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DECODING IMPLANTATION AND MENSTRUATION: THE TALE OF TWO OPPOSING SIGNALS

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1. ABSTRACT

Human endometrium is a unique tissue that undergoes sequential phases of proliferation, and secretory changes followed by tissue shedding and bleeding during menstruation. Tissue remodeling is a distinct feature of human endometrium in the secretory phase which prepares endometrium for implantation during the "receptive phase" of the cycle. A discrete dissolution of extracellular matrix (ECM) by a host of enzymes called matrix metalloproteases (MMP) is required for a successful implantation. In the absence of implantation, as a result of progesterone withdrawal, human endometrium loses its receptive state in the premenstrual period and subsequently undergoes a generalized breakdown of ECM by MMPs during menstruation. The homeostasis of ECM of endometrium and the delicate balance between its synthesis and degradation appear to be mediated by reciprocal interaction between TGF-beta and ebaf (lefty) signaling. While TGF-beta acts as a pro-fibrogenic cytokine and maintains the integrity of ECM in endometrium, expression of lefty is associated with events that lead to destruction of ECM facilitating tissue shedding.

2. INTRODUCTION

Human endometrium undergoes sequential morphological and biochemical changes in each menstrual cycle in preparation for implantation (Figure 1). This includes a period of proliferation (proliferative phase) followed by changes that collectively characterize a progesterone (P) dominant secretory phase. These changes are initiated immediately after ovulation and prepare the endometrium for implantation several days later. In humans, the ovum is fertilized in the Fallopian tube. Shortly after fertilization, the fertilized ovum starts to divide, migrates through the Fallopian tube and enters the uterine cavity around day 3-4 after ovulation. The blastocyst remains free floating within the endometrial cavity for a day but it starts to implant on day 5-10 after ovulation (1-5). If implantation does not take place, the fall in the serum level of P in the late secretory phase initiates the process of menstrual bleeding. Under these conditions, shedding of the endometrium and bleeding is a normal physiologic response. In some conditions, however, bleeding from endometrium is abnormal. In these situations, menstrual bleeding may increase in amount (menorrhagia), duration, or frequency. In other instances, the patient may experience intermenstrual spotting or breakthrough bleeding. The endometrial bleeding in these conditions is also associated with dissolution of endometrial ECM. The data reviewed below demonstrate that the robust and generalized tissue breakdown of endometrium during menstruation

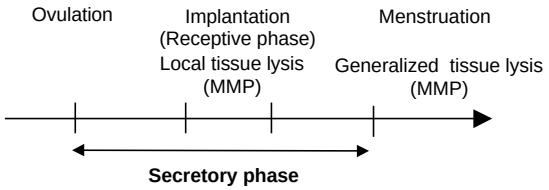

Figure 1. Tissue remodeling during secretory phase in human endometrium. Tissue remodeling is a distinct feature of secretory endometrium. While a trivial and localized breakdown of ECM is essential to embryo implantation, a robust and generalized tissue dissolution leads to menstruation. These events appear to be mediated by MMPs.

results from secretion and activation of a class of proteinases which digest the ECM constituents. It is becoming increasingly clear that the same host of enzymes, however, contribute to a discrete digestion of endometrial tissue which is essential to the blastocyst implantation (Figure 1).

3. THE ROLE OF P AND P WITHDRAWAL IN IMPLANTATION AND ENDOMETRIAL BLEEDING

The molecular events that keep the secretion and activation of MMPs in endometrium in check during implantation window and prevent tissue shedding remained obscure until recently. The current operative hypothesis is that P greatly suppresses the expression of MMPs during the "implantation window" and that upon P withdrawal, these enzymes are secreted by endometrial cells and lead to tissue dissolution (6-9). Using endometrial explants, Marbaix *et al* showed that breakdown of endometrial matrix upon sex steroid hormone withdrawal was completely and reversibly inhibited at all stages of the menstrual cycle by specific inhibitors of MMPs, but not by inhibitors of cysteine and serine proteinases (10). Others have provided more direct evidence that P inhibits MMP expression and that P withdrawal leads to secretion of MMPs. P inhibited activation of latent MMP-2 by membrane-type 1 MMP (11) and P withdrawal increased MMP-2 in endometrium and led to the dissolution of ECM and dissociation of stromal cells (7). Using endometrial explants, Osteen *et al* showed that the expression of MMP-7 is under the control of P (8). Consistent with these *in vitro* findings, the expression of endometrial MMP-7 was suppressed when the level of P was experimentally elevated in ovariectomized rhesus monkeys (12). It was subsequently reported that administration of anti-progestins to reproductively intact baboons increased endometrial MMP-7 during the mid-secretory phase (13). Once released and activated, MMPs degrade the ECM, which is comprised of collagens, fibronectin, gelatins, proteoglycans, elastin and basement membrane components. Collectively, these findings show that MMPs are crucial to endometrial tissue shedding and bleeding (14-17).

The initial clue of what accounts for endometrial tissue shedding and bleeding was gleaned over half a century ago. By transplanting endometrial tissues into the anterior chamber of the eyes of rhesus monkeys, Markee showed that menstrual tissue shedding and bleeding is initiated by steroid hormone withdrawal (18). It was later realized that abnormal uterine bleeding also results from endogenous estrogenic activities unopposed by progesterone (P), progestogenic activities, and by exogenous administration of steroid hormones such as that occuring during the use of contraceptives made of female steroid hormones (19-25). Markee hypothesized that the endometrial tissue shedding and bleeding induced by steroid hormones must be controlled by local factors. This idea has received significant support from various basic research and clinical studies reviewed below.

4. THE ROLE OF MMPS IN IMPLANTATION

In mammals, blastocysts invade the endometrial tissue in a manner similar to invasion of tissues by tumors. It is becoming increasingly clear that MMPs are primary contributors of the tissue invasion both by blastocysts and tumors. These enzymes, by virtue of inducing a localized dissolution of endometrial tissue, facilitate the implantation of blastocyst. The ECM in endometrium is comprised of an argyrophilic network of so-called "reticular fibers" and collagen (26-28). This network undergoes remodeling in response to decidualization and implantation. The process of decidualization is associated with a vanishing collagen type I, III, V, and VI content in the pregnant rat uteri (29-30). Although, the dissolution of ECM in decidualized stroma is independent of an embryo (29), the collagen concentration is reduced more around the implanting blastocyst as compared to the non-implanting sites (30). Collagen type I is virtually absent around the rat embryos on day 5 of pregnancy but re-appears on day 8 around the developing placenta (31). These modifications of tissue occur in a background of significant alterations in protein synthesis and secretion which are associated with a positive protamine blue reaction at the site of embryo implantation (32). Thus, the implantation requires both modification of synthesis as well as degradation of proteins. The expression of MMPs also undergo distinct changes throughout pregnancy in ovine, mouse, rat and human endometrium (33-36). MMP-1 immunoreactivity can be detected locally in close proximity of implanting embryo (35). The mRNA encoding MMP-9 is detected in uteri which are undergoing oil-induced decidualization and immunoreactivity for MMP-9 and MMP-2 is detectable during early pregnancy and in oil induced decidualized endometrium (37). Injection of peptide hydroxamate, which is MMP inhibitor, retards the decidual development. Similarly, the development of decidua is inhibited in transgenic mice that overexpress TIMP-1 (34). Besides the secretion of MMPs by endometrium, the invasion of blastocyst is further enhanced by the release of the same enzymes by trophoblasts which is stringently controlled in terms of time and space by TIMPs (38). When placed in culture, mouse blastocysts adhere to ECM, and trophoblast giant cells invade and degrade ECM (39). Collectively, these findings show that the dual release of MMPs by the endometrium and the embryo jointly drive the implantation process.

5. THE ROLE OF MMPS IN ENDOMETRIAL BLEEDING

Although a localized expression of MMPs is essential to implantation, it appears that aberrant, dysregulated or superfluous expression of MMPs around the time of implantation by endometrium or blastocyst would be an undesirable event. A generalized activation of these enzymes in human endometrium can lead to menstruation or abortion causing significant tissue shedding, so that by the end of the process, most of functionalis is lost. These are predictable outcomes if implantation fails to proceed normally or does not occur at all. For these reasons, to insure that the endometrial tissue breakdown does not occur unnecessarily or excessively, the expression of various MMPs such as MMP-3, MMP-7 and MMP-9 and their inhibitors, TIMPs, is stringently controlled at the maternal-embryo interface. Transcripts of these TIMPs (TIMP-1, TIMP-2, TIMP-3) can be detected throughout the pre and peri-implantation development in the mouse uteri (40). Careful analysis has shown that these inhibitors have a distinct temporal pattern of expression. For example, inhibitory activity is highest on day 1 of pregnancy in mouse endometrium and this activity progressively decreases so that minimal levels are detectable on day 12 (41). Most of such alterations are attributable to the changes in the expression of TIMP-1 while TIMP-2 expression levels do not change but TIMP-3 expression progressively increases during the same time period (41).

6. ENDOMETRIAL BLEEDING IS ASSOCIATED WITH DISSOLUTION OF ECM BY MMPS

If implantation does not take place or is defective, endometrium is shed during the normal physiologic menstrual process but this tissue can also bleed abnormally. Abnormal uterine bleeding, is observed during and at both ends of the reproductive years around the time menarche or menopause (19, 1993, 21,24,25,42-45). Menstrual breakdown of endometrium has been traditionally viewed to result from vasospasm of endometrial spiral arteries followed by tissue ischemia and lysis (20). However, the molecular events that underlie this tissue breakdown remained obscure until recently. Human endometrium contains an argyrophilic network of so-called "reticular fibers" which contain both type I and type III collagen (26-28). When the serum concentration of progesterone falls at the end of the menstrual cycle, this interstitial fibrillar meshwork undergoes focal breakdown and is extensively lysed at menstruation. Basement membranes around glands and blood vessels also undergo fragmentation and disruption. Breakdown and collapse of this framework leads to shrinkage of the endometrium, piecemeal loss of the functionalis layer, and subsequent bleeding (26). Regression of endometrium also occurs in mammals that do not menstruate. For example, in cycling rats, the wet weight and the collagen content of endometrium decreases during metestrus to 20% of their proestrus values (46). These findings indicate that proteolysis is an essential feature of the endometrial cycle (47). The involvement of lysosomal enzymes in proteolytic events at menstruation was first proposed in late 1960's and early 1970's by Wood *et al*, 1969, and Henzl *et al*, 1972 (48-49). However, more recent studies have failed to provide conclusive evidence in support of this hypothesis (50). A significant body of work shows that MMPs that degrade collagen, fibronectin, gelatin, elastin as well as the constituents of basement membrane including laminin, participate in digestion and breakdown of the endometrial matrix during menses and abnormal uterine bleeding (51-52). Various lines of evidence form the basis of this belief. These include 1. spatio-temporal expression of MMPs during normal menstrual and abnormal uterine bleeding, 2. their secretion from endometrial cells in culture, and 3. the evidence that MMPs degrade endometrial extracellular matrix.

The first key evidence for the role of MMPs in endometrial tissue shedding is their spatio-temporal expression during menstruation. Virtually all known MMPs are expressed in human endometrium during menstrual bleeding (51-52), and expression of some endometrial MMPs such as MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 and MMP-10 is enhanced around the time of menstrual bleeding (7, 14, 53). While the expression of some potent stromelysins such as MMP-7 is maintained at a low level in human endometrium during early and mid-secretory phases, its expression is significantly increased during late secretory and menstrual phases (54). In a related species with menstrual cycle such as baboons on artificial cycles, MMP-3 and MMP-7 were also expressed during menses (13). These studies show that the expression of MMPs in human endometrium is exquisitely controlled during menstrual cycle and that the expression of key MMPs is increased during menses. Moreover, MMPs are also highly expressed in the endometria of patients who experience steroid hormone-induced bleeding (55-57). In women using progestin-only contraceptives, endometrial MMP-1 and MMP-3 were found to be highly expressed, to the extent present in the endometria of menstrual phase controls (56). In patients using subdermally implanted slow-release levonorgestrel (Norplant), positive MMP-9 immunostaining was observed extracellularly in areas of tissue lysis and in the surrounding stromal and intravascular leukocytes (56). The number of MMP-9 positive cells was significantly increased in the endometrial biopsies of normal control subjects at menstruation and endometria of Norplant users which displayed a shedding morphology (55). The close temporal and spatial correlation between the expression of MMPs and endometrial tissue shedding provide supportive evidence for the role of MMPs in tissue breakdown at menstruation. Once released and activated following P withdrawal, MMPs degrade the extracellular matrix and for this reason, it is believed that MMPs are crucial to endometrial tissue shedding and bleeding (10, 14-17).

Besides the endometrial extracellular matrix, a second target which participate in endometrial bleeding are the superficial endometrial arteries. Ultrastructural analysis have shown that the endometrial vessels undergo significant changes during menstrual cycle (58). In the early proliferative phase, the basal lamina of endometrial capillaries is loosely formed and discontinuous. In the late proliferative phase, basal

lamina becomes more distinct and in the early to mid-secretory phase, whorled extensions that include pericytes develop. In the late secretory phase, the endometrial stroma and the basal lamina undergo widespread degeneration, and the cell-to-cell contacts sharply decrease. Collectively, these events make vessels fragile and susceptible to bleeding. Essentially, similar changes occur in the endometria of patients who use progesterone containing contraceptives such as Norplant. The vessels in these patients appear to be extremely fragile and easily bleed during hysteroscopic examination (59-60). Petechiae and ecchymoses are frequently observed in the endometria of these patients (60). In the initial months of exposure to Norplant, when bleeding problems are most common (59), the amount of basement membrane components such as laminin, collagen type IV, and heparan sulfate proteoglycan is reduced in the endometrial vessels (59, 61). The degradation of the extracellular matrix components in these patients appears to be due to higher MMP activity (56). Collagenase-1 mRNA, focal stromal breakdown and lysis of collagen fibers are evidenced in most bleeding endometria, but never in the nonbleeding ones. In the areas showing breakdown, immunolabeling for gelatinase A is strongly increased (62). The number of perivascular mural cells which contain smooth muscle actin also decreases in patients using progestin-only contraceptives further leading to the vessel fragility (63). The underlying basis of such changes appears to be a state of P withdrawal not necessarily due to reduced progesterone but presumably due to reduction of both progesterone receptor A and progesterone receptor B isoforms (62, 64).

7. THE ROLE OF TGF-BETA IN IMPLANTATION AND ENDOMETRIAL BLEEDING

The current operative hypothesis is that P controls the expression of MMPs through local endometrial cytokines (8-9, 65-66). Keller *et al* recently showed that progesterone inhibits the IL-1 mediated stimulation of MMP-3 (65) and Bruner *et al* identified TGF-beta as the principal mediator of MMP-7 (matrilysin) suppression by progesterone (9). During the secretory phase, endometrial stromal cells were found to be the source of this TGF-beta (9). On day 1 to 4 of pregnancy, a major portion of this TGF-beta resides in the epithelium but the decidual cells and ECM also remain a substantial source of TGF-beta in the mouse and rat endometria (67-69). Likewise, in pregnant mares, TGF-beta mRNA is found in the epithelial cells (70) and in humans, TGF-beta is found in the stromal and decidual cells and within ECM (9, 71-72). Taken together, these finding show that TGF-beta supports implantation by regulating the expression of MMPs. Since TGF-beta knockout was embryolethal, Das *et al* studied the effect of the TGF-beta by down-regulating its receptors in the uteri of TGF-alpha transgenic mice (73). This down-regulation delayed the blastocyst attachment reaction and delayed parturition. These findings support the view that TGF-beta plays important roles in homeostasis of ECM of endometrium and in implantation.

TGF-beta is one of the leading cytokines that regulates the homeostasis of ECM. TGF-beta controls the tissue integrity by two complementary mechanisms, one involving up-regulation of collagen mNA, and the other by inhibition of activity of MMPs. (74-75). TGF-beta increases the ECM accumulation by signaling along the Smad pathway and by inducing connective tissue growth factor (CTGF). CTGF, a heparin binding 38 kD cysteine-rich peptide, is considered to be an immediate early growth responsive gene and a downstream mediator of TGF-beta actions in fibroblasts (76). CTGF induces collagen synthesis, in a number of *in vitro* and *in vivo* models (77-79). CTGF is present in epithelial cells of human endometrium throughout the entire menstrual cycle but its expression in the stroma co-incides with the duration of "implantation window" (80). Thus, expression of CTGF in human endometrium appears to be part of the molecular pathways involved in TGF-beta actions and required for the implantation (81-84). These findings have provided clues as to how the fate of ECM in endometrium during the critical period of implantation need to be controlled both by regulation of expression of collagen and the key MMPs. The last but a critical final event in the intricate circuit that regulates the homeostasis of tissue matrix might rely on regulation of TGF-beta expression and/or function. We have identified ebaf/lefty, a novel member of the TGF-beta superfamily, that suppresses TGF-beta activity by inhibiting the phosphorylation of Smad2 (86-89). Based on this important evidence coupled with other data reviewed below which show that lefty inhibits both CTGF and collagen mRNA synthesis and enhances secretion of collagenase, we have formulated the following hypothesis: Lefty is the key endometrial factor, that regulates the breakdown of ECM both by inhibition of collagen synthesis and enhancement of its degradation by MMPs. These actions of lefty are mediated by inhibition of TGF-beta through the Smad signaling pathway.

8. LEFTY IS AN ENDOMETRIAL BLEEDING ASSOCIATED FACTOR

Abnormal uterine bleeding is one of the most common disorders that occurs in women throughout the span of their reproductive years. During this time period, nearly 20% of women exhibit menorrhagia (90-91) and virtually every woman, at some point in her lifetime, experiences episodes of abnormal uterine bleeding (19). Extensive bleeding from endometrium accounts for 70% of the hysterectomies performed annually (92). One of the more effective contraceptives is made of progestins. A high incidence of irregular uterine bleeding is the primary patient complaint that has limited the long-term use of progestin-only contraceptive agents (21,24-25, 93). This is the major reason for discontinuation of use and acceptability of these common contraceptives (94-96). Abnormal uterine bleeding is a major medical problem not only for women but also for their families and health care services (23). In spite of the widely appreciated magnitude of the problem, and the understanding that endometrial bleeding is directly related to the effect of steroid hormones on endometrium, it is not clear that when progesterone level falls what local mediator(s) triggers endometrial tissue shedding and bleeding.

We argued that factors that participate in endometrial bleeding, should be maximally expressed in human endometrium around the time of menstruation. Moreover, we reasoned that the endometrial factor(s) that is responsible for conferring a state of non-responsiveness to endometrium and induces MMPs should be detectable at a high level in late secretory and menstrual phase endometrial tissues. To identify such an endometrial bleeding associated factor, we carried out differential display on RNA of endometrial tissues from all phases of the menstrual cycle of normal healthy menstruating women. The amplified products of differential display were resolved in sequencing gels. Amongst hundreds of amplified bands examined, the intensity of one band representing a novel human gene was found to be most intense during late secretory and menstrual phases. For this reason, we named this factor ebaf (endometrial bleeding associated factor) (85). The band was cloned and sequenced. A GenBank search identified a mouse gene which showed a significant degree of homology to the human gene. In view of its asymmetric expression in the left side of the mouse embryo, the mouse gene was designated lefty (lefty-1) (97). Ebaf is also named lefty-A by Kosaki *et al*, who showed mutation in the gene in individuals with L-R axis malformation (98). For simplicity, we will refer to ebaf as lefty. We argued that if lefty is involved in endometrial tissue breakdown, it should be maximally expressed in human endometrium around the time of menstruation. Northern analysis of endometrial tissues of various phases of menstrual cycle gave credence to this line of reasoning (85). *In situ* hybridization, was used to identify the cells that express lefty in human endometrium. Lefty was expressed both in the endometrial stromal fibroblasts and epithelial cells (85-86, 99). Careful examination of expression of lefty in endometria of women with abnormal uterine bleeding showed that lefty was also highly expressed in these endometria (85). Taken together, These data provide support for the hypothesis that lefty is an endometrial bleeding associated factor.

9. LEFTY EXPRESSION IS NEGATIVELY CONTROLLED BY STEROID HORMONES

We reasoned that demonstration of negative regulation of lefty expression by steroid hormones would be supporting evidence of lefty being the cause of endometrial bleeding. In other words, steroid hormones are negative regulators of lefty and when the negative regulation is removed, lefty induces endometrial bleeding. We showed that the expression of lefty mRNA is negatively regulated by steroid hormones including P alone (100). We quantified, the concentration of lefty mRNA during menstrual cycle *in vivo* and in organ cultures of endometrium. In noncultured endometria, lefty mRNA concentration was dramatically (100-fold) increased in tissues which exhibited signs of matrix breakdown. A similar increase was seen in proliferative endometria, cultured for 24 h in the absence of ovarian steroids. This increase was inhibited by the addition of progesterone, alone or in combination with estradiol. In this model system, increase in lefty mRNA preceded the release of MMPs. These findings show that lefty expression is negatively regulated by P.

10. LEFTY IS A CYTOKINE

We identified lefty protein in the endometrial tissue, endometrial fluid, serum and urine of women around the time of menstrual bleeding. Three protein bands were immunoreactive with an affinity purified rabbit polyclonal antibodies generated against a peptide at the C terminus of lefty protein (87-88). To gain additional insight on the identity ot these bands, we characterized the mode of processing and post-translational modification of lefty proteins. Presence of a signal peptide suggested that lefty might be a secreted protein (85). Two potential cleavage sites exist within the lefty?precursor. Transfection of 293, BALB/3T3 and other mammalian cells with lefty showed synthesis and release of proteins bands of 42, 34 and 28 kD into the culture media of transfected cells (88). Transduction of GP+E86 fibroblasts with retroviral vectors transducing lefty also showed presence of the same bands in the culture medium (101). Thus, it seems that the processing of the lefty doe not depend on the cell type that expresses the protein. Using transfection and a number of mutations introduced into lefty, we showed that lefty is synthesized as a 42 kD precursor and is cleaved at Lys^{77} and Arg^{135} to produce 34 and 28 kD mature secreted proteins (88).

The deduced amino acid sequence of lefty showed a great amount of identity and similarity with the known members of the TGF-beta?superfamily. A motif search revealed that the predicted lefty protein contains most of the conserved cysteine residues of the TGF-beta related proteins (85) which are necessary for the formation of the cysteine knot structure (102-103). The lefty sequence contains an additional cysteine residue, 12 amino acids upstream from the first conserved cysteine residue. The only other family members, known to contain an additional cysteine residue, are TGF-beta, inhibins and GDF-3 (104). Lefty, similar to GDF-3/Vgr2 and GDF-9, lacks the cysteine residue necessary for the formation of intermolecular disulfide bond (104-105). Whereas the carboxy terminus of the TGF-beta family is usually CX1CX1, lefty has a longer C terminal sequence, CX1CX19 (85). Therefore, lefty appears to be an additional member of the TGF-beta superfamily with an unpaired cysteine residue which may not exist as a dimer.

11. LEFTY IS AN INHIBITOR OF TGF-BETA SIGNALING

Lefty and its related proteins are poised to act as inhibitors of TGF-beta family members (97, 106-110). Thus, we considered that lefty might cause extracellular matrix remodeling by inhibiting TGF-beta actions. A number of carefully executed studies showed the validity of this hypothesis (89). Lefty perturbs the TGF-beta signaling by inhibiting the phosphorylation of Smad2 following activation of the TGF-beta receptor (89). Moreover, lefty inhibits the events which lie downstream from R-Smad

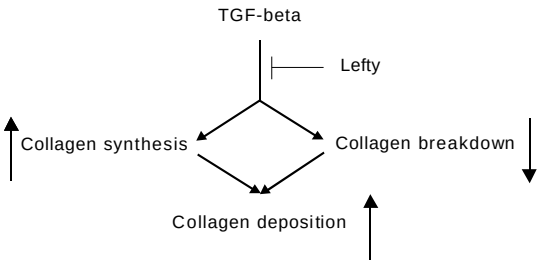

Figure 2. A model for the role of TGF-beta and lefty in implantation and tissue shedding. TGF-beta, is one of the major mediators of ECM homeostasis. This protein acts through two complementary pathways, one which stimulates ECM accumulation and the other which reduces ECM degradation. We hypothesize that lefty, by virtue of inhibiting the TGF-beta activity, leads to a negative balance in the amount of collagen deposited in tissues and therefore can support tissue dissolution during implantation. Overexpression of lefty leads to menstruation and potentially can lead to abortion and loss of embryo.

phosphorylation including heterodimerization of R-Smads with Smad4 and nuclear translocation of R-Smad-Smad4 complex. Lefty opposes the effect of TGF-beta on the expression of reporter genes for major cell cycle factors p21, and Cdc25. Smad3 and Smad4, both have domains that bind the 5'-TCTGAGAC-3' termed Smad Binding Element or (SBE). Lefty inhibits the TGF-beta induced promoter acitivity driven by SBE or promoter activity of a constitutively active TGF-beta RI (89). Moreover, it was recently shown that the expression of CTGF that induces proliferation of fibroblasts and collagen synthesis is mediated by Smad3 and Smad4 (111). Lefty is also capable of inhibiting the promoter activity of CTGF mediated by TGF-beta (89). Thus, lefty provides a repressed state of TGF-beta responsive genes and participates in negative modulation of TGF-beta signaling by inhibition of phosphorylation of R-Smads (89).

12. LEFTY INHIBITS DEPOSITION OF COLLAGEN *IN VIVO*

TGF-beta is a profibrogenic cytokine that induces collagen synthesis and suppresses MMPs (Figure 2). Since lefty inhibits TGF-beta signaling, we entertained the possibility that lefty might oppose the TGF-beta actions *in vivo* and lead to the loss of ECM. Using *In situ* hybridization, we showed that lefty is markedly expressed in endometrial stromal fibroblasts around the time of menstrual bleeding (85). To simulate this *in vivo* expression for characterizing the lefty actions, we constructed two retroviral vectors, a control vector (LG) enabling cells to express GFP and the vector, LEIG, that transduces the expression of both GFP and lefty-A (101). The success of these transduction experiments was assessed by analysis of GFP fluorescence, immunostaining and demonstrating that lefty is secreted by the LEIG transduced and not LG transduced cells (101). These cells were then subcutaneously introduced into athymic mice and the amount of collagen deposited in the stroma of the tumors developed from the fibroblasts was quantitated. As expected, the LG tumors contained abundant collagen fibers. In contrast, in the LEIG tumors, there was little intervening stroma containing collagen. To further validate that the extracellular matrix observed in these tumors was collagen, sections were stained with trichrome, which in view of its affinity, casts a blue color onto collagen fibers. While collagen fibers were detected in large amounts both at the center and periphery of the LG tumors, the LEIG tumors exhibited a paucity of these fibers. These findings show that lefty suppresses collagen synthesis and prevents collagen deposition.

13. LEFTY INHIBITS COLLAGEN AND CTGF MRNA EXPRESSION *IN VIVO*

The RNA from LG and LEIG transduced cultured cells and tumors derived from them were subjected to reverse transcription (RT) followed by polymerase chain reaction for mouse collagen type I (101). Although the collagen type I mRNA was detected in both LG and LEIG transduced cells *in vitro*, the LG tumors had more collagen mRNA that found in the LEIG tumors (101). We attribute lack of effect of lefty *in vitro*, to absence of any stimulating signal such as TGF-beta that lefty is able to inhibit. The inhibitory actions of lefty can be observed in presence of a stimulating signal such as TGF-beta which is ubiquitously expressed *in vivo*. Persuaded by these observations, we then carried out real time quantitative RT-PCR to determine that amount of CTGF and collagen type I mRNA in the same tissues. These studies confirmed the results of RT-PCR and showed 4.7 fold reduction in the expression of collagen type I mRNA in the tumors derived from lefty$^+$ cells. Moreover, there was 2.8 fold reduction of CTGF mRNA expression in the same tumors as compared with the control tumors. These results are consistent with the histologic data on reduced deposition of collagen in tissue sections of lefty$^+$ tumors and show that such reduction is the result of reduced CTGF and collagen mRNA transcription. Collectively, the available data support the hypothesis that lefty inhibits collagen deposition *in vivo*. The findings show that lefty, by acting on the CTGF promoter, inhibits expression of CTGF and consequently reduces the deposition of collagen by a mechanism which includes transcriptional control of collagen type I mRNA expression.

14. LEFTY INDUCES COLLAGENOLYTIC AND ELASTOLYTIC ACTIVITIES *IN VIVO*

A critical step in building the hypothesis that lefty is involved in tissue dissolution would be induction of collagenolytic activity by lefty. Proteolytic activity was not significantly different in the tissue culture media of LG and LEIG transduced cells. However, *in vivo*, there was a significant increase in the proteolytic activities in the LEIG transduced tumors. This difference was noted using collagen, gelatin or elastin as substrates (101). At least five different species of enzyme, induced by lefty causing collagenolysis and caseinolysis were seen by zymography. These findings show that lefty actively participates in the dissolution of ECM by inducing collagenlysis and elastolysis.

15. INDUCTION OF MMPS BY LEFTY IS SUPPRESSIBLE BY STEROID HORMONES

The above findings provide the evidence that lefty causes the breakdown of the ECM components by induction of collagenolytic, gelatinolytic and elastolytic activities. Since such activities are inducible by MMP-3 and MMP-7, two major enzymes that degrade a wide variety of ECM components such as laminin, fibronectin, gelatin, proteoglycan, procollagenase and collagen type IV, V, IX, and X (reviewed in Tabibzadeh and Babaknia 1995), we measured the ability of lefty to induce these enzymes in endometrial explants. Lefty induced significant levels of both MMP-3 and MMP-7 in the explants (100). These findings suggest that lefty causes degradation of ECM by induction of MMPs. The extent that steroid hormones counteract the MMP inducing effect of lefty was also studied. Endometrial explant cultures were treated with lefty in the absence and presence of steroid hormones and the amount of MMP-3 and MMP-7 in the culture media was analyzed by Western blotting. The experiments showed that steroid hormones significantly down-regulate the amount of MMPs induced by lefty (100).

16. LEFTY IS OVER-EXPRESSED IN ENDOMETRIA OF PATIENTS WITH INFERTILITY

Infertility is a common clinical problem. As shown in the classic Guttmacher's table, about 7% of couples can be considered infertile after they have tried for two years to attain pregnancy (112). In the US, in 1982, nearly one in five married women of reproductive age reported that, during their lifetime, they had sought professional help for infertility (113) and in 1988, 8.4% (a total of 4.9 million) of women, ages 15-44, had impaired fertility (114). After all the standard clinical investigations are done and known causes of infertility attributable to other identifiable pathologies are ruled out, a substantial number (10%) of infertility cases remain of unknown etiology ("unexplained infertility") (115).

In normal endometrium a state of non-responsiveness is attained after day 10 post-ovulation shortly before menstruation begins. Nearly 50% of women who become pregnant after day 10 post-ovulation, lose their embryos (116). The possibility exists that in some infertile women, the endometrium attains this state of non-responsiveness earlier as compared with normal fertile women, causing a state of infertility. A second possible scenario is that infertile women might become pregnant but might lose the blastocyst by abortion. This is also a likely possibility since about 30% of normal women experience a subclinical pregnancy which is ended with abortion (117-118). An accentuation of this event could potentially be the cause of infertility in some patients (119). Thus, it appears that there is a close relationship between the attainment of a non-receptive state in endometrium in the secretory phase and a subsequent menstruation or loss of embryo by abortion (120-123).

Any imbalance in regulatory mechanisms that drive endometrium during the secretory phase may lead to lesions within the molecular repertoire that drives the events in the secretory phase. For example, during an anovulatory cycle, the production of the systemic steroid hormones is aberrant. In luteal phase defect, this aberrancy is subtle and leads to a lag in the maturation of endometrium. The means by which the disease processes such as endometriosis, or pathologies within the Fallopian tube lead to infertility are not well understood. However, in women with infertility, the treatment of these processes increases the chance of conception, suggesting the endometrium as the target organ for the effects of these diseases. In some instances, the underlying basis for the infertility remains unclear (unexplained infertility). From the members of the molecular repertoire of the "endometrial receptivity" period, with the exception of $\alpha_v\beta_?$ integrin (124), no other gene has been described thus far whose aberrant expression is associated with or results in infertility. It has been suggested that the expression of the immunoreactivity for $\alpha_v\beta_?$ and $a_v\beta_?$ integrins co-incides with the "implantation window" (125). Immunostaining for α_v increased throughout the menstrual cycle, while the $\beta_?$ subunit appeared abruptly on cycle day 20 on luminal and glandular epithelial cells (124). Discordant luteal phase biopsies (≥3 days out of phase) from infertile patients exhibited delayed epithelial $beta_3$ integrin immunostaining (21). Later, the abnormal $beta_?$ immunostaining was also found in infertility associated with tubal factor (126) and unexplained infertility (127).

We reasoned that since lefty is highly expressed at the time that endometrium is non-receptive, lefty over-expression might be a molecular lesion in the endometria of patients with infertility. In normal fertile subjects, lefty proteins were present at a low level in endometrium during the "implantation window", and high levels could be found only around the time of menstruation (Tabibzadeh *et al*, 2000). Consistent with this hypothesis, Northern blot analysis showed that the expression of the lefty 2 mRNA is up-regulated in the endometria of infertile patients during the "implantation window". In over 50% (14/26) of endometria from infertile patients, the lefty mRNA was up-regulated during the "endometrial receptivity period". The infertility in these women was associated with endometriosis, polycystic ovary, bilateral tube occlusion, anovulatory cycle, luteal phase defect, premature ovarian failure and habitual abortion. In some women, the underlying basis of infertility remained unknown (unexplained infertility). Therefore, the dysregulated expression of the lefty 2 mRNA in endometrium seems to be a common event in diverse types of infertility. An additional, smaller lefty mRNA was also detectable in endometria of some of infertile women. Consistent with these findings, the production/secretion of the endometrial lefty was found to be perturbed in the endometria of infertile women. As compared to the endometria of normal fertile women, there was relatively more lefty 2 protein in the endometria of infertile women during the "implantation window". Such dysregulated expression of the lefty by the endometrium could be reversed by an appropriate treatment strategy. When such expression was optimally reversed, 4/4

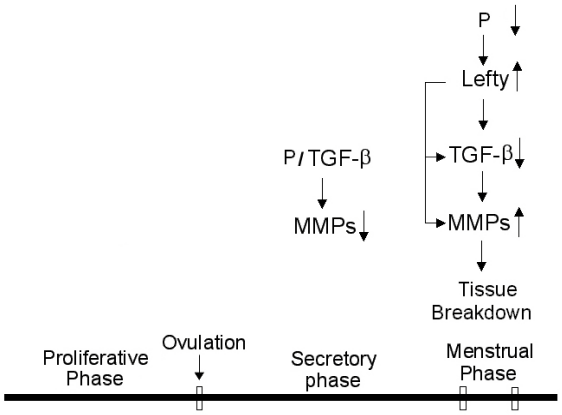

Figure 3. A model for regulation of MMPs and tissue shedding and bleeding in endometrium. During the secretory phase, progesterone (P) and TGF-beta suppress expression of MMPs. P withdrawal during the late secretory phase or in bleeding conditions leads to up-regulated expression of endometrial lefty which leads to menstrual shedding and bleeding.

patients became pregnant after such treatment. However, both women in whom the treatment failed to adequately suppress the dysregulated expression of the lefty by the endometrium, failed to become pregnant. Therefore, dysregulated expression of the lefty mRNA and protein in infertility patients during the "endometrial receptivity period" may result in premature closure of the "implantation window" and may account for their inability to conceive.

17. LEFTY OVER-EXPRESSION LEADS TO IMPLANTATION FAILURE

The production and/or release of the lefty protein in human endometrium and the serum was dependent on the phase of the menstrual cycle. The amount of the protein was lowest during the "implantation window". Based on these findings, we speculated that successful implantation may occur in presence of a low level of lefty protein in human endometrium and that Implantation can not take place shortly before menstrual bleeding when lefty expression is high. Because lefty causes collagenolysis, we thought that lefty overexpression might be inconsistent with embryo implantation. For this reason, LG and LEIG retroviral particles were injected into the endometrial cavity of mice on day 1 of pregnancy. Nine days later, there was no viable embryo in the uterine cavities of animals injected with LEIG retroviral particles whereas the uterine cavities of control mice and mice injected with LG retroviral particles showed the expected number of viable embryos (unpublished data). These findings show that lefty over-expression is inconsistent with implantation.

18. CONCLUSIONS

Because abnormal endometrial bleeding and infertility are common clinical problems, there is a need to examine the intricate pathway that leads to breakdown of fibrovascular meshwork of endometrium. The body of the work reviewed here shows that many different conditions including implantation, abortion, menstruation and abnormal uterine bleeding, all are associated with remodeling of ECM. This remodeling, by and large, is due to either discrete or disseminated secretion and activation of MMPs in human endometrium. This activation which might be due to P withdrawal or steroid hormone treatment targets the ECM by virtue of participation of two major signaling molecules, TGF-beta and lefty. The opposing actions of these proteins sustains the endometrial tissue integrity or leads to destruction of ECM, depending on their level of expression (Figure 3).

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