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Increased Uterine Vascular Permeability at the Time of Embryonic Attachment in the Pig[1]

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ABSTRACT

The temporal relationship between embryonic attachment and endometrial vascular permeability was investigated in the gilt. Light and electron microscopy failed to reveal structural differences between Day 10 cycling and pregnant maternal epithelia, including evidence of blastocyst contact. Chorionic adhesion was preserved at mesometrial regions in 3 of 5 Day 13 pregnant animals and appeared to be related to localized differentiation of the underlying maternal epithelium.

In order to study uterine vascular permeability, 44 gilts between Days 11 and 19 of the cycle and pregnancy were injected i.v. with a 0.5% solution of Evans Blue (2.5 ml/kg body weight). Examination of excised uteri under ultraviolet light revealed a well-defined zone of endometrial fluorescence corresponding to extravascular content of the dye. Exclusive to pregnant gilts, this response appeared in conjunction with blastocyst elongation at Day 12, and was consistently confined to areas of embryonic membrane contact thereafter.

The changes in endometrial morphology and vascular permeability suggest involvement of some embryonic factor(s) acting in a localized manner. Increased histotrophe production is probably facilitated by the flux of plasma constituents to maternal epithelial cells. Coincidence of increased uterine vascular permeability at the site of attachment with elevated blood flow would enhance transport of nutrients toward the conceptus and allow access of blastocyst-induced products to the maternal circulation.

INTRODUCTION

Placentation represents the culmination of a complex series of biochemical and structural interactions between the blastocyst and maternal system, most of which remain ill-defined. Although the extent and nature of uterine responses to the implanting embryo vary dramatically among species, one event that has been suggested to be a universal prerequisite for the success of this process is increased uterine capillary permeability (Psychoyos, 1973). The extravasation of albumen-bound Pontamine or Evans Blue dye from endometrial capillaries adjacent to the conceptus results in macroscopically identifiable implantation sites in rats (Psychoyos, 1960), hamsters (Orsini, 1964), mice (Finn and McLaren, 1967), guinea pigs (Orsini and Donovan, 1971), and rabbits (Hoffman et al., 1978). A positive response has also been observed in the caruncular regions of sheep uteri following systemic injection of Pontamine Blue on Day 15 of gestation (Boshier, 1970). This localized endometrial response to the blastocyst occurs before or concomitant with modifications in uterine histology for all species examined.

Based on uniform staining of the uterine mucosa of a Day 11 pregnant gilt injected with Pontamine Blue via the uterine artery, Crombie (1972) concluded that demonstrable areas of increased vascular permeability did not exist in the pig. However, the earliest dates cited for initial adhesion of the porcine chorion to the uterine epithelium are 14 (Perry et al., 1976) to 15 (Crombie, 1972) days of gestation, indicating that

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this study may have been conducted too early in pregnancy.

A preliminary trial in our laboratory revealed that the systemic treatment of cyclic or early pregnant gilts with Pontamine Blue resulted in ubiquitous dye distribution throughout the endometrium at several stages of the estrous cycle and early pregnancy (Days 11 to 19). Treatment with Evans Blue, which has fluorescent properties (van der Kooy and Kuypers, 1979), and subsequent evaluation of tissue fluorescence under ultraviolet exposure provided a more sensitive and specific method for gross demonstration of increased uterine vascular permeability. An attempt was made to relate the timing of permeability changes to structural evidence of attachment.

MATERIALS AND METHODS

Animals

Nulliparous crossbred gilts (Yorkshire × Landrace) of similar age and weight were checked daily for estrus in the presence of a vasectomized boar, and assigned to cycling or bred groups for killing following their second exhibited estrous cycle. Animals to be bred were artificially inseminated with fresh unextended semen on the first day of standing estrus and 24 h later. Kill dates were based on designation of the first day of standing estrus and mating as Day 0. Animals were included in the pregnant group only if normally developing blastocysts were subsequently found within their uteri.

Experiment I: Morphologic Study of Attachment

Four or 5 gilts were killed on each of Days 10 and 13 of the estrous cycle and pregnancy. The reproductive tract was removed immediately upon exsanguination. Each uterine horn was trimmed free of the mesometrium and then opened along the antimesometrial aspect. Segments of uterine horn were pinned endometrial side up in a wax-filled tray containing 0.2 M cacodylate buffer (pH 7.4) and then examined under a magnifier-illuminator to locate the embryonic disc and extraembryonic membranes. Upon removal of the buffer, the tissue was gently flooded with 1.5% gluteraldehyde in 0.2 M cacodylate buffer and fixed for 30 min.

Since blastocysts contained within pregnant uteri at Day 10 floated free of the uterine mucosa, tissue was randomly selected along the mesometrial region of these animals. Sampling sites were chosen along each of 2 embryos within each uterus at Day 13 and included specimens from regions with membranes adhered to the uterine wall and from regions lacking trophoblastic contact. Endometrium was taken randomly from the mesometrial area in cycling gilts for histologic comparison with uteri from gilts on equivalent days of gestation.

Light-microscopic specimens were fixed for 24 h in Bouin's fixative, dehydrated through a graded ethanol series, embedded in glycol methacrylate (Sorvall JB-4 embedding medium), and examined as 2-μm sections stained with hematotoxylin and eosin. For transmission electron microscopy, samples of endometrium were cut into 1-mm cubes, fixed for 2 h with 3% gluteraldehyde in 0.2 M cacodylate buffer, postfixed for 2 h in 1% OsO_4, stained "en bloc" with saturated aqueous uranyl acetate (UA), dehydrated, and embedded in Araldite CY212. Thin sections were poststained with 2% UA in 50% ethanol and lead citrate.

Experiment II: Vascular Permeability

A 0.5% (w/v) solution of Evans Blue (J. T. Baker Chemicals, Phillipsburg, NJ) in Dulbecco's phosphate-buffered saline (PBS; pH 7.4, 264 mOsm) was prepared and administered (i.v.) at a dosage of 2.5 ml/kg. Each of the 44 animals included in this study were immobilized in a squeeze and the dye was infused over a 5-min period through a 19-gauge butterfly infusion set inserted into the ear vein. After 10 min, the gilt was killed and exsanguinated, and the uterus was gently removed with manual contact restricted to the cervix and mesometrium.

In order to control for the possibility that fluorescence might represent dye contained within the lumens of capillaries just below the epithelium and not that which was extravascular, the arterial supply of one horn for each uterus was cannulated and cleared of residual blood and dye by repeated flushing with PBS. Both uterine horns were then trimmed and pinned out on wax as outlined above.

Portions of flushed and unflushed horns were examined and photographed under two laterally suspended ultraviolet (UV) light sources (46 cm, 15 W, peak emission at 365 nm). Correspondence between the course of fluorescent tracts and that of the embryonic membranes was investigated by inserting flag pins into the fluorescent zone under UV light and comparing their position relative to that of the embryonic vesicle under visible light. Blastocysts at Day 12 and thereafter could be visualized as convoluted

filamentous forms at the mesometrial region under a magnifier-illuminator by gently spreading apart the endometrial folds. Satisfactory fluorescent images were obtained by exposure of Kodak 35-mm Daylight Ektachrome (100 ASA) through a Kodak Wratten 2A lens barrier filter.

RESULTS

Morphologic Observations

No clear structural differences were detected between Day 10 cycling and Day 10 pregnant maternal epithelia. Contact between blastocysts and the uterine mucosa, if present, was not maintained. In contrast, contact between the chorion and luminal surface was preserved in 3 of 5 Day 13 pregnant gilts (Fig. 1). The nature of adhesion at this stage of gestation was tenuous, consisting primarily of close apposition between the overlying chorion and contours of the maternal apical plasmalemma (Fig. 2).

Vascular Permeability Changes

The number of gilts examined on each day from Day 11 through 19 of the cycle and gestation and the pattern of endometrial fluorescence observed are presented in Table 1. A positive response was considered to be a well-defined zone of intense fluorescence that persisted after spreading of the endometrial folds, and therefore was not simply a reflection of the additive fluorescence of overlapped endometrial tissue (see Fig. 3F). In all cases, positive responses were confined to the mesometrial area of pregnant uteri.

No differences in the pattern of fluorescence were found for arterially flushed versus unflushed uterine horns within individual animals. These findings confirmed that the fluorescence observed reflected extravascular Evans Blue indicative of increased vascular permeability, rather than intravascular dye. In one nonpregnant gilt mildly afflicted with metritis (excluded from study), fluorescence was confined to petechial hemorrhages. One gilt on Day 12 and another at Day 15 of the cycle exhibited randomized but discrete areas of more diffuse and less intense fluorescence not strictly localized to the mesometrial region. Two gilts, one killed on each of Days 18 and 19 postestrus, exhibited a gelatinous, edematous endometrium characteristic of estrual swine uteri that fluoresced in a pale, generalized manner. These patterns were distinctly different from the restricted response obtained in pregnant pigs.

A consistent pattern of fluorescence emerged that was highly correlated with the reproductive status of the animal. In none of the cycling or Day 11 pregnant gilts examined was a positive response observed. (Figs. 3A-C, E). Three of four gilts killed on Day 12 and all of those killed on subsequent days of gestation displayed positive responses (Figs. 3D and F). The definite fluorescent line apparent at Day 12 had increased in intensity by Day 15 (Fig. 3F).

On Day 11 of pregnancy, spherical blastocysts were found within the uterine lumen of 3 gilts while the fourth animal's uterus contained elongating blastocysts that were easily detached from the endometrium. Filamentous blastocysts adhering closely to the uterine epithelium and consistently confined to the mesometrial region characterized all but one of the Day 12 pregnant gilts. It is possible that conception occurred at a later time in the latter animal since the embryonic membranes lifted off the uterine wall readily and the fluorescent response was minimal (classified as negative in Table 1).

With the exception of the aforementioned gilt, animals bred 12 or more days prior to killing that

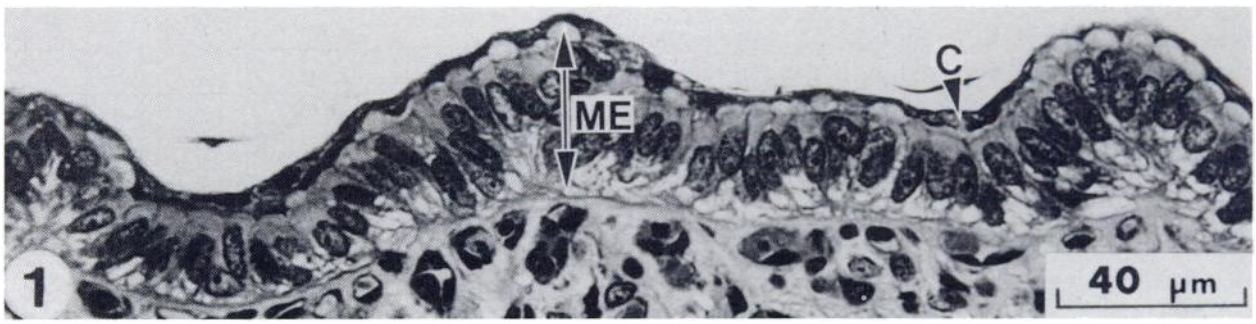

FIG. 1. Relationship between the overlying fetal chorion (*C*) and apical contours of the uterine epithelial cells (*ME*); mesometrial region, Day 13 of gestation.

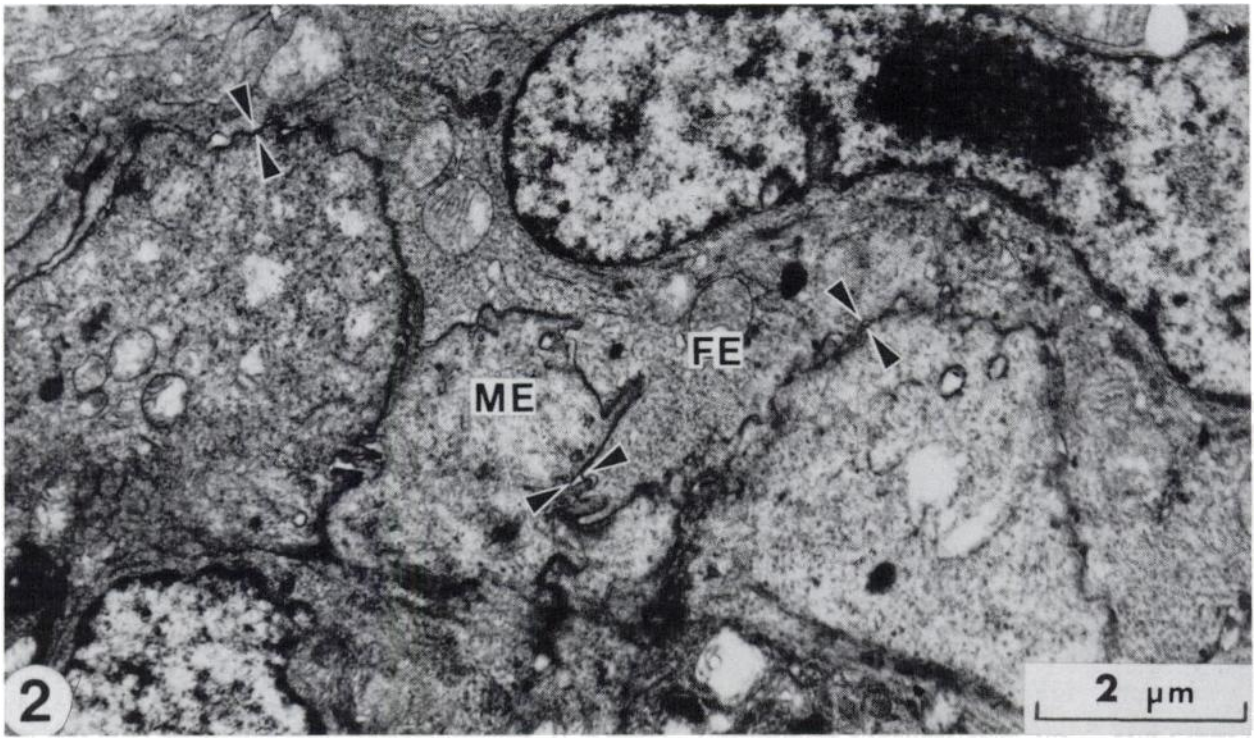

FIG. 2. Close apposition of fetal epithelial (*FE*) and maternal epithelial (*ME*) plasma membranes (*arrowheads*) at the endometrial-trophoblast interface; mesometrial region, Day 13 of pregnancy.

lacked a positive endometrial response were confirmed nonpregnant. The presence of a fluorescing line along the mesometrial region allowed accurate "mapping" of elongated blastocysts without the aid of visible light. In some cases at Day 12, it was possible to determine the extent of individual blastocysts because uterine tissue between chorionic tips lacked fluorescence.

DISCUSSION

The crucial period for the appearance of increased endometrial vascular permeability in the pig, as evidenced by the temporal consistency of localized fluorescence following systemic injection of Evans Blue, was Day 12 of gestation. By Day 13 in pregnant gilts, histologic changes associated with attachment of the blastocyst to the uterine mucosa were usually detectable. The coincidence of increased vascular permeability with histologic modifications parallels the chronology of early implantation events in the sheep (Boshier, 1970; Guillomot et al., 1981) as well as in decidua-forming species (Psychoyos, 1973). In all cases, cytologic changes associated with initiation of attachment occur within 24 h of a positive dye response.

The extravasation of albumen-bound dye, presumably evidence of vascular modification, appeared to be correlated with the metamorphosis of blastocysts from tubular to filamentous morphology and was confined specifically to zones of direct blastocyst adhesion along the mesometrial region. These observations provide clear evidence that attachment-associated changes in vascular permeability and endometrial

TABLE 1. Endometrial fluorescence observed in cyclic and early pregnant gilts following intravenous injection with Evans Blue.

	Response			
	Cycling[a]		Pregnant[a]	
Day	Yes	No	Yes	No
11	–	6	–	4
12	–	4	3	1
13	–	3	3	–
14	–	3	3	–
15	–	3	2	–
16	–	1	1	–
17	–	1	1	–
18	–	3	1	–
19	–	1	–	–

[a]Figures represent numbers of animals examined on each day.

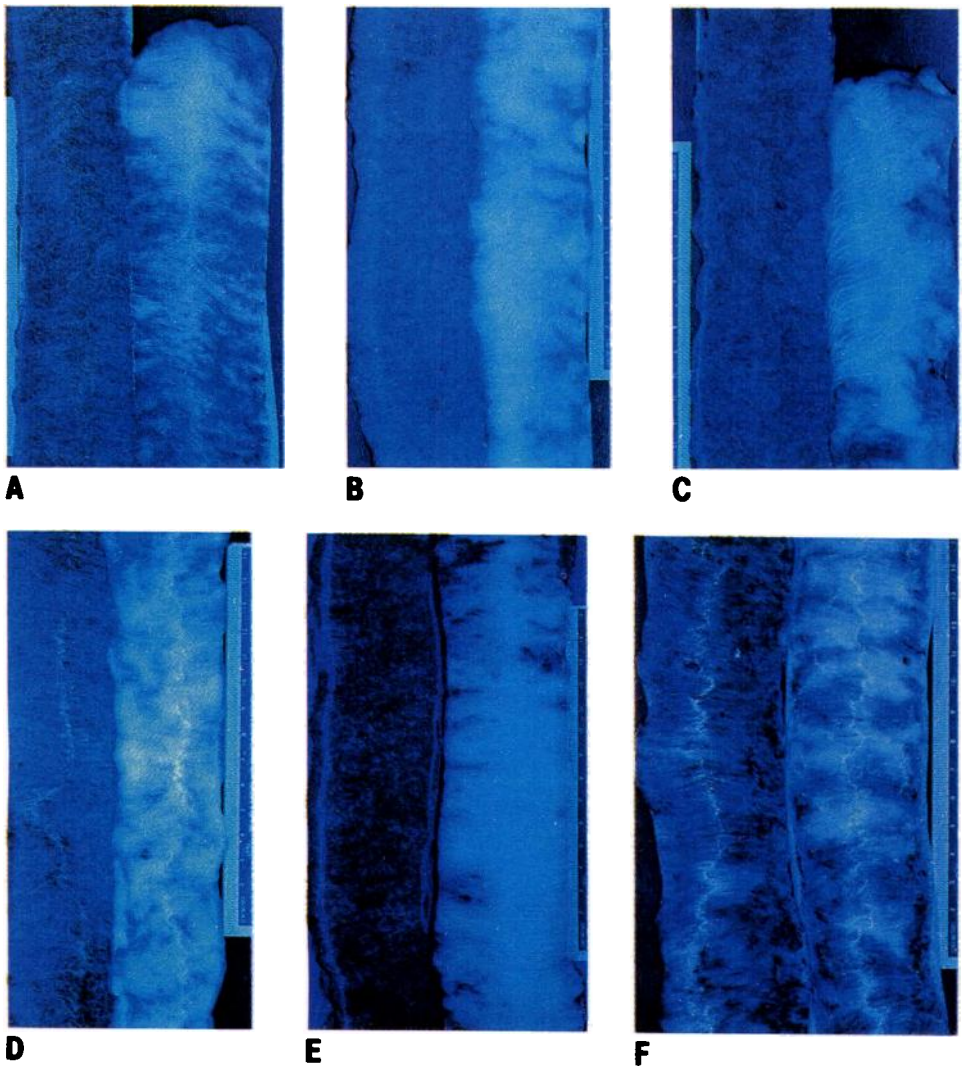

FIG. 3. Endometrial fluorescence following systemic treatment of gilts with Evans Blue. Tissue segments on the right in each image were selected from arterially flushed portions of the uterine horns; the vasculature of the tissue on the left was not cleared. A 15-cm scale is represented by the rule in each photograph. Negative responses were obtained on Day 11 of the cycle (*A*) and pregnancy (*B*), and Days 12 (*C*) and 15 (*E*) of the cycle. A well-defined zone of fluorescence (positive response) was observed along the mesometrial region at Day 12 of pregnancy (*D*) and had increased in intensity by Day 15 of gestation (*F*).

morphology are directed, at least in part, by some embryonic factor(s) acting in a localized manner. Similar alterations in uterine structure and capillary function are restricted to the region of the attaching blastocyst in other mammals having estrous cycles that have been examined to date.

A structural basis for this localized vascular phenomenon has been established in the rat and rabbit. Abrahamsohn et al. (1983) described morphologic signs of endothelial metabolic activation and the development of fenestrations and gaps between these cells, confined to the implantation sites of the rat. Intravenously administered carbon tracer could be found within such gaps and entering the uterine stroma adjacent to implanting embryos in this species and in rabbits (Hoffman and Hoos, 1984). It seems probable that gaps form in the endothelial lining of capillaries in response to some locally produced agent, thereby facilitating increased permeability of the affected vessels.

The mechanism inducing this change in vascular permeability remains to be elucidated. Its timing and appearance are constant on a relative scale for animals as diverse as deeply implanting murine rodents and

non-decidua-forming species such as the sheep and pig where placentae form through attachment of the chorion to uterine epithelium rather than invasion into the stroma. The localized nature of the permeability response may reflect the direct action of embryonic products on the endometrium, or indirect action, by inducing endometrial production of the effective agent(s). The specific mediators of this response are unknown, but histamine, prostaglandins (PGs), and estrogen have been implicated. To date, no data on histamine production by pig blastocysts or endometrium have been published, but the possible involvement of histamine mediation in vascular permeability changes in other species has been reviewed (Dey and Johnson, 1980).

Kennedy (1983) has reviewed evidence in favor of an obligatory role of PGs in endometrial vascular permeability changes in rodents and rabbits. Little is known about the type(s) of PGs involved, their site of production, or their mode of action. Most recently, Kraeling et al. (1985) presented evidence that inhibition of PG synthesis by indomethacin administration during early pregnancy results in pregnancy failure in the pig, suggesting a role for these compounds. Davis et al. (1983) have demonstrated that, although the PGE content of swine blastocysts was consistently greater than that of PGF, the difference only became significant on Days 11 and 12 of pregnancy. The timing of this differential increase in PGE corresponds to the initial appearance of a positive dye response in the gilt.

Observations on nonpregnant animals may indicate the involvement of estrogen in increased vascular permeability within the uterus. Ovine uterine capillary beds are most permeable to trypan blue during estrus (Hawk et al., 1963). This finding and the generalized endometrial fluorescence of two dye-treated gilts near estrus (Day 19), may be interpreted to reflect an increase in vascular permeability throughout the organ under estrogen domination. Systemic injection of estradiol-17β caused transient interendothelial gap formation in uterine capillaries and small venules in immature rats (Ham et al., 1970), and the development of fenestrations in mouse uterine capillaries (Martin et al., 1973). Estrogens have been demonstrated in the trophectoderm of swine blastocysts between Days 10 and 12 (King and Ackerley, 1985), and measureable production is closely correlated with blastocyst elongation (Perry et al., 1976) and apposition to the uterine mucosa. Therefore, onset of estrogen synthesis could explain both the timing and localized nature of this response.

It seems unlikely that any of these chemical agents would act in isolation to produce this response. Estradiol-17β has been shown to increase synthesis of uterine PGs in the ewe (Ford et al., 1975). Catechol estrogens, but not estradiol, stimulated prostaglandin production by both preimplantation rabbit blastocysts and endometrial cells cultured in vitro (Pakrasi and Dey, 1982). It has been hypothesized that conversion of estradiol to catechol estrogens may stimulate PG production in either or both of these tissues, triggering implantation. Uterine flushings from Day 13 pregnant pigs contain significant amounts of these compounds, and catechol, but not free, estrogens have profound effects on baseline perfusion pressures and ^{45}Ca uptake by uterine arteries (Ford, 1985).

The increased vascular permeability detected on Day 12 and commencement of attachment by Day 13 coincide with two previously documented localized responses to estrogens: increased blood flow to the gravid uterine horns and histotrophe production (Ford and Christenson, 1979; Geisert et al., 1982a, b). These effects undoubtedly facilitate passage of nutrients toward the rapidly developing conceptus and transfer of blastocyst-induced products into maternal circulation.

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