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Apolipoprotein J/Clusterin Expression Defines Distinct Stages of Blastocyst Implantation in the Mouse Uterus

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ABSTRACT

The endometrium is a dynamic tissue that responds to hormonal cues and growth factors to accommodate, regulate, and nurture developing embryos. To provide clues about the molecular mechanisms underlying the responsiveness of this tissue, we have begun to identify genes that are expressed at specific stages of early pregnancy. One such gene, apolipoprotein J (apoJ), encodes a secretory glycoprotein capable of binding lipids and membrane-active proteins. Uterine apoJ gene activity was not detected immediately following fertilization, but glandular epithelial expression of apoJ mRNA appeared just before the time of blastocyst implantation and persisted postimplantation. During implantation, uterine luminal epithelial cells also expressed apoJ, but expression was excluded from luminal cells adjacent to the sites of attached blastocysts. ApoJ protein accumulated in the glandular and uterine lumens in proximity to the epithelial cells that expressed apoJ mRNA. We suggest that apoJ expression is a marker of uterine receptivity to blastocyst implantation. Subsequent expression of apoJ message in uterine stromal cell types and in circular muscle myocytes coincided with the onset of decidualization. During this period the myocytes of the longitudinal muscle layer showed no evidence of apoJ mRNA. ApoJ protein was localized to nondecidualized tissue but was not evident in decidualized cells. In contrast, the protein was dispersed throughout both the circular and longitudinal myometrium. In the uterus of hormone-treated females stimulated with oil, apoJ was also expressed during decidualization in stromal cells and in circular myocytes, indicating that signals specifically transmitted from the embryo itself are not responsible for apoJ mRNA accumulation.

INTRODUCTION

The capacity of a tissue to be remodelled is essential for embryonic development and, in the adult, for regenerative responses to injury and stress. Death of specific cells, proliferation and migration of other cells, and reorganization of the extracellular matrix must occur with precise timing so that degeneration and renewal are balanced. The mechanisms regulating these processes to ensure correct temporal sequence for tissue remodeling are not well understood. A central event in tissue remodeling is the capture and neutralization of released amphipathic and/or hydrophobic cellular and extracellular constituents so that inappropriate destruction of bystander cells is prevented. An ideal inactivator of amphipathic debris is one that is locally available, binds amphipaths, and either neutralizes or facilitates their catabolism. Apolipoprotein J (apoJ), an amphipath-binding secretory glycoprotein present in most, if not all, biological fluids [1] has been implicated as one mediator of tissue remodeling and repair [2-4].

ApoJ is an 80-kDa glycoprotein composed of two nonidentical, disulfide-linked subunits generated in vivo by proteolytic cleavage of the precursor, proapoJ [5]. In plasma, apoJ is associated with lipids and is a marker of specific subsets of high density lipoproteins (HDL) [1, 6]. ApoJ can also bind extracellular matrix proteins, such as fibronectin [7, 8] and heparin [7-9], as well as membrane-active molecules such as apolal [6, 10, 11], carboxy-esterases [12], and membrane-associated proteases [13]. ApoJ mRNA is constitutively expressed predominantly in epithelial cells at barrier-fluid interfaces [2], but can be dramatically induced in epithelial and other cell types under a variety of conditions, such as hormone depletion in the regressing mammary gland and prostate [3, 14-16] and during ischemic injury in the kidney [17] and neurodegeneration in the brain [18]. The fact that apoJ can be up-regulated, without organ restriction, at sites of tissue injury and reorganization suggests that apoJ plays an important and generalized role in tissue remodeling.

Limited evidence predicts a role for apoJ in tissue remodeling specifically in the uterus, a physiologically and structurally dynamic organ in which expression of the apoJ gene has been reported [2-4, 19]. Uterine structure and function are governed by steroid hormone fluctuations, and apoJ gene expression in other tissues is known to be exquisitely sensitive to such fluctuations. In the rat, for example, testosterone ablation by castration results in massive apoptotic epithelial involution of the ventral prostate with concomitant and dramatic induction of apoJ mRNA [20, 21]. We [19] recently demonstrated that induced apoJ expression is associated with tissue remodeling in the mouse and human uterus following uterine involution during estrous and menstrual cycling, respectively, and also in the mouse in response to hormonal priming similar to that required for blastocyst implantation. This study defines temporal and spacial patterns of apoJ expression in the uterus during actual implantation.

The molecular participants in maternal uterine tissue remodeling in response to an invading embryo are not well defined. We propose that a functional analysis of a putative marker of tissue remodeling, apoJ, will yield clues that will provide insight into this complex process. As a first step toward this goal, this investigation was undertaken to determine whether apoJ expression is associated with a specific stage of the implantation process. We compared the expression patterns of apoJ, in relation to morphologic evidence of uterine tissue remodeling, during implantation...
and during the induction of decidualization in hormone-treated mice. The expression of apoJ in the uterus was analyzed before, during, and after blastocyst implantation at half-day time intervals from 0.5 to 9.5 days postcoitum (p.c.). ApoJ mRNA was detected by in situ hybridization; protein, by immunohistochemistry.

**MATERIALS AND METHODS**

**Implantation Analysis**

Adult (12–14 wk) C57BL/6 mice were used. Mice were housed under 12L:12D cycles and provided with standard lab chow and water ad libitum. Half-day time intervals were based on detection of a copulatory plug, designated as Day 0.5 p.c., following cohabitation of two females and one male 16 h prior to examination. Whole-day time intervals were similarly established after 3 h of cohabitation. Preimplantation was defined as the time from detection of the copulatory plug to implantation at approximately 4.5 days p.c. Implantation sites were identified by injection of 250 μl of 1% pontamine sky blue (Chicago Blue) in water into the tail or jugular vein of mice anesthetized with metofane [22]. The mice were removed from anesthesia for 15 min and killed by cervical dislocation. Identification of implanted embryos was determined by the characteristic blue band at the site of implantation [22]. Uteri, including embryos, were excised, snap frozen, and embedded in O.C.T. embedding compound (Baxter Scientific, McGaw Park, IL). Results for individual time points were obtained from at least 5 pregnant females. A minimum of 8 tissue sections were evaluated per mouse.

**Induction of Decidualization**

C57BL/6 mice, which had been ovariectomized for 14 days, were treated as previously described [22]. Briefly, mice were injected with 100 ng of estradiol-17β for 3 consecutive days. After 3 days of rest, the mice were injected daily with 500 μg progesterone and 10 ng of estradiol-17β (designated Day 1). Stimulation of a decidual reaction was initiated in one uterine horn by intruterine administration of 10 μl of corn oil on Day 3 [22]. Uteri were analyzed from Days 1 to 7 after the addition of oil. The unstimulated horn served as the control. At least 2 animals (4 uterine horns) and 4 sections per horn were analyzed per time point.

**In Situ Hybridization**

Tissue sections (6 μm) were cut on a cryostat, air dried, and fixed in 4% paraformaldehyde/single-strength PBS for 1 h at room temperature and processed as previously described [2, 3]. Hybridization was performed at 42°C for 18 h, using HindIII- and BamHI-generated mouse apoJ sense and antisense cRNA probes, respectively (5 × 10⁶ cpm/ml), from clone 5–1 [2, 3].

**Immunohistochemistry**

Tissue sections (6 μm) were cut on a cryostat, air dried, fixed in cold acetone for 10 min, and processed for immunohistochemistry by using the peroxidase-antiperoxidase method [23], as previously described [3]. Incubation with the primary antibody (10 μg/ml), rabbit anti-rat SGP-2 IgG [24] or nonimmune rabbit IgG, was performed for 1 h at room temperature. The secondary and tertiary antibodies were goat anti-rabbit IgG (1/25 dilution) and rabbit peroxidase-antiperoxidase complex (0.2 mg/ml), respectively.

**RESULTS**

After mating, passage of the fertilized egg through the oviduct to the uterus requires 0.5–2.0 days in the mouse. Our results reveal two distinct cell-type and temporal patterns of apoJ expression: the preimplantation induction and subsequent maintenance through decidualization of epithelial cell apoJ mRNA vs. the transient expression of apoJ mRNA during decidualization in stromal cells and in circular smooth muscle myocytes. The earliest time that apoJ was detected in the uterus was at 0.5 days p.c., when the protein was evident on sperm in the uterine lumen (Fig. 1A). At this time, no apoJ mRNA or protein (Fig. 1B) was detected in the uterine lumen, stroma, or myometrium or in endometrial glands. ApoJ mRNA was first detected in the uterus between Days 2.5 and 4.5 p.c., restricted to glandular epithelial cells (Fig. 1D); apoJ protein was similarly localized with accumulation evident in the glandular lumens (Fig. 1C). No apoJ mRNA or protein was detected in luminal epithelial, stromal, or myometrial cells.

In the mouse, hatched blastocysts implant at approximately 4.5–5.0 days p.c. Sites of implantation were identified by pontamine sky blue uptake. Figure 2A illustrates a representative implantation site, with the blastocyst indicated by the asterisk in the uterine lumen. Glandular epithelial cells continued to express and secrete apoJ. In contrast to the results from Days 0.5–4.5 p.c., apoJ mRNA was now abundant in the luminal epithelial cells, but not in those located adjacent to implantation sites (Fig. 2B, arrowheads). ApoJ protein was localized in the uterine lumen in proximity to the cells expressing the mRNA (not shown). No apoJ message was detected in the luminal epithelial cells at sites where blastocysts had implanted (Fig. 2C), and no protein was detected in the adjacent uterine lumen (not shown).

Decidualization at 5.0–7.5 days p.c., when uterine stromal tissue undergoes reorganization as fibroblast-like cells assume an epithelial-like pattern of morphology, marked the first appearance of apoJ in nonepithelial cells. ApoJ mRNA was expressed in decidualized but not undecidualized stromal cells (Fig. 2, C and E). ApoJ protein, in contrast, was abundant throughout areas of undecidualized stromal cells with little or none in the vicinity of obviously decidualized cells (Fig. 2, D and F). A detailed examination of numerous sections obtained over this time period revealed that apoJ mRNA was expressed at high levels in decidual cells as early as morphological decidualization was noted on Day 5.0. ApoJ mRNA first appeared in a band of decidualized cells in proximity to the embryo. As the decidual region expanded and the border separating decidualized from undecidualized tissue moved away from the embryo, the band of apoJ-expressing cells moved similarly. Abundant apoJ mRNA was also detected in myocytes of the circular, but not longitudinal, layer of smooth muscle during this same period of time (Fig. 2E), and apoJ protein was distributed throughout the myometrium (Fig. 2F). As noted previously, apoJ mRNA was expressed in the glandular epithelial cells (Fig. 2E), and the protein was evident in glandular lumens (Fig. 2F). Expression of apoJ mRNA on Day 7.5 p.c. in mouse uterine decidual cells was first reported by French et al. [4]; also, Ahuja et al. [25] detected apoJ message in glandular and luminal epithelial cells on Days 5.5–6.5 p.c. However, these investigators did not re-
port apoJ message in smooth muscle myocytes or evaluate apoJ protein localization relative to the cells expressing the mRNA.

Once implantation and decidualization were completed, between 8.0 and 9.5 days p.c., no apoJ message was detected in the cells of the fully decidualized region (Fig. 2G), although low-level apoJ protein was randomly dispersed in the stroma (Fig. 2H). At this time, epithelial cells of the uterine lumen were the predominant cell type expressing apoJ mRNA (Fig. 2G). ApoJ protein was evident in the lumen (Fig. 2H). It was not possible to determine unequivocally whether glandular epithelial cells continued to express apoJ message, since uterine glands were difficult to distinguish because of the expansion of the decidua. Weak apoJ signal persisted in the myocytes of the circular muscle, and low-level diffuse apoJ protein signal appeared throughout the myometrium (not shown). We were unable to detect apoJ mRNA or protein in the placenta.

To determine whether the induced expression of apoJ at 5.0–7.5 days p.c. in decidual cells and circular myocytes

FIG. 1. ApoJ localization during preimplantation. A) Immunohistochemistry showing apoJ protein present on sperm in the uterine lumen (arrowhead) on Day 0.5 p.c. B) ApoJ protein was not detected in uterine tissue from 0.5 to 2.5 days p.c., shown here as a representative Day 1.5 p.c. section. D) From Days 2.5 to 4.5 p.c. (Day 4.5 shown), apoJ mRNA was expressed only in the glandular epithelial cells with (C) apoJ protein localized to the glandular lumens (arrowhead). A, x300; B-D, x150.

FIG. 2. Expression of apoJ during blastocyst implantation and decidualization. A) A Day 5.0 p.c. tissue section stained with hematoxylin and eosin, showing an implantation site (asterisk). B, C) ApoJ mRNA was abundantly expressed in luminal epithelial cells distal (B, arrowhead) to the implantation site (asterisk), shown here at 5.5 days p.c., and in decidualized stromal cells. C) ApoJ mRNA was not expressed, however, in the luminal epithelial cells at the implantation site. D) ApoJ protein was detected in the undecidualized stroma; a Day 6.0 p.c. section is shown, representative of Days 5.0–6.0 p.c. E) ApoJ message was expressed in decidualizing stromal cells and circular muscle myocytes during decidualization from Days 5.0 to 7.0 p.c.; a representative sample at Day 6.5 p.c. is shown. F) ApoJ protein was abundant from Days 5.0 to 7.0 p.c. throughout undecidualized stroma and myometrium; a representative sample at Day 6.5 p.c. is shown. G) From Days 8.0 to 9.5 p.c., apoJ mRNA was primarily present in luminal epithelial cells, with (H) protein present in the lumen; Day 8.5 p.c. is shown as a representative sample. Low-level apoJ protein scattered throughout the decidua was evident. A, B, and D, ×100; C and E–H, ×200.
FIG. 3. Expression of apoJ during artificially induced decidualization. A) No apoJ mRNA was detected in uterine luminal epithelial cells 3–4 days following stimulation (Day 3 shown), although abundant apoJ signal was present in glandular epithelial cells (arrowheads). B) ApoJ mRNA was strongly expressed in decidual stromal cells and myocytes of the circular muscle band in hormone-treated females on Day 5 following stimulation. C) A control section of the decidua on Day 6 probed with an apoJ sense cRNA, indicating background hybridization. D) A serial section to (C) probed with apoJ
required the embryo and was consistent with natural implantation, we evaluated hormone-treated females for apoJ from 1 to 7 days following artificial initiation of the decidual reaction. Many of the cellular and morphological changes that occur in the uterus during implantation are recapitulated in hormone-treated females in response to a nonembryonic stimulus such as the corn oil [22] used here. In the oil-stimulated uterine horn, no apoJ expression was detected in epithelial cells of the uterine lumen at any time after stimulation, although glandular epithelial cells expressed abundant apoJ message. Figure 3A, showing a section of a uterus obtained on Days 3–4 poststimulation, is representative of this response. By Day 5, apoJ expression in stimulated, hormone-treated femaled cells was induced in decidualizing cells and circular myocytes (Fig. 3B), a response identical to that in pregnant animals (Fig. 2, C and E). Decidual apoJ expression continued through Day 6 (Fig. 3D). No decidual apoJ expression was detected later, however, on Days 7 and 8 (Fig. 3E). Figure 3C, a serial section to that shown in Figure 3D but stained with control sense apoJ riboprobe, is representative of the low level of background signal. In the control unstimulated uterine horn, apoJ mRNA was expressed in both the luminal and glandular epithelial cells, whereas there was no decidual reaction or apoJ expression in the stroma or myometrium (Fig. 3F).

DISCUSSION

The induction of apoJ expression defines discrete stages in the time surrounding blastocyst implantation in the mouse. Prior to blastocyst hatching in early (< 2.0 days p.c.) preimplantation, apoJ protein is attached to sperm in the uterine lumen, although no expression of apoJ message in the uterus is evident. Observation of the absence of apoJ mRNA in uterine cell types immediately after fertilization extends the reports of Morales and colleagues [26, 27] that sperm-associated apoJ is derived from the male. ApoJ mRNA is first detected in the uterus in glandular epithelial cells at 2.5 days p.c., just prior to blastocyst implantation, and these cells express apoJ message continuously through pre- and perimplantation. At the approximate time of implantation (Day 4–5 p.c.), the apoJ message accumulates in epithelial cells of the uterine lumen, which are believed to be contiguous with those of the endometrial glandular structures. Strikingly, where implanted blastocysts can be identified by pontamine sky blue staining, the apoJ message is localized exclusively to undecidualized stroma. Although morphologically normal luminal epithelial cells at these implantation sites express apoJ mRNA. Nevertheless, the gene is expressed in distal but nearby uterine epithelial cells, allowing for possible interaction between secreted apoJ and the blastocyst.

There is a remarkable transient association of apoJ expression with the developmental process of decidualization. No apoJ expression was detected in cells undergoing apoptosis, consistent with the finding of Gu et al. [32]. As the embryo invades the maternal stroma from 5.0 to 7.5 days p.c., a morphological and functional differentiation occurs as fibroblast-like cells become epithelial-like [22, 28–30]. The cells in the zone surrounding the invading embryo decidualize initially, with the resultant decidualized tissue situated immediately adjacent to the embryo [28–30]. More distal to the embryo, the cells remain undecidualized. As the embryo continues to invade and enlarge, the zone of decidualized cells enlarges. During this process, apoJ mRNA remains localized primarily to the cells in the boundary zone between obviously decidualized and undecidualized tissue. ApoJ protein, in contrast, appears to be localized exclusively to undecidualized stroma. Although there is a transient increase in permeability to maternal blood components early postimplantation, the striking complementarity between apoJ mRNA expression and protein localization suggests that undecidualized cells express receptors or binding sites for decidualy produced apoJ.

During the time of decidualization, apoJ mRNA is also expressed transiently in the myocytes of the circular, but not longitudinal, smooth muscle layer, although apoJ protein is present throughout the myometrium. Little is known about the physiology of the myometrium during early pregnancy. In keeping with the association between epithelial and stromal apoJ expression and tissue reorganization, the expression of apoJ in circular myocytes may indicate that architectural changes occur in this tissue layer early following implantation. The uterus is known to have increased in size by 5–10-fold 5–9 days after implantation. The initial myometrial response to increased decidual volume and pressure may involve changes in cell shape or architecture and may thus be considered a form of tissue remodeling [33]. ApoJ expression has been shown to be coincident with morphological differentiation in cultured vascular smooth muscle cells [9, 34]. ApoJ expression may be useful as a marker to distinguish circular from longitudinal myocytes and as a tool to understand physiological changes at the molecular level in the myometrium during early pregnancy.
The findings that apoJ is associated with uterine tissue remodeling and that its expression in glandular and luminal epithelial cells coincides with implantation suggest that apoJ not only is a marker of uterine receptivity for blastocyst implantation but also plays an active role in the implantation process. This work may have important clinical implications in the study of human infertility. Since apoJ is a membrane policeman, it serves as a regionally restricted energy and metabolite transporter, providing temporal and cell-type pattern of apoJ expression in the uterus: lipid transport [1, 6, 10, 11], cytoprotection [1-3], and injury repair [39-41]. The abundance of apoJ is suggestive of a fundamental metabolic role, and we favor a function in tissue remodeling by localized (vs. systemic) lipid transport. We propose that initially glandular and luminal epithelial cells provide apoJ-associated lipid to the blastocyst, consistent with the luminal localization of the apoJ protein, evident in this study. Subsequently, specialized secretory stromal and myometrial cells, distinguished from their nonsecretory counterparts by apoJ expression, provide lipid for expansion of the decidua and myometrium. Even the absence of apoJ message in epithelial cells localized at blastocyst implantation sites can be explained within this framework. We cannot exclude the possibility that these cells do not express the apoJ gene because they have been terminally altered by contact with the blastocyst. The possibility must be considered that the steady-state level of apoJ message is low and therefore undetectable because of blastocyst-induced accelerated translation of the apoJ mRNA. It is relevant in this context that the trophoectoderm cells of the blastocyst express abundant gp330 [42]. Gp330, a member of the low density lipoprotein receptor gene family, is responsible for binding and internalizing apoJ [43]. Moreover, a striking feature of epithelial cells in contact with a blastocyst during implantation is their loss of triglyceride deposits [44], and we [45] have shown that nascent apoJ can be secreted as a triglyceride-rich lipoprotein. Secreted apoJ may therefore be a lipoprotein that serves as a cytoprotectant, serving simultaneously as a receptor-mediated endocytosis, serving simultaneously as a cytoprotectant.

Differences between circular and longitudinal myocytes are intriguing and warrant further investigation. A protein that is induced near the implantation site but does not require a living embryo as a stimulant may play a role in receptivity for implantation by committing the uterine lining to blastocyst attachment and/or invasion. Other molecules are expressed with hormone dependence near the implantation site, such as heparin-binding epidermal growth factor [36], leukemia inhibitory factor [37], and tenascin [38]. The function of apoJ is not known with certainty. The predominant theme underlying apoJ expression in the uterus and in many other organs is its association with dynamic interfaces that separate tissue from harsh biological fluids [2] and normal tissue from reorganizing tissue [39]. A number of roles for apoJ have been suggested by biochemical and anatomical data. Three are consistent with the temporal and cell-type pattern of apoJ expression in the uterus: lipid transport [1, 6, 10, 11], cytoprotection [1-3], and injury repair [39-41]. The abundance of apoJ is suggestive of a fundamental metabolic role, and we favor a function in tissue remodeling by localized (vs. systemic) lipid transport. We propose that initially glandular and luminal epithelial cells provide apoJ-associated lipid to the blastocyst, consistent with the luminal localization of the apoJ protein, evident in this study. Subsequently, specialized secretory stromal and myometrial cells, distinguished from their nonsecretory counterparts by apoJ expression, provide lipid for expansion of the decidua and myometrium. Even the absence of apoJ message in epithelial cells localized at blastocyst implantation sites can be explained within this framework. We cannot exclude the possibility that these cells do not express the apoJ gene because they have been terminally altered by contact with the blastocyst. The possibility must be considered that the steady-state level of apoJ message is low and therefore undetectable because of blastocyst-induced accelerated translation of the apoJ mRNA. It is relevant in this context that the trophoectoderm cells of the blastocyst express abundant gp330 [42]. Gp330, a member of the low density lipoprotein receptor gene family, is responsible for binding and internalizing apoJ [43]. Moreover, a striking feature of epithelial cells in contact with a blastocyst during implantation is their loss of triglyceride deposits [44], and we [45] have shown that nascent apoJ can be secreted as a triglyceride-rich lipoprotein. Secreted apoJ may therefore be a lipoprotein that serves as a cytoprotectant, serving simultaneously as a cytoprotectant, serving simultaneously as a cytoprotectant.

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