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REGULAR ARTICLE

Lois A. Salamonsen · Svetlana Shuster · Robert Stern

Distribution of hyaluronan in human endometrium across the menstrual cycle

Implications for implantation and menstruation

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Abstract Hyaluronan is a molecule with many known roles in cellular physiology and is often associated with tissue remodeling. The human endometrium undergoes dramatic remodeling during the course of the normal menstrual cycle but its regulation is not well understood. This study examined the levels of deposition of hyaluronan in human cycling endometrium by histochemical localization, using a highly specific hyaluronan-binding peptide. This was facilitated by avoiding conventional formalin fixation, which results in serious loss of the water-soluble hyaluronan. Peaks of hyaluronan deposition were observed in the stromal compartment during the mid-proliferative (days 5–10) and the mid-secretory phase (days 19–23) of the cycle. In physiological terms, the first phase corresponds to the time of rapid cellular proliferation of undifferentiated cells, whereas the second phase coincides with the time when the implantation of the conceptus would be initiated in a fertile cycle. By the time menstruation starts, very little hyaluronan remains in the stroma. In contrast with the stromal staining, hyaluronan deposition around blood vessels was constant throughout the cycle. The dramatic changes in hyaluronan deposition and their correlation with the cyclic growth and remodeling in the human endometrium suggest a major role for hyaluronan in the physiology of this tissue.

Keywords Endometrium · Hyaluronan · Hyaluronic acid · Tissue remodeling · Menstrual cycle · Human

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L.A. Salamonsen (✉)
Prince Henry's Institute of Medical Research, PO Box 5152, Clayton, Victoria 3168, Australia
e-mail: lois.salamonsen@med.monash.edu.au
Tel.: +61-3-95944373, Fax: +61-3-95946125

S. Shuster · R. Stern
Department of Pathology, School of Medicine, University of California San Francisco, San Francisco, CA 94143–0506, USA

Introduction

Hyaluronan (hyaluronic acid, HA), is a prominent component of the extracellular matrix (ECM), particularly in rapidly growing and remodeling tissues undergoing regeneration and repair (Toole 1991), while additional roles for it are increasingly being described in other basic biological processes (Lee and Spicer 2000; Toole 2000). HA is a nonsulphated, linear glycosaminoglycan (GAG) that exists as a high-molecular weight (10^4–10^7 Da) polymer. It is unique amongst GAGs in that it is nonsulphated and the only GAG not synthesized covalently linked to protein to form a proteoglycan molecule. Its strong negative charge attracts a large associated volume of water, capable of expanding its solvent domain up to 10,000 times its actual polymer volume. This hydration causes voluminous expansion of tissues. Clearing of HA by hyaluronidase enzymes reduces hydration of the ECM, causing compaction of cells and tissues. This appears to facilitate cell-cell contacts, contributing to the initiation of programs of differentiation (Frost and Stern 1997; Nicoll et al. 1999).

HA plays an important role in a number of biological processes by providing a provisional matrix facilitating cell migration (Laurent and Fraser 1992). It can also function as a cellular signaling molecule binding to receptors such as CD44 (Aruffo et al. 1990; Underhill 1992) or the receptor for HA-mediated motility (RHAMM; Hall and Turley 1995). HA can also exist in a number of lower molecular weight forms that exhibit different biological effects on cells and tissues (Forrester and Balazs 1980; West et al. 1985).

The architecture of the endometrium undergoes extensive remodeling during the reproductive cycle. The cyclical changes occur in response to the ovarian steroid hormones and in preparation for implantation of a conceptus if the cycle is fertile. If no blastocyst implants, the functionalis layer is shed at menstruation and regrowth occurs in anticipation of this event during the next cycle. The remodeling includes phases of cellular proliferation, cellular differentiation, and tissue breakdown along with

alterations in the composition of the ECM. During the preovulatory phase, re-epithelialization is followed by extensive cell division, along with production of ECM components (Wienke et al. 1968; Wynn 1989), which leads to recovery of functional thickness. Following ovulation, there is considerable cellular differentiation, the tissue becomes more hydrated, the stromal collagen more loosely organized and the production of collagen-I and fibronectin is slowed. Ultrastructural studies suggest that the highest rate of production of new matrix occurs in the proliferative and early secretory phases (More et al. 1974). Two phases of tissue edema are identifiable in human endometrium, one during the mid-proliferative phase and one in the mid-secretory phase. Late in the cycle, tissue regression precedes the onset of menstruation.

The tissue loosening required for implantation and subsequent invasion of trophoblasts into decidual locations may be associated with swelling and expansion of the extracellular space. Highly hydrated space-filling molecules would thus be required to stabilize the interstitium (Aplin et al. 1988). In the mouse uterus, HA increases suddenly on the day of implantation (Carson et al. 1987; Brown and Papaioannou 1992) and appears to be associated with regions of the endometrium that contain proliferating stromal cells (Brown and Papaioannou 1992). A similar correlation between HA expression and cell proliferation has been noted in a wide variety of different cell types (reviewed in Toole 1991): thus HA may also have an important function during the phase of rapid cellular proliferation within the endometrium.

Given the nature of the remodeling of the human endometrium, we examined the expression of HA across the normal menstrual cycle, to determine its cyclical changes and whether these are related to known morphological and physiological events. Such information could provide possible functions for this versatile molecule in the normal endometrium.

Materials and methods

Tissue collection and fixation

Endometrial tissue (functionalis) was obtained at curettage from women with regular menstrual cycles and no apparent endometrial dysfunction, who gave informed consent for collection of tissue. Approval was given by the Human Ethics Committee at Monash Medical Centre, Melbourne, Australia. The women were either of proven fertility and were scheduled for tubal ligation or were undergoing testing for patency of the fallopian tubes. Patients with leiomyomas or endometriosis, or those who had received any steroid treatment during the past 12 months were specifically excluded from the study. The tissue samples were fixed in Carnoy's fixative for 2–4 h, washed in 90% ethanol, and processed to paraffin wax blocks. Specimens were histologically dated by an experienced gynecological pathologist, according to the method of Noyes et al. (1950). Sections were cut at 6 μm and mounted on polylysine-coated glass slides for histological staining. A total of 38 specimens taken across the cycle were stained. These included: one tissue from each of days 1, 3, 4, 5, 6, 7 9, 10, 13, 14, 15, 16, 17, 18, 19, 22, 23, 24; two tissues from each of days 8, 12, 20, 21, 25; four tissues on day 26; three tissues on each of days 27 and 28.

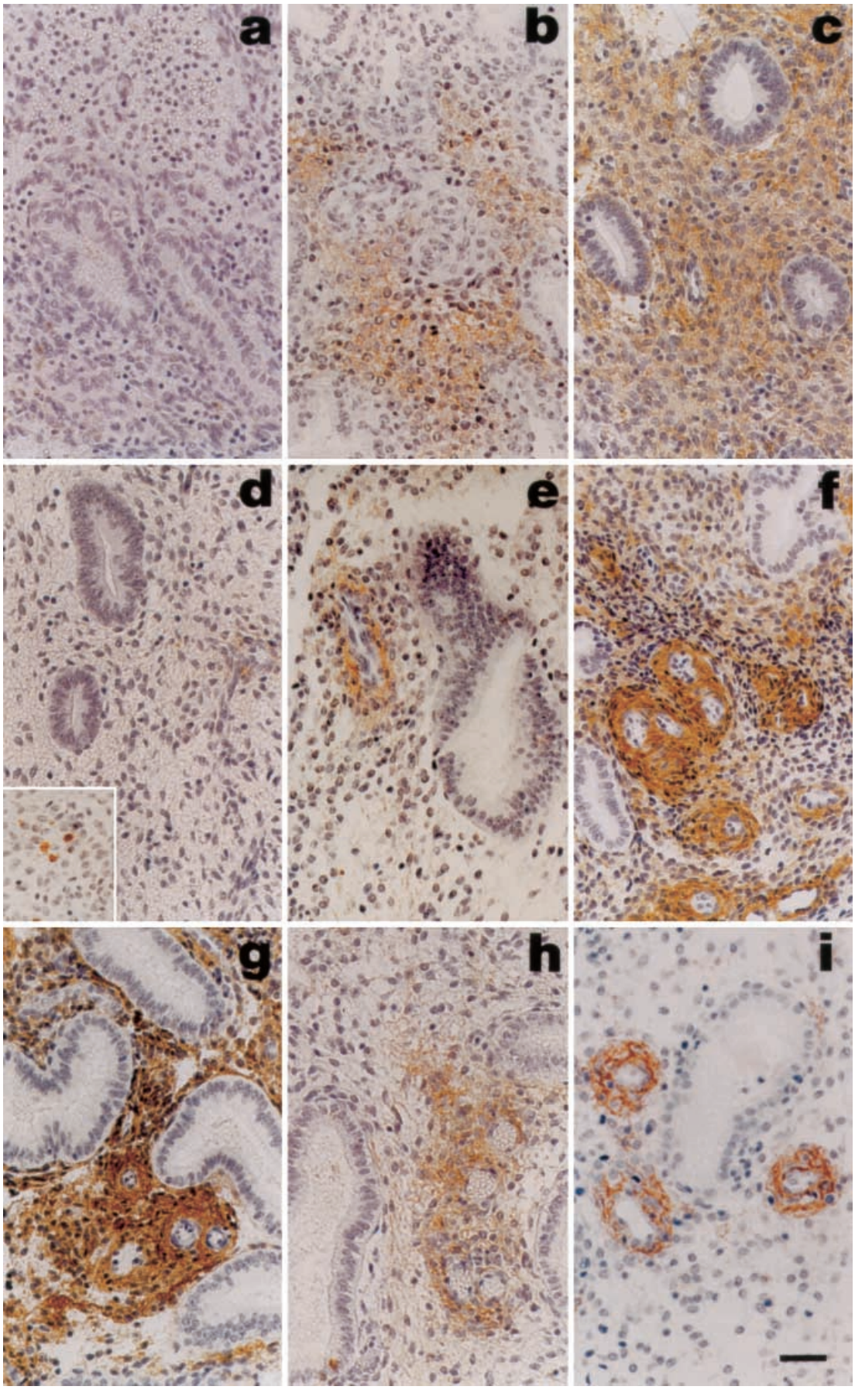
HA histochemistry

Histological staining for HA was performed utilizing a highly specific, biotinylated HA-binding peptide (bHABP), derived from a tryptic digest of bovine aggrecan, the chondroitin sulfate-rich proteoglycan of cartilage, and isolated by affinity chromatography using a column of HA-sepharose (Tengblad 1979). The distribution of HA in human skin has been described using this technique (Meyer and Stern 1994). The critical nature of the fixation procedure in preserving HA has been emphasized previously (Lin et al. 1997). In the present study, tissue was fixed in Carnoy's fixative (chloroform, acetic acid, methanol, 30:10:60 v/v), the components of which appear to hinder the diffusion of HA into the fixative solution during the process. Sections were subject to deparaffinization followed by rehydration. They were then incubated in 3% normal goat serum (Vector, Burlingame, Calif.) for 30 min at 37°C as a blocking solution before incubation with the diluted bHABP (1:200 of a 0.5 mg/ml preparation) overnight at 4°C. Slides were then washed for 10 min with phosphate-buffered saline that was calcium- and magnesium-free (PBS-CMF). Endogenous peroxidase activity was blocked by incubating for 30 min at room temperature with 3% H_2O_2 in PBS-CMF; the sections were rinsed with PBS-CMF and then incubated for 45 min with the avidin-labeled horseradish peroxidase solution prepared as specified by the manufacturer (Vectastain ABC Peroxidase Kit PK 4000; Vector). After washing for 15 min in PBS-CMF, the slides were incubated for 5 min in the peroxidase substrate solution (Peroxidase Substrate Kit, DAB SK-4100; Vector), washed for 5 min in tap water, counterstained with hematoxylin, cleared, and mounted with Permount. Control sections were stained with bHABP that had been incubated overnight at 4°C in a solution containing 0.3 mg/ml HA (ICN, Irvine, Calif.), or were pretreated with 50 U/ml *Streptomyces* hyaluronidase (Calbiochem, San Diego, Calif.) in PBS at 37°C for 24 h before staining for HA. An additional control section was preincubated with PBS at 37°C for 24 h before staining for HA. In every staining run, a section from a single tissue block (day 22) was included to provide quality control. Photomicrographs were taken using an Olympus Vanox AHBT3 microscope (Olympus, Woodbury, NY) with an integrated Olympus C-35AB-Y camera. Subjective scoring of stromal staining intensity was carried out by two independent investigators on a scale of 0–5.

Results

Histolocalization of HA within the endometrium was examined in samples taken across the menstrual cycle. Samples (1–4) from each day of the normalized 28-day cycle were obtained, and eight representative samples are shown in Fig. 1. Staining of the quality-control tissue did not alter between runs. Samples taken from different

Fig. 1a–i Histochemistry for hyaluronan (HA) deposition in human endometrium across the normal menstrual cycle. Positive staining is *brown* and the nuclear counterstain is *blue*. Tissue is shown on: **a** day 1, demonstrating overall absent staining; **b** day 3, showing low level of staining in stroma, but stronger around remaining spiral arterioles; **c** day 7, showing intense and regular stromal staining; **d** day 12, demonstrating reduced stromal staining. The *inset* shows positive staining in polymorphs; **e** day 15, showing only low levels of patchy staining; **f** day 19, showing a marked increase in stromal staining, particularly around blood vessels; **g** day 21, displaying strong positive staining between glands and around blood vessels; **h** day 24, demonstrating that stromal staining is markedly reduced; **i** day 27, showing dramatically reduced positivity for HA, except in perivascular areas. Magnification is the same for all photomicrographs. *Bar* 20 μm ▶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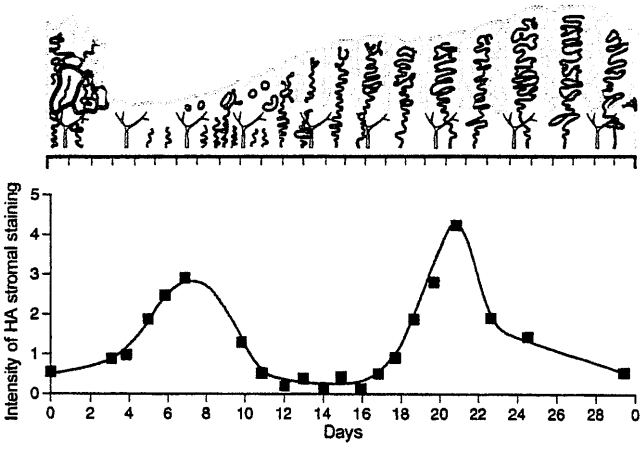

Fig. 2 Levels of HA deposition in the human endometrial stroma across the normal menstrual cycle. Estimates of HA deposition were made independently by two investigators, using a scale of 0–5. A representation of the human endometrial cycle is shown for comparison

patients on the same day of the cycle showed remarkable consistency. In general, HA was localized within the endometrial stroma but with considerable cyclical changes. Staining was quite variable during the menstrual phase; some areas of stroma remained completely negative (Fig. 1a; day 1), while in other areas, even within the same tissue sample, it was relatively strong. Maximal staining was observed around those spiral arterioles still remaining from the previous cycle (Fig. 1b; day 3). By day 4, staining was quite intense immediately under the luminal epithelium, seemingly in areas where reepithelialisation was complete. Staining was also apparent around some but not all glands and around blood vessels. However, approximately 50% of the tissue at this time was devoid of immunoreactive HA.

As the cycle progressed through the proliferative phase, staining of HA in the endometrial tissue became more regular throughout the stroma (Fig. 1c; day 7) and was often observed immediately surrounding the blood vessels. Staining was negative in epithelium. However, by the late proliferative phase (from day 12; Fig. 1d), stromal staining became faint again, although HA was occasionally seen around blood vessels. Some polymorphonuclear leukocytes stained positive (Fig. 1d; inset). This faint and patchy stromal staining continued into the early secretory phase, although areas around arterioles overall stained more strongly than other areas of stroma (Fig. 1e; day 15).

By the mid-secretory phase (Fig. 1f; day 19), there was again a marked increase in stromal positivity, and on day 21 (Fig. 1 g) there was strongly positive HA around and between the glands and around blood vessels. This started to decrease again on day 24 (Fig. 1 h), becoming much less around blood vessels and patchy in the remainder of the stroma. By day 25, HA staining was negative except for the immediate areas around blood vessels. Positive reactivity was no longer apparent around the glands, and this continued to be the case until the end of the cycle (day 27; Fig. 1i). However, perivascular staining was maintained and indeed remained throughout the cycle.

Subjective scoring performed by the two investigators was in close agreement and the consensus results are shown diagrammatically in Fig. 2. The marked cyclical variation in HA content emphasizes that the peak levels of HA stromal deposition correspond with the two phases of stromal edema in the endometrium. Furthermore, the time when HA staining is at a minimum, prior to menstruation, coincides with the well-documented tissue regression, and this is assumed to result from the loss of the HA-associated water of hydration.

Discussion

This paper demonstrates for the first time that the GAG HA undergoes substantial variation with the menstrual cycle in the human endometrium. Peaks of stromal deposition of HA coincide with phases of profound cellular proliferation and with the time when the tissue is receptive to embryo implantation.

Interstitial collagens, including types I, III, V, and VI are localized diffusely in the stroma throughout the menstrual cycle. Components of the basement membrane, particularly type IV collagen and laminin, are found exclusively in the basement membrane of the endometrial glands and in the walls of the blood vessels though the cycle. Collagen IV, laminin, and heparan sulphate proteoglycan can also be identified within the stroma late in the cycle, when they become organized into a pericellular aura around decidualized stromal cells (Aplin et al. 1988; Iwahashi et al. 1996). Collagen VI is abundant in endometrium in the proliferative phase but is progressively lost in the secretory phase, being extensively degraded and eventually disappearing (Aplin et al. 1988; Aplin 1989). The tissue loosening required for implantation and subsequent invasion of trophoblasts into decidual locations may be associated with this selective breakdown of type VI collagen fibrils, allowing swelling and expansion of the extracellular space.

Bursts of HA deposition in the ECM coincide with rapid cell proliferation (Tomida et al. 1974; Brecht et al. 1986; Mian 1986; Toole 1991). As shown in the present studies, a positive correlation of HA deposition occurs with the period of cellular proliferation during the proliferative phase (approximately day 7; Fig. 1b). The level of HA reaches a nadir prior to ovulation and remains low during the early secretory phase, when cellular differentiation is initiated. However, it rises again to reach a peak around the time when the embryo would attach and start to implant in a fertile cycle (approximately days 19–23). It is of interest to note the observation (Toole 1991) that, during embryogenesis, HA peaks during embryonic cell proliferation and migration and falls in order for differentiation to commence. In the present study, the level of HA deposition also decreases prior to

menstruation, when tissue regression is occurring but earlier than hypoxia would be expected. The levels of HA are likely to be regulated indirectly by the cyclical alterations in the steroidal milieu which result in alterations in the expression of many cytokines and other regulatory molecules during the menstrual cycle (reviewed by Salamonsen and Woolley, 1996). These in turn are likely to directly or indirectly regulate the expression of the hyaluronan synthases and hyaluronidases whose relative activities determine HA levels. Surprisingly a negative correlation of HA deposition occurs with the influx of leukocytes prior to the onset of menstruation. HA and its expansive water of hydration provide a permissive environment for cell motility, as well as conferring motility directly upon cells through receptor-mediated interactions with the cytoskeleton (Hall and Turley 1995; Entwistle et al. 1996; Collis et al. 1998; Zhu and Bourguignon 2000). This is clearly not the case in the migration of leukocytes in the premenstrual period. Other mechanisms supporting cell motility, including the release of chemokines by the endometrial cells (Salamonsen and Lathbury 2000), are likely to be operative in this situation.

HA's ability to form hydrated gels facilitates the diffusion of small molecules, including growth factors, through the intercellular space. HA also facilitates invasion by separating cellular or fibrous barriers. What happens to HA in the fertile cycle is not shown in our study – the loss of HA occurs along with the falling progesterone levels so, with the maintenance of progesterone in early pregnancy, the HA may be maintained. Alternatively, if the HA were to fall in a fertile cycle, the clearing of the HA after mid-cycle may serve to restrict the subsequent trophoblast invasion, which in the human occurs some 8–9 days after ovulation (Enders 1997). Observations using in vitro studies with mouse embryos on artificial matrix support the contention that an HA-rich ECM may facilitate invasion of the stroma during implantation (Carson et al. 1987). Furthermore, human trophoblast proliferation and differentiation on either collagen-1 or Matrigel in vitro demonstrates the need for a permissive ECM, with interaction between fibronectin and integrin-$\alpha_V\beta_1$, stabilizing the developing cytotrophoblast column (Aplin et al. 1999, 2000). Whether the in vitro studies reflect the situation in vivo, whether HA is a necessary component of the ECM at implantation, and whether interaction between HA and its receptors is also important remains to be established.

Many studies have implicated HA and its binding proteins, the hyaladherins, in facilitating tissue penetration by migrating cells. Brown and Papaioannou (1992) find HA around proliferating stromal cells during implantation in the mouse. However, it is absent from the decidual zone, and the HA-negative decidual cell may be involved in restricting the invasion of trophoblast cells during embryo implantation. Interestingly, decidual cells in the mouse produce tissue inhibitor of metalloproteinase-3 (TIMP-3), which is also proposed to limit trophoblast invasion (Reponen et al. 1995; Leco et al. 1996), while, in the human endometrium during the latter part of the cycle, the decidualized stromal cells also produce large amounts of TIMP-1, -2, and –3 (Higuchi et al. 1995; Zhang and Salamonsen 1997). It is important to note that, in the mouse, decidualization occurs only in response to a blastocyst, whereas in the human it occurs spontaneously during the late secretory phase of each cycle. The enzymes which degrade HA are the hyaluronidases (Stern and Csóka 2000): HYAL-1 has recently been identified in human endometrium (Tellbach, van Damme, J.J. Brown, L.A. Salamonsen, unpublished data). It will be important to establish whether one or more of these enzymes are synthesized around the sites of decidualization during the late secretory phase of the cycle, when the present study demonstrates disappearance of HA from the stroma. The very intense staining for HA in the stroma in the vicinity of the blood vessels around day 19–21 is lost as the cycle progresses. Interestingly, this loss coincides both in time and location with the start of decidualization, which is initiated from about day 23 of the cycle and which occurs first in the vicinity of the spiral arterioles. It would be of considerable interest to determine the HA distribution in tissue taken in early pregnancy when trophoblast migration is occurring: if there is indeed an absence of HA in decidual tissue in human early pregnancy as in the mouse, it is likely that factors other than HA are of most relevance to trophoblast migration during placental development.

The HA receptor CD44 is expressed by epithelial and stromal cells of endometrium at all phases of the menstrual cycle and in decidualized stromal cells in pregnancy (Behzad et al. 1994). In the latter, CD44E is present in epithelium and CD44H in stroma. It is the CD44H, also known as CD44s, that acts as a receptor for HA and can mediate attachment and migration of HA substrates, as well as internalize extracellular HA (Behzad et al. 1994). However, data from other investigators (Yaegashi et al. 1995) conflicts with this as they find CD44, both the standard form and the variant form, bearing the v6 exon. These CD44 isoforms are found only in the mid-late secretory phases on epithelium, at all but the luminal epithelial cell surfaces. To date, there is no information on RHAMM expression in the human endometrium. Explication of the total array of HA receptors is not yet complete, and other receptors that are perhaps more prominent in the endometrium await description. Campbell et al. (1995) show that CD44 is present on preimplantation human trophoblasts, but they are down-regulated on the trophoblast surface at 8–11 weeks following implantation. This may contribute to the control of invasiveness, making it a self-limiting process. Goshen et al. (1996) have also shown expression of CD44v7 on human trophoblasts.

At this time, nothing is known of the influence of steroid hormones on the net deposition/degradation of HA in any hormone-responsive tissue, and thus the human endometrium will provide an excellent model for examining the role of both estrogen and progesterone on control mechanisms.

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