**In vivo gene transfer of lefty leads to implantation failure in mice**

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BACKGROUND: Endometrium is a unique tissue that is prepared for implantation of blastocyst during each menstrual cycle. In humans, if implantation does not occur or fails, endometrium is shed. METHODS and RESULTS: We identified ebaf/lefty, as a key cytokine, highly expressed in human endometrium during the non-receptive phase of tissue remodelling. Lefty was increased in the endometria of a number of patients with ‘unexplained infertility’ during the receptive phase, suggesting dysregulation of lefty as a potential factor contributing to infertility. Here, we showed that induction of a similar state of lefty overexpression in endometrium, by in vivo gene delivery, decreased implantation in pregnant mice. This state of overexpression could be induced by a retroviral vector transducing lefty or by liposome-mediated introduction of a lefty expression vector. Analysis of endometrium showed increased lefty after in vivo gene transfer. CONCLUSION: These findings suggest that induction of a state of lefty overexpression in endometrium leads to reduced implantation.

Key words: ebaf/endometrium/lefty/mouse/pregnancy

Introduction

Infertility is a common clinical problem. As shown in the classic Guttmacher’s table, ~7% of couples can be considered infertile after they have tried for 2 years to attain pregnancy (Guttmacher, 1956). 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 aetiology (‘unexplained infertility’) (Gurgan, 1992, 1994; Taylor et al., 1994, 1999). Abnormal expression of αβ3 integrin and HOXA10 was associated with infertility in women (Lessey et al., 1992; Taylor et al., 1999). Immunoreactivity for αβ1 and αβ3 integrins coincided with the ‘implantation window’ (Lessey et al., 1994). Immunostaining for α increased throughout the menstrual cycle, while the β3 subunit appeared abruptly on cycle day 20 on luminal and glandular epithelial cells (Lessey et al., 1992). Discordant luteal phase biopsies (∼3 days out of phase) from infertile patients exhibited delayed epithelial β3 integrin immunostaining (Lessey et al., 1992). Later, abnormal β3 immunostaining was also found in infertility associated with tubal factor (Meyer et al., 1997) and unexplained infertility (Lessey et al., 1995). Similarly, HOXA10 was increased in the endometrium in the receptive phase. Blocking uterine HOXA10 expression reduced endometrial receptivity in mice (Bagot et al., 2000). Women with decreased rates of implantation had decreased HOX expression, and women with endometriosis, polycystic ovary syndrome and hydrosalpinx had an abnormal endometrial HOXA10 expression (Taylor et al., 1999a,b; Daftary and Taylor, 2002; Cermik et al., 2003). HOXA10 directly regulated β subunit gene expression, suggesting a link between HOXA10 and integrin deficit in infertile patients (Daftary et al., 2002).
Despite advances in understanding the molecular mechanisms that underlie development of a receptive state in endometrium, factors and pathways that render endometrium non-receptive prior to menstruation are less well characterized. In normal endometrium, a non-receptive state is attained after day 10 post-ovulation, shortly before menstruation begins. This state of endometrium appears to be related to the expression of the molecules that prime endometrium for menstruation and tissue shedding. We identified a factor, eba/lefety, which is minimally expressed during the receptive phase but is highly expressed during the non-receptive phase in endometrium of normal fertile women (Kothapalli et al., 1997). In a previous study, we showed that the amount of lefty proteins was low in the endometria and sera of normal fertile women during the ‘receptive phase’ (Tabibzadeh et al., 2000). Lefty proteins were abundant in the endometria of normal fertile women in ‘non-receptive’ and in infertile women during the ‘receptive’ phase of the cycle (Tabibzadeh et al., 2000). During this phase, lefty proteins were more abundant in patients with endometriosis who did not conceive than in patients who became pregnant. Pregnancy occurred in infertile women with endometriosis when treatment was associated with downregulation of lefty proteins in endometrium. On the other hand, pregnancy did not occur in infertile women with endometriosis when the treatment failed to decrease lefty proteins in endometrium. Together, these findings showed that a dysregulated increase of lefty in human endometrium during the receptive phase of the cycle was associated with infertility and that treatment of infertility led to decreased endometrial lefty. To investigate whether infertility might be caused by lefty overexpression, in this report, we examined whether the induction of overexpression of lefty, by in vivo gene delivery, in endometrium during pregnancy led to implantation failure in mice.

Materials and methods

Materials

Reproductive aged, albino, female and male and time pregnant CD1 mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). All chemicals were from Sigma-Aldrich company (St Louis, MO) or Fisher Scientific (Pittsburgh, PA). The avidin–biotin–peroxidase kit was from Vector Laboratories (Burlingame, CA). The goat polyclonal antibody to lefty peptide (M-20) mapped to the C-terminus of mouse lefty was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The antibody is reactive with the goat polyclonal antibody to lefty peptide (M-20) at a concentration of 2 μg/ml. The antibody to actin was from Abcam, Inc. (Cambrige, MA). Material was also obtained by cloning full-length lefty (ebaf) and β-galactosidase (β-gal) into pcDNA3.1(+) vector.

Retroviral and pcDNA3.1(+) vectors

We recently developed two retroviral vectors, LG and LEIG (Mason et al., 2002). LG vector acts as a control for LEIG vector that transduces lefty. Viral particles were obtained by transduction of the GP + E86 fibroblastic cell line. These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.). For transduction, cells were seeded into 6-well plates (Falcon, Franklin Lakes, NJ), at a concentration of 1.3 × 10^5 cells/ml and maintained in a CO₂ chamber at 37°C for ~16 h. When 50% confluent, cells were transduced with amphotropically packaged retroviral vectors, LG and LEIG, in the presence of 8 μg/ml of polybrene as previously described (Ulloa et al., 2001). Serum-free medium was collected 20–24 h after transduction. We also developed two expression vectors by cloning full-length lefty (ebaf) and β-galactosidase (β-gal) into pcDNA3.1(+) vector.

In vivo gene delivery

Female mice were mated with male mice overnight, and animals were checked and examined every 8 h until detection of a vaginal plug. Injections of retroviral particles were performed on the day the plug was found (day 1). Viral particles were diluted in minimum essential medium (MEM) before injection to give a final titre of 5 × 10⁶ viral particles/10 μl. Mice were anaesthetized by intraperitoneal injection of 250 μl of 5% xylazine/10% ketamine mixture. A laparotomy was performed to expose both uterine horns. Each uterine horn received a total of 10 μl of solution introduced by using a 27-gauge needle. The incision site was closed in two layers (peritoneal and cutaneous) with 4-0 Vicryl sutures and animals were returned to their cages. On day 9 of pregnancy, animals were sacrificed by neck dislocation and the number of embryos in both uterine horns was determined. Samples of each uterine horn were fixed in formalin and embedded in paraffin for sectioning. These experiments were conducted in accordance with an approved protocol issued by the Yale Animal Care and Use Committee.

In a second set of experiments, mice received lefty or β-gal in pcDNA3.1(+) expression vector and lipofectamine on day 2 of pregnancy. A final concentration of 16 μg/ml DNA and 40 μg/ml liposome was obtained by dilution in 1 × Dulbecco’s phosphate-buffered saline (PBS). Animals were anaesthetized by 5% xylazine/10% ketamine mixture and an incision was introduced in the left flank to expose the left uterine horn. A 25 μl aliquot of DNA–liposome mixture was introduced into the horn by using a 27-gauge needle. The solution was injected under stereomicroscopic guidance at the tube–horn border directly into the horn lumen. The site of incision was closed and animals were returned to their cages. Pregnant animals were sacrificed on day 9 of pregnancy by neck dislocation and the number of embryos in each uterine horn was counted. Samples of right and left uterine horns were frozen for western blot analysis. Other samples were processed for β-gal staining. These experiments were conducted in accordance with an approved protocol from the SUNY Animal Care and Use Committee.

SDS–PAGE and western blotting

The tissue lysates (250 μg protein/lane) were fractionated in a 12% denaturing gel together with a pre-stained protein ladder (Life Technologies, Inc.) and subsequently were blotted onto a nitrocellulose membrane in a Mini-Trans-Blot apparatus (Bio-Rad Laboratories, Hercules, CA). The blots were stained with the goat polyclonal antibody to lefty peptide (M-20) at a concentration of 2 μg/ml. The secondary antibody used was donkey anti-goat IgG–horseradish peroxidase (HRP) (Santa Cruz Biotechnology). Bands were detected by chemiluminescence as described by the manufacturer.

Histochemical staining

Tissues were fixed in 10% buffered formalin and were transferred through a graded series of alcohol and embedded in paraffin. Paraffin sections were stained with haematoxylin and eosin and viewed under a light microscope. For staining the β-gal-positive cells,
tissues were fixed for 30 min in a solution of 1.25% glutaraldehyde diluted in PBS. After fixation, tissue blocks were rinsed twice in 20 ml of PBS and placed in X-gal staining solution [PBS containing 2 mmol/l MgCl$_2$, 0.02% NP-40, 0.01% sodium deoxylylate, 5 mmol/l K$_2$Fe (CN)$_6$, 5 mmol/l K4 (CN)$_6$ and 1 mg/ml X-gal] for 24 h at room temperature. Counterstained sections of β-gal-stained tissues were prepared and visualized under the microscope.

**Statistical significance**

The analysis of variance (ANOVA) was used to compare the number of embryos in the non-treated and treated groups. Statistical significance of data was obtained with Sigma-stat at the 5% level ($P < 0.05$).

**Results**

Lefty overexpression was first induced in the uteri of pregnant mice by using retroviral particles. We recently reported on development of two retroviral vectors, a control vector (LG) and a second vector (LEIG), enabling transduced cells to express lefty in vitro and in vivo (Mason et al., 2002). We used these vectors to transduce lefty in uterine horns of pregnant mice. Viral particles were generated, titrated and tested for the presence of lefty in culture medium of LEIG- and LG-transduced GP + E86 cells before use as described previously (Mason et al., 2002). Using transfection and a number of mutations introduced into lefty, we showed that lefty precursor was a 42 kDa protein which was cleaved at Lys77 and Arg135 to produce 34 (long form) and 28 (short form) kDa mature proteins (Ulloa et al., 2001). Lefty proteins were present in the culture media of LEIG- and not LG-transduced cells (data not shown). Tested lots of retroviral particles were used for administration to four sets of mice. When retroviral vectors are used, vector toxicity should be considered. According to Paracelsus’ first rule (poison is a question of dose), it can be predicted that any transgene product has a defined therapeutic window compatible with the desired function and without the predominance of unwanted effects (Borzelleca, 2000). For this reason, initially, a dose response study was carried out to determine whether an increased dose of retrovirus may be toxic due to the increase in viral protein. Various titres of viral particles ranging from $5 \times 10^5$ to $5 \times 10^6/10^{10} \mu l$ were used. When low titres of viral particles were used, no significant difference between the number of embryos in animals receiving LG and LEIG retroviral particles was noted and higher titres of LG retroviral particles reduced the number of embryos. This strengthens the view that control virus showed a toxic effect at high dosage. However, when used at $5 \times 10^6$ viral particles/10 μl, the LG retroviral particles did not significantly affect the number of embryos as compared with the control group. Since the optimal dosage of retrovirus and the dosage in the control virus were similar, the implantation defect detected was not due to the toxicity of viral proteins. For this reason, studies were carried out with this titre for both LG and LEIG retroviral particles. Four sets of mice were used in the study. The first set of mice were not pregnant. The second set of mice were pregnant but did not receive retroviral particles. The third and fourth sets of mice were pregnant. The third set of mice received LG and the fourth set of mice received LEIG retroviral particles on day 1 of pregnancy in a total of 10 μl per uterine horn. Mice were sacrificed on day 9 of pregnancy and the number of embryos in both uterine horns was counted. The number of embryos in the control animals that were pregnant and did not receive retroviral particles was consistent with that expected in this strain of mouse (Table I). Introduction of LG retroviral particles was associated with a slight decrease in the number of embryos detected (Table I). However, the number of embryos found in the uterine horns of mice injected with LEIG retroviral particles was significantly reduced and was $< 50\%$ of that detected in the control mice (Table I).

Haematoxylin and eosin-stained sections of uterine horns were examined under light microscopy. The uterine sections of non-pregnant mice showed the typical slender lumen lined by a surface epithelium (Figure 1A). The uterine horns of pregnant mice and those injected with LG retroviral particles showed the presence of a dilated lumen containing embryos, surrounded by a decidualized stroma (Figure 1B and C). The sections of the uterine horns injected with LEIG retroviral particles also showed a dilated lumen, and embryos were present at a reduced number. However, in three animals, there were no embryos present. In these animals, the surface epithelium had a folded and papillary appearance and decidualized stroma was absent (Figure 1D). The surface epithelium of the uterine horn in non-pregnant mice exhibited tall columnar epithelial cells (Figure 1A). The surface epithelium, however, had a flat appearance in pregnant mice that received no injection or LG retroviral particles adjacent to the sacs (Figure 1B and C). The surface epithelium of uterine horns injected with LEIG retroviral particles had tall columnar cells similar to those found in the non-pregnant mice and showed apoptotic bodies (Figure 1D and A). A large number of these bodies were also noted in the underlying glands in the LEIG-treated animals and not the controls (Figure 1A–D).

Because there was some decrease in the number of embryos injected with LG vector potentially due to toxicity of the vector, we further validated these results with an independent approach. We generated β-gal and lefty expression vectors. These vectors were transfected into HeLa cells. As expected, β-gal activity was detected in transfected cells and lefty was present in culture media of cells transfected with lefty expression vector (data not shown). β-Gal expression vector was introduced into the left uterine lumen of day 2 pregnant mice with and without lefty expression vector. Animals were sacrificed on day 9 of pregnancy and the number of embryos in each uterine horn was counted. Table II shows

<table>
<thead>
<tr>
<th>Vector injected</th>
<th>No injection</th>
<th>LG</th>
<th>LEIG</th>
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<tr>
<td>No. of embryos</td>
<td>84</td>
<td>70</td>
<td>40</td>
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Number of mice = 8. $P = 0.018$ (no injection group versus LEIG group).
the total number of embryos in right and left uterine horns injected with expression vectors. As compared with the number of embryos detected in the left uterine horns of animals injected with β-gal expression vector, there was a significant decrease in the number of embryos in the left uterine horn injected with lefty expression vector (Table II). The right uterine horn in the same animals showed a slight decrease in the number of embryos detected as compared with those found in the left uterine horns injected with β-gal (Table II).

We considered that the reduced number of embryos in the right horn of mice injected with lefty expression vector might be attributable to transfer of fluid from the left to the right uterine horn during injection. To show this directly, we injected Chicago blue into the left uterine horn after exposing both uterine horns by laparatomy. As the volume of injectate increased and approached 25 μl, transfer of Chicago blue to the right uterine horn became evident (Figure 2). To analyse further that lefty transfection resulted in an increased amount of lefty in the left uterine horn, the tissue lysates from uterine horns were subjected to western blot analysis and the blots were incubated with goat anti-lefty M-20 antibody (Figure 3). As compared with the amount of lefty present in the uterine horns of the control mice, there was an increased amount of lefty protein in the uterine horn of animals injected with lefty expression vector (Figure 3). There was more lefty in the left uterine horn as compared with the right uterine horn in the same animal (Figure 3). To show β-gal, sections of uterine horns were stained for β-gal activity. β-Gal activity was not present in non-injected uterine horn but was detected in the glands of horn injected with β-gal (Figure 4A and B).

Discussion

We identified ebafl/lefty during a search for genes highly expressed during the non-receptive phase in human endometrium. Northern blot analysis of endometrial tissues of various phases of the menstrual cycle showed lefty to be highly expressed during perimenstrual and menstrual phases of the cycle (Kothapalli et al., 1997). Lefty mRNA was increased ~100-fold in human endometrium at the perimenstrual phase (Cornet et al., 2002). Lefty proteins were present in low amounts during the ‘receptive phase’ in the endometria and sera of normal fertile subjects, but were abundant in the endometrial tissue and endometrial fluid around the time of menstruation (Tabibzadeh et al., 2000). Similar amounts of lefty proteins were found in endometria of a number of infertile patients during the receptive phase of the cycle, suggesting dysregulated lefty to be contributing to the infertility in these women (Tabibzadeh et al., 2000).

Our current study was carried out to show that maternal overexpression of lefty in endometrium could reduce the chance of implantation in pregnant mice similarly to that observed in infertile women. Commonly, viral vectors have
been used for delivery of foreign genes into animal cells in vivo. While retroviral vectors can only enter dividing cells, adenoviruses enter both dividing and non-dividing cells. However, adenoviruses cause activation of innate immune responses shortly after administration in vivo, which leads to substantial loss of vector genomes in the first 24 h (Liu and Muruve, 2003). Non-viral methods that utilize a mixture of DNA and liposomal complexes have also been used recently in the reproductive tract (Bagot et al., 2000; Relloso and Esponda, 2000). We used both retroviral gene delivery and liposome-mediated DNA transfer in the current study to introduce lefty into the uterine horns of pregnant mice. In vivo gene transfer of lefty and not the control vectors led to a significant decrease in the number of embryos implanted in the uterine horns. Recently, Bagot et al. (2000) showed that introduction of HOXA10 antisense oligonucleotides at the time of implantation led to a significant reduction in the number of embryos which implanted in the uterine horns. Increasing expression of HOXA10 by delivery of pcDNA3.1(+) expression vector, however, led to larger litters and increased number of live births as compared with controls that received β-gal expression vector. Together, these findings show that the rate of implantation in endometrium is controlled by a number of genes which appear to include HOXA10 and lefty.

The reason that in vivo gene delivery of lefty did not lead to complete loss of implantation in pregnant mice might be due to several factors including, but not limited to, the low efficiency of transfection, stability of the proteins in vivo, type of cells transduced or transfected, and accessibility of proteins to the binding proteins. Charnock-Jones et al. (1997) found a low percentage of transfected cells in the reproductive tract following DNA–liposome complex transfer, ~6% in the oviduct epithelium and ~9% in the uterine glands. Relloso and Esponda (2000) also noted a very small percentage of cells transfected in the tube and uterine horn when DNA–liposome complexes were introduced into the oviduct. Such a low rate of transfection has also been noted in other tissues including respiratory epithelium and muscle, with a variable transfection rate of 1–30% (Acsadi et al., 1991; Grubb et al., 1994). The limitation of the retroviral gene

Figure 2. Trans-uterine flow of Chicago blue. Chicago blue (1%) was injected into the left uterine horn and its flow to the right uterine horn was followed over time (A–C). (D–F) The cross-sections of both horns (D), left horn (E) and right horn (F) after injection of 25 μl of fluid.

Figure 3. Western blot analysis of lefty in uterine horns of mice injected with β-gal and lefty vectors. The blot was re-probed for actin to show equal loading.
Figure 4. β-Galactosidase activity in the uterine horn not injected with β-gal (A) and that injected with β-gal vector (B).

transfer is that these vectors can only enter dividing cells, have a short life span (6 h) and do not enter the non-dividing cells. To overcome these limitations of in vivo gene delivery, a conditional transgenic approach is required to induce lefty in a regulated fashion in cells that normally express lefty. Only such an approach allows us to gain full insight into the role of lefty overexpression on implantation.

The in vivo gene delivery used in the current study led to gene expression, which lasted the course of this study similar to that reported previously (Bagot et al., 2000). Bagot et al. showed that β-gal activity was still detected 18 days following liposome-mediated gene transfection with pcDNA3.1(+) LacZ, the same type of vector used in this study (Bagot et al., 2000). Following in vivo gene delivery, Relloso and Esponda (2000) found maximum expression of β-gal activity to be reached in 7 days in oviducts and 14 days in the uterus. Consistent with these previous findings, in the current study, high levels of lefty were detected in endometrium 9 days after introduction of lefty by pcDNA3.1(+) vector.

The reduced implantation in this report appears to be due to lefty overexpression in endometrium for several reasons. First, only the retroviral vector transducing lefty and not the control vector led to a significant reduction in implantation rate. There was a smaller decrease noted in the implantation rate with the control vector, probably due to toxicity of the vector. We noted that an increased number of viral particles introduced into the uterine horn led to reduced implantation regardless of whether LG or LEIG vectors were used (data not shown). Secondly, the findings obtained with retroviral vector could be simulated with pcDNA3.1(+) expression vector and not the control pcDNA3.1(+) β-gal expression vector. Thirdly, the decreased implantation was more evident in the horn injected with pcDNA3.1(+) lefty expression vector than the contralateral horn. The reduced implantation in the horn not injected with lefty expression vector is probably due to trans-uterine horn transfer of fluids as seen with Chicago blue injection. Fourthly, the pcDNA3.1(+) expression vectors were functional since β-gal was detected in cells transfected in vitro and in the uterine horns injected with pcDNA3.1(+) β-gal expression vector. Similarly, lefty proteins were present in the culture media of transfected cells and in the uterine horns injected with pcDNA3.1(+) lefty expression vector.

Potentially, lefty effects by in vivo gene delivery to the uterine horn might be directed at the endometrium, the tube and the embryo. Lefty effects on the tube are unlikely, however, since the narrow utero-tubal junction acts as a barrier for the retrograde flow of fluid from uterine horn to the tube (Hunter, 1988). Lefty knockout was embryo-lethal, suggesting an important function for lefty during embryogenesis (Meno et al., 1998). Thus, lefty overexpression might alter embryogenesis by targeting the embryo. Lefty has been shown to inhibit the function of several members of the transforming factor-β (TGF-β) family which are dependent on epidermal growth factor (EGF)–CFC (Cripto, Fr11, and Cryptic) for signalling, such as nodal and Vg1 (Cheng et al., 2004). Lefty binds EGF–CFC or directly binds nodal (Chen and Shen, 2004). Although this action might be significant for the effect of lefty on the embryo, the presence of nodal or EGF–CFC in human or mouse endometrium has not yet been established. DNA microarray and northern blot analysis showed that lefty was expressed during decidualization (Popovici et al., 2000; Brar et al., 2001). Expression of lefty was increased by 70- to 123-fold in 24–36 h after human stromal cells were treated with cAMP (Tierney et al., 2003). These findings show lefty to be an important mediator during stromal decidualization and that aberrant expression of this gene might cause defective implantation due to faulty decidualization. Consistent with this, decidualization was absent in endometria of mice treated with the LEIG vector that did not contain any implanted embryo. We recently showed that lefty increased matrix metalloproteinase (MMP)-3 and MMP-7 in human endometrium and led to inhibition of collagen synthesis and collagenolysis in vivo (Cornet et al., 2002; Mason et al., 2002). Cornet et al. (2004) recently showed that lefty also induced MMP-9 in endometrial explants. It is becoming increasingly clear that MMPs are required for both embryo implantation and menstruation. These enzymes, by virtue of inducing a localized dissolution of endometrial tissue, facilitate the implantation of blastocyst. However, their generalized expression and activation can lead to menstruation. Thus, it is tempting to speculate that the effect of lefty on implantation might be directed by modulation of endometrial MMPs.

One possibility is that reduced implantation as reported here might be due to lefty overexpression in embryos. However, this is quite unlikely for several reasons. First, lefty is a natural endometrial protein and, because it is secreted into the uterine cavity, blastocysts are exposed to lefty (Tabibzadeh et al., 2000). Secondly, retroviral vectors were injected on day 2 of pregnancy into the uterine horn, 2 days before the arrival of the blastocysts in the uterine horns and long after the retroviral vectors have lost the ability to transduce cells. This reduces the possibility that overexpression of
lefty in blastocyst leads to infertility and suggests endometrium rather than blastocyst to be the main target in infertility.

In summary, we showed here that lefty overexpression led to implantation failure. The findings reported here are similar to the clinical observation in infertile patients of dysregulated endometrial lefty expression during the receptive phase of the menstrual cycle. However, the exact mechanism of how lefty overexpression interferes with implantation may be more complex than induction of a pure implantation defect. An effect of lefty on the embryo or a systemic effect of lefty cannot be ruled out based on the presented evidence.

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