids separated from the respective endometrial fragments even with gentle pipetting, i.e. gentle overlaying with buffer or fixative. This observation was made with all tumor cell lines used. Thus none of the studied tumor cell spheroids was able to attach to intact rabbit uterine epithelium from the apical cell pole under the conditions used (table 1).

In the rare cases when single tumor cell spheroids did remain attached to an endometrial fragment during application of such shear forces histology revealed that this had occurred either in the area of a former cut edge where the regenerated epithelium still appeared unusually flat or via contact to degenerating symplasms that were just in the process of being sloughed off (fig. 1). This sloughing of uterine epithelial symplasms was previously noticed to start, in endometrial explants without confrontation, after 2 days in culture with progesterone [cf. Hohn et al., 1989]. As a rule, however, before symplasms were sloughed off completely an intact epithelial lining of flat cells had reformed underneath. In this case, no penetration of tumor cells through the epithelial lining nor invasion into the underlying stroma was observed.

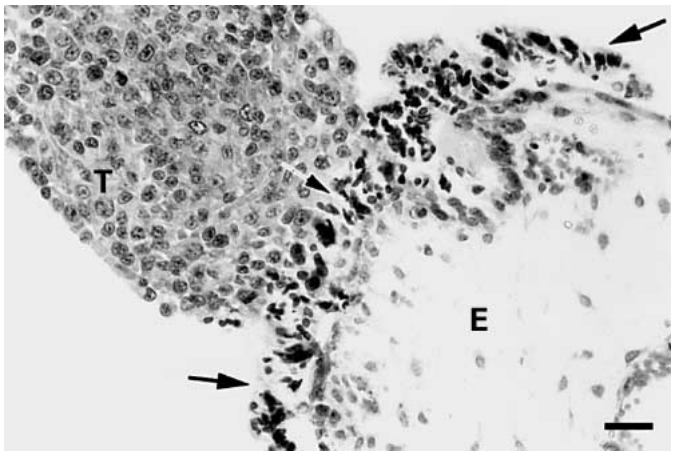

Fig. 1. Endometrial fragment (E) explanted from a rabbit at 4 d 16 h p.i. hCG, precultured for 2 days (6 d 16 h) and confronted with MO_4 cell spheroids (T = tumor spheroid). Most of the multicellular spheroids fail to attach to intact uterine epithelium and are lost already after the stationary phase of confrontation. Here, a single spheroid has attached exceptionally to degenerating parts of the epithelium (symplasms: arrows). Direct contact to intact epithelium is not observed. New epithelium is regenerating underneath the symplasm. Bar = 25 μm.

Table 1. Behavior of the different cell lines in in vitro experiments

Cell type	Attachment to epithelium	Attachment to stroma	Invasion into stroma	Migration onto stromal surface
MO_4	–	+	+	+[1]
MCF7	–	+	–	–
NBT-II	–	+	+	–
12R1c-RK	–	+	–	+[2]
LLC-H61	–	+	+	–
V2	–	+	+	+
Jeg-3	–	++	++	+

1 Formation of epithelium-like cover.
2 Few cells.

Variation of the Confrontation Period

This particular series of experiments was performed in order to make sure not to have missed the receptive state of the endometrium due to an artificial shifting of the timeframe in culture. The stationary phase of confrontation was varied in a way providing that endometrial epithelium was in contact with MO_4 cell spheroids over four hour periods covering 6 d 2 h to 8 d 4 h p.i. hCG. Otherwise, the conditions of the experiment were the same as just described. Also in this series of experiments, adhesion of MO_4 spheroids to uterine epithelium or even penetration into the stroma were never observed.

Confrontation with Fresh Uterine Explants

When larger segments of the whole uterine wall (comprising all layers including endometrium plus myometrium) were explanted from pregnant rabbits on 6 d 16 h p.c. and cultured (mucosa up) in Petri dishes for 24 h, small and even larger areas of necrosis developed in the

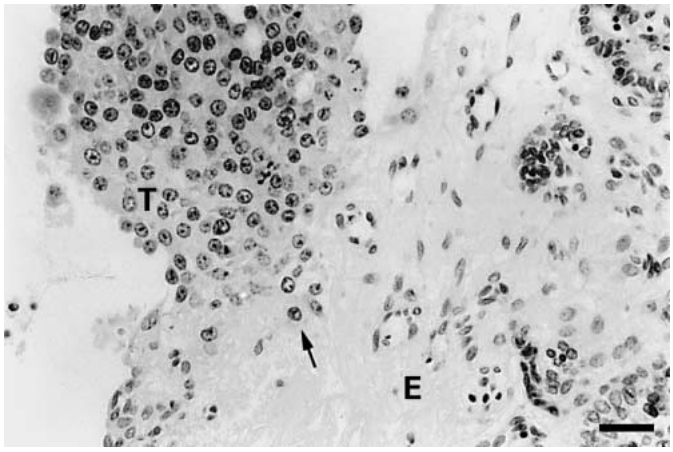

Fig. 2. MO_4 cell spheroid (T) attached where endometrial stroma (E) was exposed after artificial removal of epithelium. In this experiment a large patch of uterus was explanted on 6 d 16 h p.c. and cultured immediately with spheroids for 24 h. Single tumor cells are invading the connective tissue (arrows). Bar = 25 µm.

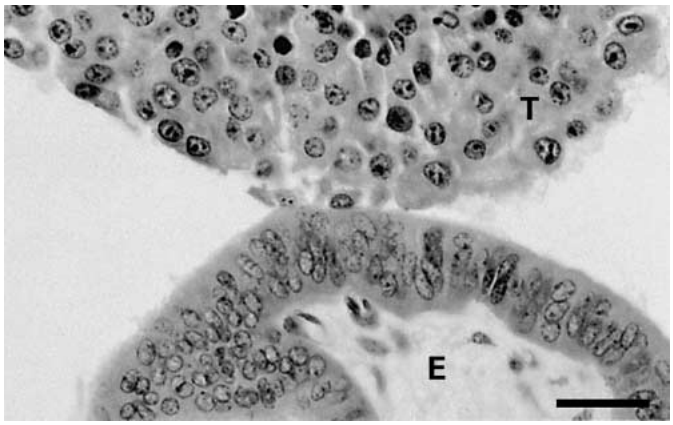

Fig. 3. MO_4 cell spheroid (T) after transfer to the uterine lumen of a pseudopregnant rabbit for 32 h in vivo (from 6 d 16 h to 8 d p.i. hCG). The spheroid was found located between two mucosal folds. The contact to epithelial cells appears to be very loose, and there is no indication of adhesion and invasion into the endometrium (E). Note pycnotic nuclei in the spheroid. Bar = 25 µm.

myometrium, in deeper areas of the stroma, and in bottom parts of the glands. Occasionally, small such foci of necrosis were detected in the luminal epithelium (not shown). In tissue taken from implantation chambers symplasms formed in the luminal epithelium. For confrontation, only MO_4 cells were used in this limited series of experiments. When transferred onto the epithelium-covered surface of the endometrium, they were never observed to adhere during an incubation period of 24 h. Frequently, however, spheroids slipped off the surface onto lateral regions of the explant and were later found to have attached to exposed stroma of the cut edge. Here the MO_4 cells invaded the connective tissue (fig. 2).

Transfer of MO_4 Cell Spheroids into the Uterine Lumen in vivo

In order to avoid any organ culture artifacts with respect to the receptive state of the uterine epithelium, suspensions of MO_4 cell spheroids were injected into the uterine lumen of laparatomized pregnant or pseudopregnant rabbits on 6 d 16 h p.c or p.i. hCG, respectively. When spheroid-bearing segments were examined after 48 h (pregnant) or 32 h (pseudopregnant), spheroids were found either free in the lumen, in punctual contact with luminal epithelium (fig. 3), or engulfed by endometrial folds with intimate contact to the epithelium (fig. 4). When tumor cells were found in contact with epithelial cells the epithelium was never observed to be invaded but appeared to be intact (fig. 3). Interestingly, the presence of MO_4 cell spheroids seemed to elicit an epithelial cell fusion reaction (fig. 4), i.e. epithelial cells formed larger symplasms here. Only in the area of incisions where the epithelium had been damaged were tumor cells found invading the stroma (not shown).

Confrontation of Tumor Cells with Endometrial Stroma in vitro

As just mentioned, the in vivo experiments as well as confrontation with fresh endometrial explants suggest that MO_4 cells are able to adhere to and invade into rabbit uterine stroma if this is exposed to them. The uterine epithelium, in contrast, appears to provide a barrier for tumor cell attachment and invasion. In order to corroborate this assumption a systematic in vitro series was designed in order to bring endometrial stroma into contact with multicell spheroids from all of the cell lines used. In these experiments, spheroids were confronted with exposed stroma of endometrial fragments that were either explanted freshly on 6 d 16 h p.i. hCG or explanted on 4 d 16 h p.i. hCG and precultured for 2 days. In the latter case the (regenerated) epithelial lining was partially removed, before confrontation with spheroids, by cutting the endometrial fragments with a scalpel. As compared to spheroids confronted with epithelium, a remarkable difference was seen already at the end of stationary confrontation on agar: most of the spheroids (from all cell lines) remained

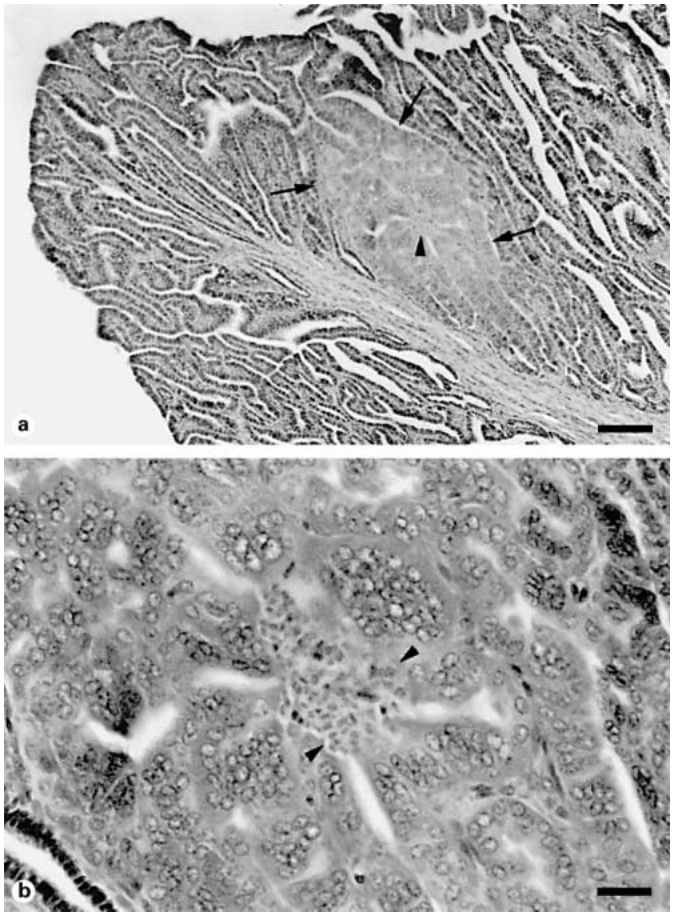

Fig. 4. Epithelial reaction induced by a small MO_4 cell spheroid in a pregnant rabbit in vivo. **a** After spheroids had been transferred to the uterine cavity on 6 d 16 h p.c. a small MO_4 aggregate (arrowhead) is located deep between minor endometrial folds. The stage shown is 8 d and 16 h p.c. The neighboring epithelium displays reduced staining intensity and symplasms have formed as typically seen in the implantation chamber (between arrows). **b** Higher magnification from (**a**) with arrowheads indicating the location of the spheroid: There is no sign of invasion of tumor cells through the uterine epithelium into the endometrium, but some small regions of the epithelium are transformed into syncytia although not yet degenerated or sloughed. The nuclei of the tumor cells appear to be condensed and are probably already undergoing necrosis/apoptosis at this stage. Bars represent 100 μm (**a**) or 25 μm (**b**).

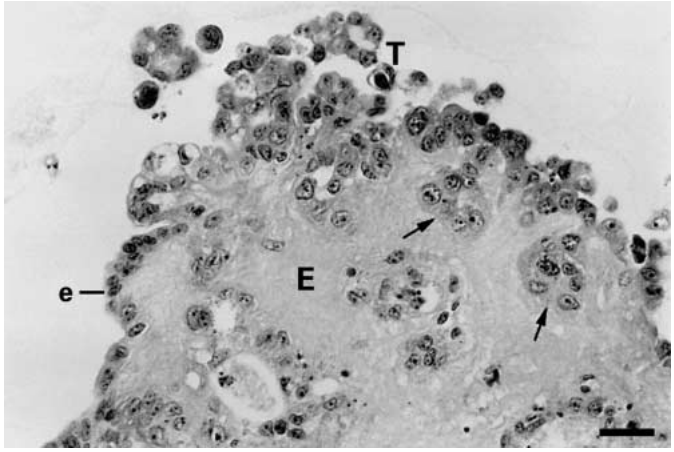

Fig. 5. Confrontation of NBT-II cells with exposed endometrial stroma (E) in vitro. After stationary confrontation on 6 d 16 h p.i. hCG the confronted tissues had been kept in shaker culture for 96 h. Rests of one of the spheroids (which in case of NBT-II cells are rather unstable) are still attached to the stroma and tumor cells (T) have invaded the connective tissue individually or in small groups (arrows). Stroma cells in this area show signs of degeneration. The sheet of epithelium (e) in the proximity of the tumor cell aggregate probably has formed by outgrowth from the original epithelial covering on the opposite site of the endometrial fragment. Bar = 25 μm.

attached to endometrial fragments even during harsh washing with PBS (table 1). Histological examination after 2 days in shaker culture confirmed that one to several tumor cell spheroids had attached to the stroma but none to epithelium of endometrial fragments. With respect to intimacy of the interaction between tumor cells and host stroma a broad variety was observed ranging from attachment without invasion to attachment followed by vigorous infiltration of tumor cells into stroma.

In the case of MO_4 spheroids individual cells were found leaving the aggregate at the contact front and invading the stroma whereas others migrated onto the stromal surface covering it with a kind of monolayer after 24 h in shaker culture. At the invasion front the endometrial stroma appeared necrotic (not shown). MCF-7 cells attached to stroma but maintained their aggregated (spheroid) interrelationship throughout shaker culture without any sign of invasion into stroma (not shown). NBT-II cell spheroids were rather unstable in shaker culture and disaggreated. However, numerous cells attached to exposed stroma and were found to invade connective tissue in form of tongues resulting in a focal distribution (fig. 5) surrounded by altered host tissue (stroma appearing to have lost distinct structures and to have degenerated homogeneously). 12R1c-RK cell spheroids remained as more or less rounded aggregates after attaching to endometrial fragments but developed central necrosis during poststationary culture. Cells appeared not to leave the spheroid except for only a few cells that migrated onto the stromal surface next to the spheroids. LLC-H61 cells exhibited infiltrating growth as individual cells or cells

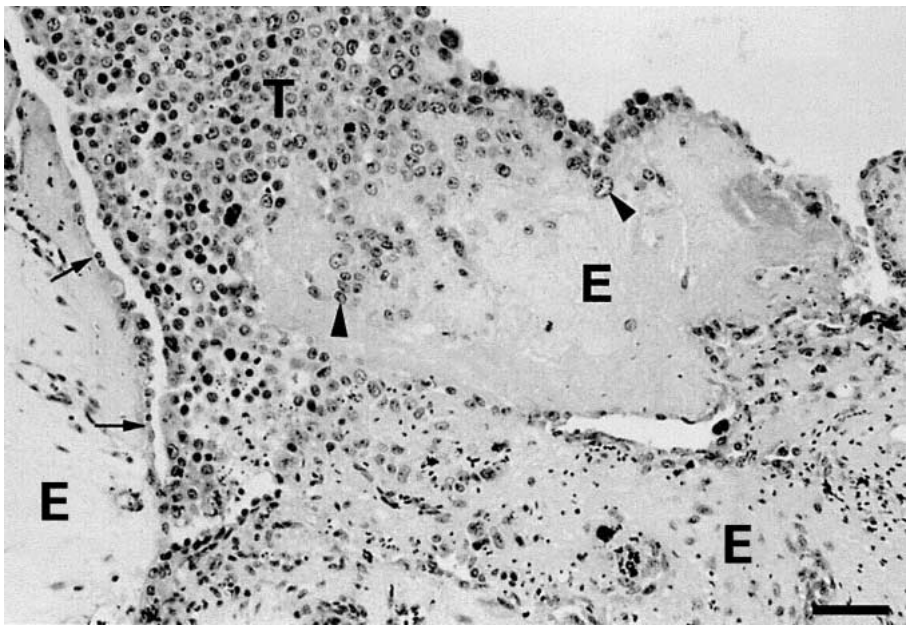

Fig. 6. Endometrial fragment with spheroid of V2 cells adhering to stroma that had been exposed artificially before confrontation was started (at 6 d 16 h p.i. hCG). Stage shown is after 48 h in shaker culture. From the spheroid, cells have migrated onto the surface of the endometrial fragment (E) from where they are invading the stroma (arrowheads). Note that the tumor cells (T) have not attached to surface epithelium present on part of the endometrial fragment (as outgrowth from original epithelium, arrows). Bar = 50 μm.

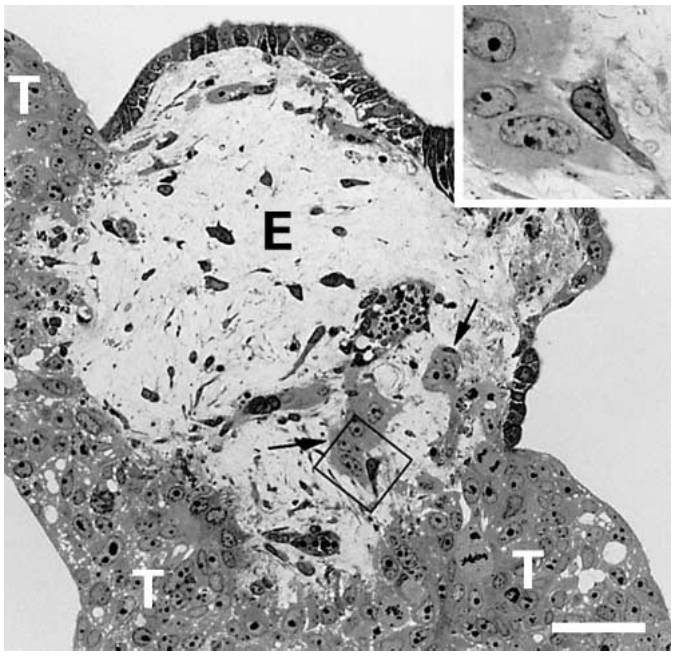

Fig. 7. Jeg-3 (human choriocarcinoma) cells confronted with exposed endometrial stroma after 72 h in shaker culture. One or more spheroids have attached to the endometrial fragment (E) and cover all surfaces that are not coated by epithelium. Tumor cells (T) are found invading the stroma in different areas (arrows). The connective tissue does not display obvious signs of degeneration. Inset: Higher magnification of the boxed area showing Jeg-3 cells that appear to be in close contact with a fibroblast of the host tissue. Bar = 50 μm; inset: bar = 17 μm.

migrating in small groups into the connective tissue. V2 cells (derived from a rabbit carcinoma) formed stable spheroids which attached to stroma but again not to epithelium. Cells leaving the aggregate moved onto the exposed stromal surface while others invaded the endometrial stroma in the contact zone (fig. 6). Jeg-3 cells displayed very extensive attachment to exposed stroma as well as deep invasion of cell columns into the host stroma (fig. 7). In the stroma Jeg-3 cells were found in close proximity to vital-appearing host tissue cells (inset in fig. 7). Interestingly, when these Jeg-3 cells attached to exposed stroma in the immediate neighborhood of retained uterine epithelium they formed close contacts here (fig. 8), i.e. the two epithelia (Jeg-3 = human trophoblast tumor; rabbit uterine epithelium) formed macula or zonula adherens type junctions with each other and membranes were run-

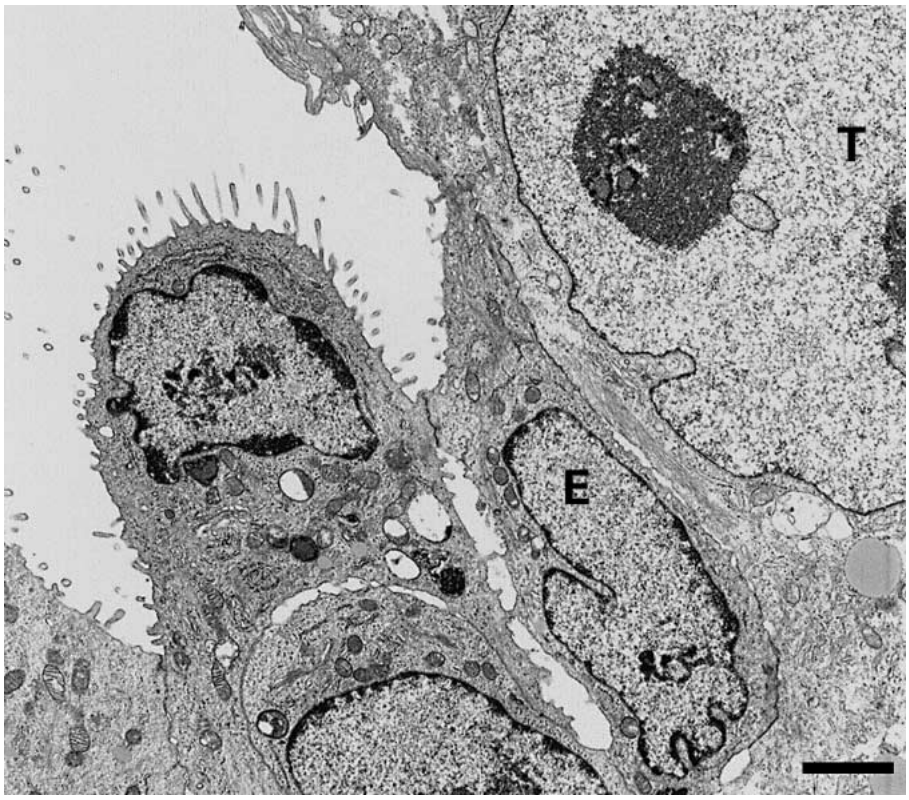

Fig. 8. Jeg-3 cells form close contact with basolateral but not apical membranes of endometrial epithelial cells. After confrontation with artificially exposed stroma followed by seven days of shaker culture Jeg-3 cells (T) have approached epithelial cells from the side and from underneath and membranes of both cells have aligned over wide stretches. The epithelial cells (E) appear to be vital. Although the tumor cell rises over the surface level of the epithelium contact to the apical plasma membrane domain has not been made. Bar = 2 μm.

ning in parallel at a distance of 20 to 30 nm (fig. 9, 10). Such contacts were always found only at the baso-lateral domains of uterine epithelial cells. In contrast, contact at the apical cell pole was never seen (not shown). Where tumor cells approached the basal side of the epithelium the basement membrane was no longer apparent (fig. 9).

Discussion

Contact formation at the apical cell pole of uterine epithelium was probed, in this investigation, for any degree of specificity that can possibly be detected here. This was done by confrontation with multicellular spheroids of tumor cells from different origins with respect to species as well as to tissue. The rabbit in vitro model used here had been shown previously to allow progestational differentiation of uterine epithelium to proceed in organ culture [Hohn et al., 1989]. Under the conditions used it seems to be possible to maintain in vitro the receptive state of the endometrium since adhesion and invasion of trophoblast via the apical surface of the uterine epithelium was observed during confrontation with blastocysts in vitro [Hohn and Denker, 1990]. The tumor cell lines investigated in the present study had been shown previously to be highly invasive in various assay systems including an embryonic chick heart model [Mareel, 1979; Graf et al., 1981; Biswas et al., 1982; Kasid et al., 1985; Grümmer and Denker, 1989; Madsen and Briand, 1990; Grümmer et al., 1994]. It came as a surprise, therefore, to see that, in contrast to trophoblast cells of rabbit blastocysts, none of the tumor cells – not even the rabbit carcinoma cell line V2 – was able to establish stable adhesion to the apical surface of rabbit uterine epithelium. These results were confirmed by various modifications of the in vitro model as well as by in vivo experiments. In contrast to the unsuccessful confrontation with uterine epithelium, all tumor cell types used did attach well to exposed uterine stroma with about 50% of them also showing invasion of the connective tissue. This result points to a special role that has to be ascribed to the uterine epithelium in conferring specificity to and in regulating the process of implantation initiation.

Experiments that are comparable to those performed in this study had previously been made in rodents in vivo where tumor cell suspensions had been transferred into

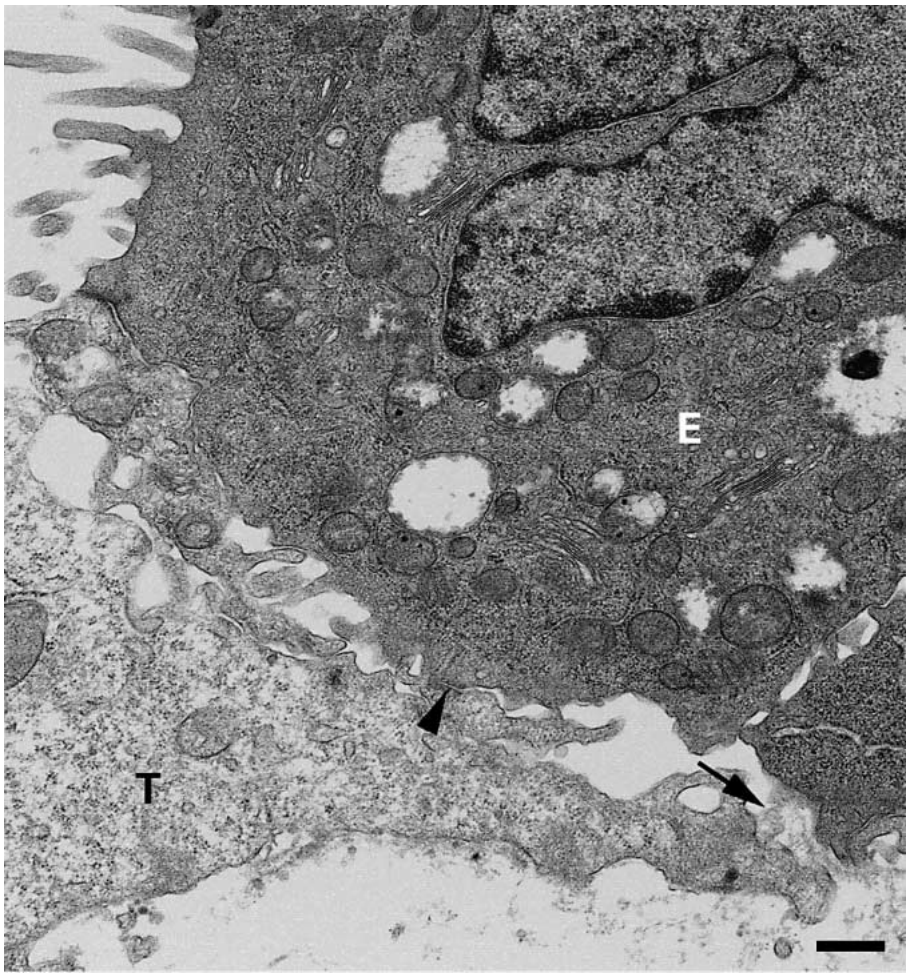

Fig. 9. Jeg-3 cell in contact with basolateral plasma membrane of epithelial cells after stromal confrontation as in figure 8. While the tumor cell (T) has made various punctual plasma membrane contacts with uterine epithelium (E) the basement membrane appears to be displaced or even locally dissolved (arrow). A primitive desmosome has formed between both cells (arrowhead). The apical plasma membrane of the uterine epithelial cell remains free (see microvilli in the left upper corner). Bar = 0.5 μm.

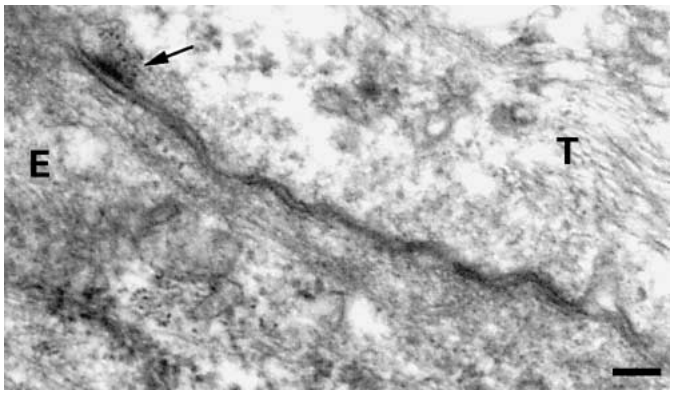

Fig. 10. Desmosomal contact (arrow) formed between a uterine epithelial cell (E) and a Jeg-3 cell (T). Cell membranes run in parallel over long stretches. Higher magnification from figure 8. Bar = 0.1 μm.

the uterine lumen of mice or rats. The results of the different studies were somewhat heterogeneous: Generally, transepithelial invasion into the endometrial stroma was observed. Some investigators did not find differences between pregnant and nonpregnant or hormone-treated animals (mouse sarcoma/mouse uterus [Hall, 1940]; human lung carcinoma/mouse [Maharajan et al., 1989, 1990]; various tumor types in mouse, rat, hamster or guinea pig [Homburger et al., 1956; Stein-Werblonski, 1961; Schlesinger, 1962]). Among these authors, Hall and Homburger conceded to have caused larger injuries during inoculation of tumor cells denuding the stroma for tumor cell adhesion. However, other authors reported on hormone dependency, i.e. invasion through the epithelium was only found during the implantation window, when the endometrium was in the receptive state ([Lions, 1970]: rat sarcoma/rat; [Short and Yoshinaga, 1967]: rat carcinosarcoma/rat; [Mohs and Guyer, 1937]: rat carcino-

ma/mouse; [Wilson, 1963; Wilson and Potts, 1970]: mouse melanoma/mouse). However, the fact that the displacement type of implantation is found in mouse and rat [Schlafke and Enders, 1975] may pose certain problems with the interpretation of the results of such experiments, in particular with respect to extrapolation to other species. In rodents the receptive uterine epithelium detaches very easily from its basement membrane and undergoes apoptosis [Psychoyos and Casimiri, 1980; Schlafke et al., 1985]. This detachment appears to be elicited by mechanical irritation as exerted by the blastocyst or even by inert beads, oil droplets, etc. [Blandau, 1949]. Therefore, if the uterine epithelium is not a very effective barrier for trophoblast invasion in (murine) rodents, at least not during receptivity, it probably cannot be very selective either, e.g. with respect to various types of other invasive cells, i.e. tumor cells. In contrast, the non-receptive uterine epithelium does appear to be a barrier to blastocyst trophoblast as well as to (at least certain) tumor cells: In normally cycling or immature (non-receptive) mice invasion of blastocysts is impossible but occurs after mechanical alteration of the endometrium resulting in loss of the epithelium [Cowell, 1969]. Tumor cells were likewise found not to penetrate intact non-receptive uterine epithelium in rodents [Wilson, 1963; Short and Yoshinaga, 1967; Wilson and Potts, 1970]. Uterine epithelial degeneration (triggering of the apoptotic program) in the implantation chamber may be a prerequisite for implantation in those species allowing the trophoblast to adhere to exposed basement membrane. This appears to be in line with observations made in our studies insofar as in vivo or in vitro all tumor cells did adhere to exposed endometrial stroma but not to the apical domains of intact epithelium. Adhesion of tumor cells to epithelium was observed only to degenerating symplasms in the process of being sloughed off (perhaps comparable to trophoblast adhesion to degenerating epithelial cells in the mouse model described by Morris et al. [1983]). Occasional attachment of tumor cell spheroids to regenerating epithelium was most probably due to small gaps not yet covered by epithelial cells exposing patches of stroma.

Attachment of tumor cells to stroma appears to be a rather trivial nonselective process in contrast to adhesion to epithelia: tumor cells (like all cells) have basically the ability to adhere to some molecule(s) of the extracellular matrix (ECM). In contrast, the cellular interaction with epithelia appears to be much more selective, particularly via their apical plasma membrane [de Ridder et al., 1975, 1977]. This lack of selectivity of tumor cell adhesion to ECM is a phenomenon that is well-known from the literature on in vitro models for tumor cell invasion. It is considered an advantage in a well-established model for testing and quantifying stromal invasion of tumor cells [Mareel, 1983]. In that model, tumor cells attach to ECM molecules deposited on the surface of embryonic chick heart fragments by a layer of fibroblasts. It may be due to this adhesion to ECM that no interspecies barrier is observed so that this model has proven very useful as a nonselective general invasion assay. In contrast, it is known that typical epithelia are largely resistant to adhesion of other cells at their apical pole [de Ridder et al., 1975, 1977]. In the present investigation, the preservation of an intact epithelium was an important part since the focus was not on trophoblast invasiveness but on any selectivity and regulatory functions that the host tissue, the endometrium, may have.

In a previously described model spheroids of different human trophoblast tumor cell lines had been confronted with precultured human endometrial fragments [Grümmer et al., 1994]. These tumor cells were able to adhere to progestational human uterine epithelium and to penetrate it. One may argue that in this setting the interspecies problem encountered in the present studies was avoided by using human endometrial fragments and human tumor cells. But this was also taken into account by using V2 rabbit carcinoma cells in combination with rabbit endometrial fragments in the present series of experiments. An important difference to the present study, however, is that the cells confronted with human endometrial fragments [Grümmer et al., 1994] were exclusively human choriocarcinoma cell lines, i.e. malignant trophoblast cells (cell lines BeWo, Jeg-3, and JAr; for characterization of the cell lines see [Pattillo and Gey, 1968; Kohler and Bridson, 1971; Pattillo et al., 1971]). These cells have been shown in numerous studies to exhibit various trophoblast-like cytological and biochemical characteristics and have, therefore, been used as a model in many studies of trophoblast behavior [c.f. Hohn et al., 1998]. One of these choriocarcinoma cell lines, Jeg-3 cells, has also been used in the present investigations. The invasive behavior regarding interactions with denuded rabbit endometrial stroma was quite comparable to that observed with human endometrium after penetration of the epithelium. However, in contrast to observations with human endometrium, Jeg-3 cells did not interact with the apical domain of rabbit uterine epithelium. It might be argued that this could be due to lack of potential to fuse with rabbit endometrial epithelial cells. Fusion with the uterine epithelium is the peculiar mode that is employed by the rabbit trophoblast in order to overcome this barrier. While in nonhuman pri-

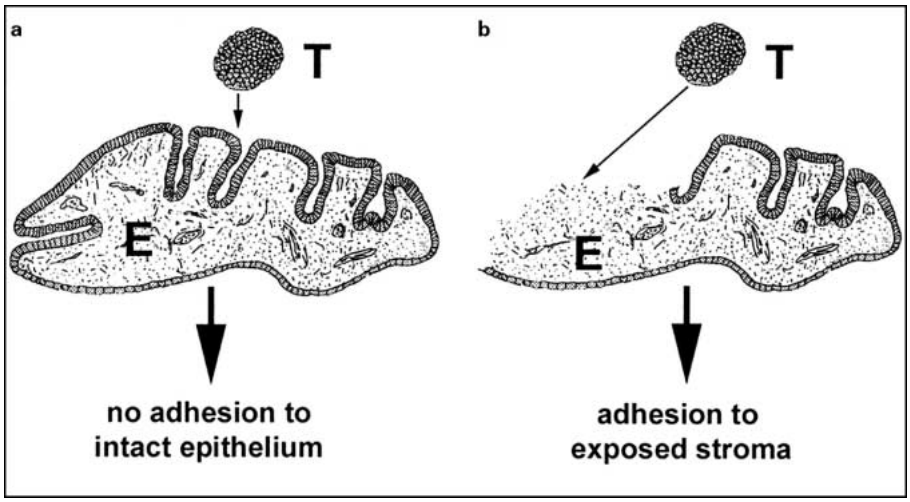

Fig. 11. Summary of the results after confrontation with intact epithelium (**a**) and with exposed stroma (**b**). T = tumor cell spheroid; E = endometrial fragment.

mates trophoblast cells seem to intrude between uterine epithelial cells [Enders et al., 1983; Enders, 1993] it is indeed an unsolved question whether human trophoblast is able to undergo (limited) fusion with uterine epithelium, as electron microscopic findings on early human implantation stages in utero are lacking and descriptions of somewhat later stages are controversial [Larsen, 1970; Enders, 1993]. Interestingly, however, after invading the stroma Jeg-3 cells were able to establish cell-to-cell interactions with the basolateral plasma membrane domains of uterine epithelial cells, i.e. from underneath, resulting in formation of macula or zonula adherens type junctions. This demonstrates that of the cell lines used here at least Jeg-3 cells are basically able to initiate an adhesive interaction with receptive rabbit uterine epithelial cells and form cell junctions here, thus bridging the interspecies barrier. However, such interaction appears to be possible only at the basolateral but not the apical plasma membrane domains, and even in the receptive state is the cross-species specificity apparently not lost at the apical cell pole.

In some cases another kind of interaction between tumor spheroids and rabbit uterine epithelium was observed in that spheroids appeared to elicit fusion of uterine epithelial cells with each other (symplasm formation) (c.f. fig. 4) as observed in vivo in the implantation chamber [Duval, 1889]. This is, however, not necessarily indicative of any specific type of signal exchange since it has been obtained even after mechanical irritation [DeFeo, 1967; Hoffman et al., 1977].

In summary, receptive rabbit uterine epithelium does not allow for adhesion of various types of invasive tumor cells via its apical cell pole while tumor cells appear to attach easily to exposed endometrial stroma (c.f. fig. 11). These results are consistent with the view that tumor cell invasion in endometrial stroma involves the same basic mechanisms as stromal invasion of trophoblast cells does. Comparison with observations made in various in vitro models of tumor cell invasion suggests that this is due to the fact that trophoblast and tumor cell penetration into connective tissue (extracellular matrix) are not excessively selective with respect to the type of host tissue encountered. The situation appears to be quite different with respect to the uterine epithelium, at least in this rabbit model. While rabbit blastocyst trophoblast can attach to and invade receptive rabbit uterine epithelium, tumor cells are not observed to adhere to the intact apical surface of this epithelium, although at least one of the tumor cell lines (Jeg-3) was able to establish adherens type junctions with basolateral but not apical domains. Consequently, the apical surface of rabbit uterine epithelium appears to develop a remarkable degree of selectivity for adhesion of trophoblast but not other invasive cells during the implantation window.

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