Short Communication

Host-Derived TGFB1 Deficiency Suppresses Lesion Development in a Mouse Model of Endometriosis

M. Louise Hull,^{*,†} M. Zahied Johan,^{*}
Wendy L. Hodge,[†] Sarah A. Robertson,^{*} and
Wendy V. Ingman^{*,‡}

From The Robinson Institute, Research Centre for Reproductive Health, and School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide; the Department of Obstetrics and Gynaecology,[†] Women's and Children's Hospital, North Adelaide; and the School of Medicine,[‡] The Queen Elizabeth Hospital, University of Adelaide, Woodville, Australia*

Transforming growth factor- β 1 (TGFB1) is a multifunctional cytokine that is abundant in both endometriotic lesions and the peritoneal fluid in women with endometriosis. However, the role of TGFB1 in the development of endometriosis is as yet undefined. In the present study, we investigated the physiologic function of TGFB1 in endometriotic lesion development, using *Tgfb1*-null mutant mice on a background of severe combined immunodeficiency. Xenotransplantation of human eutopic endometrial tissue resulted in development of endometriosis-like lesions in 63% of ovariectomized estrogen-supplemented *Tgfb1*-null mutant mice and in 68% of wild-type control mice. Median lesion weight was reduced by 11-fold in *Tgfb1*-null mice compared with wild-type control mice, and the fraction of glandular epithelium in lesions from *Tgfb1*-null mice was reduced by 32% compared with that in control mice. In lesions from *Tgfb1*-null mice, the relative abundance of both macrophages and α -smooth muscle actin-positive myofibroblasts was reduced by 66% and 47%, respectively. Deficiency of TGFB1 neither altered the percentage of proliferating cells in the epithelial or stromal compartments of the lesions nor affected blood vessel density or vessel size. Observation of this study indicates that host-derived TGFB1 deficiency suppresses endometriotic lesion development and provides proof of principle that targeting TGFB1 signaling pathways in cells that support the survival of ectopic endometrium may be an effective therapeutic approach in women with endometriosis. (*Am J Pathol* 2012, 180:880–887; DOI: 10.1016/j.ajpath.2011.11.013)

Endometriosis is a condition defined by the presence of uterine endometrial tissue, with characteristic glandular and stromal tissue structures, at an ectopic site in the peritoneal cavity. The disease affects approximately 10% of women of reproductive age, and causes pain and impaired fertility.¹ Although medical and surgical treatments are available, these have substantial adverse effects and are rarely curative. Women with endometriosis often experience recurrence of symptoms and prolonged morbidity from the disease.²

Lack of understanding of the endometriotic disease process is the key limitation to the ability to find an effective medical treatment. Although several theories about the pathophysiology of endometriosis have been postulated, Sampson's 1927 theory of retrograde menstruation³ remains the most widely accepted. Sampson postulated that during menstruation, endometrial fragments pass retrograde through the fallopian tubes into the peritoneal cavity. The endometrial fragments become implanted at ectopic sites including the surface of the peritoneum and ovaries, and are established as hormonally responsive endometriotic lesions.³

Until recently, little was understood about the cellular and molecular interactions that promote establishment of an ectopic endometrial lesion. However, gene expression profiling has led to formulation of models of endometriotic lesion development.^{4,5} These models postulate that during establishment of an endometriotic lesion, the tissue undergoes a process of central ischemia, followed by tissue breakdown, inflammation, and tissue remodeling. These processes occur sequentially, and correlate with both the histologic properties of lesions at various stages of development of the disease and the mRNA transcript profiles identified in human endometriosis.⁵

Transforming growth factor- β 1 (TGFB1) is a multifunctional cytokine that regulates a variety of biological pro-

Supported by National Health and Medical Research Council project grants 1012950 and 565313 (to M.L.H. and W.V.I.)

M.L.H. and M.Z.J. contributed equally to the manuscript.

Accepted for publication November 21, 2011.

Address reprint requests to M. Louise Hull, Ph.D., The Robinson Institute, 6th Floor Medical School North, University of Adelaide, Frome Rd, Adelaide, Australia, 5005. E-mail: louise.hull@adelaide.edu.au.

cesses including cell proliferation, extracellular matrix formation, tissue remodeling, and inflammation.^{6,7} Similar biological events occur during endometriotic lesion establishment, and although there is little understanding of the signaling events that control them, there is evidence of TGFB1 involvement.⁸ In women with endometriosis, TGFB1 is up-regulated in peritoneal fluid, endometriotic tissue, and serum,^{9–11} and has been identified as the central component of a molecular network that is active during establishment of endometriotic lesions.⁵ We hypothesized that TGFB1 promotes endometriotic lesion development and that TGFB1 deficiency would lead to suppression of lesion formation. Through analysis of lesion formation in *Tgfb1*-null mutant mice xenografted with human endometrial fragments, we demonstrate that host-derived TGFB1 deficiency suppresses lesion development, and suggest that the TGFB signaling pathway is a potential target for development of novel therapeutic strategies to prevent recurrence of endometriosis.

Materials and Methods

Approval for this study was obtained from the Animal and Human Ethics Committee of the University of Adelaide and the Animal and Human Ethics Committee of the Child, Youth & Women's Health Service (Adelaide, Australia). All animal experiments were conducted in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (seventh edition; 2004).

Collection of Endometrial Tissue

Endometrial biopsy samples were collected using suction curettes (Pipelle de Cornier; Laboratoire CCD, Paris, France) either during surgery ($n = 6$) or in the outpatient setting ($n = 1$). All women ($n = 7$) were aged between 22 and 45 years (mean, 42 years), had regular menstrual cycles (28 to 30 days), and were not taking any medication. One of the women had stage II endometriosis; the others were either confirmed to be disease free at surgery ($n = 4$) or demonstrated no symptoms of endometriosis ($n = 2$). Eutopic endometrial biopsy samples were acquired in the late follicular phase (day 10 to 14) of the menstrual cycle, as confirmed using Noyes' criteria,¹² except for one that was obtained at day 20. All biopsy samples were immediately placed in prewarmed phenol red-free Dulbecco's modified Eagle's medium/Ham's F12 culture medium (Sigma-Aldrich Pty., Ltd., Sydney, Australia) with 1% insulin-transferrin-selenium-X supplement (Gibco-BRL, Invitrogen Corp., Carlsbad, CA), 1% penicillin/streptomycin, 0.1% EX-CYTE Growth Enhancement Supplement (Celliance Corp., Kankakee, IL), and 10 nmol/L 17 β -estradiol (Sigma-Aldrich). The tissues were aseptically sectioned into 1-mm³ fragments and stored in groups of 15 to 18 in tissue culture inserts in an incubator at 37°C before being aspirated into a syringe for injection into recipient mice (see *In Vivo Injection of Human Endometrial Tissues*).

Mice

Breeding pairs heterozygous for the *Tgfb1*-null mutation on a mixed CF1/129/C3H/CB17 background produced homozygous *Tgfb1*-null mutant mice (*Tgfb1*^{-/-}), heterozygous mice (*Tgfb1*^{+/-}), or homozygous wild-type progeny (*Tgfb1*^{+/+}). All mice were homozygous for the *Prkdc*^{scid} mutation. The colony was maintained under controlled light (12L:12D photoperiod) and temperature, pathogen-free conditions, and were fed *ad libitum* a standard diet and sterilized water. All husbandry and experimental procedures in mice were performed in a laminar flow hood using sterile instruments.

The genotype of each mouse was determined using PCR of tail DNA as described previously.¹³ In brief, the PCR enabled detection of the intact and disrupted *Tgfb1* gene, using the forward primer 5'-GAGAAGAACTGCTGTGTGCG-3' and the reverse primers 5'-GTGTCCAGGCTCCAAATATAGG-3' to detect the intact *Tgfb1* gene or 5'-CTCGTCCTGCAGTTCATTCA-3' to detect the mutant *Tgfb1* gene containing a neomycin resistance gene inserted into exon 6.¹⁴

In Vivo Injection of Human Endometrial Tissues

Before all invasive procedures, the mice were anesthetized using isoflurane (Veterinary Companies of Australia Pty. Ltd., Aradrom, Australia) in oxygen. At 6 weeks, the mice underwent bilateral ovariectomy, and 30 mg/kg 17- β estradiol in peanut oil (Sigma-Aldrich) was administered subcutaneously every second day. (In a previous experiment, it had been determined that administration of 30 mg/kg 17- β estradiol maintained estrogenization of murine vaginal squames over a time frame of 4 weeks). After 6 or 7 days, 15 to 18 of the 1-mm³ fragments of human endometrial tissue were randomly allocated for subcutaneous injection at a ventral midline site, as previously described.⁵ Each patient provided enough eutopic endometrial tissue for one or two recipient *Tgfb1*^{-/-} mice and up to 3 *Tgfb1*^{+/+} control mice. The number of control mice in each experiment depended on the size of the litter. For each *Tgfb1*^{-/-} mouse (total $n = 8$), there were one to three littermate *Tgfb1*^{+/+} control mice (total $n = 19$). In some experiments, when fewer than 3 *Tgfb1*^{+/+} mice were available, recipient littermate *Tgfb1*^{+/-} mice (total $n = 4$) were included for comparison. After surgery, 0.02% mepivacaine (Nature Vet Pty. Ltd., Glenorie, Australia) local anesthetic was applied to the incision site. Ten days after xenotransplantation, mice were sacrificed at 1 hour after injection of 1 mg 5'-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich), and the lesions were dissected, fixed in 4% paraformaldehyde, and embedded in paraffin.

Analysis of Endometriosis-Like Lesions

All lesions larger than 2 mm in *Tgfb1*^{-/-} mice ($n = 5$), *Tgfb1*^{+/+} mice ($n = 13$), and *Tgfb1*^{+/-} mice ($n = 4$) were recovered and were included in the weight analysis. Lesions from *Tgfb1*^{-/-} mice ($n = 5$) were compared with those from *Tgfb1*^{+/+} mice ($n = 13$) in the weight and

morphometric analyses. Lesions were excluded unless both glandular epithelium and stroma were evident ($n = 1$ of <2 mm in the *Tgfb1*^{-/-} group, which contained only stromal cells).

Immunohistochemistry

Immunohistochemistry was performed using the following primary antibodies: rat anti-mouse F4/80 (Caltag Laboratories, Inc., Burlingame, CA) reactive with murine macrophages, mouse anti- α -smooth muscle actin (α -SMA) (clone 1A4; Sigma-Aldrich) reactive with myofibroblasts, and rabbit anti-human von Willebrand factor (vWF) (Chemicon International, Inc., Temecula, CA) reactive with endothelial cells. The BrdU In Situ Detection Kit (BD Pharmingen, BD Biosciences, San Diego, CA) was used to localize proliferative cells in the lesions, as described previously.⁵ Irrelevant isotype-matched mouse and rat control IgG antibodies (both from Sigma-Aldrich) were used as negative controls for the monoclonal antibodies, and nonspecific rabbit IgG antibodies served as control for the polyclonal rabbit vWF antibody (Chemicon International). Sections of mouse uterus and human endometrium were used as tissue controls and as a negative control for BrdU.

Five-micrometer sections were dewaxed and rehydrated in Safesolvent (Safesolvent, Inc., Ft. Lauderdale, FL) and descending concentrations of ethanol. Proteinase K (Sigma-Aldrich) was used for antigen retrieval for vWF staining. Endogenous hydrogen peroxidases (Sigma-Aldrich) were blocked in 3% hydrogen peroxide, followed by incubation in 10% to 15% normal rabbit or goat serum, depending on the secondary antibody. Anti-F4/80 (1:50) and anti-vWF (1:600) primary antibodies were incubated overnight at 4°C, and anti- α -SMA antibodies (1:500) were incubated at room temperature for 1 hour. Reactivity was detected using biotinylated rabbit anti-rat IgG (F4/80) (1:100, for 40 minutes), goat anti-mouse IgG (α -SMA) (1:4000, for 30 minutes), or goat anti-rabbit (vWF) (1:800, for 1 hour) at room temperature (all from Chemicon International). Biotinylated secondary antibodies were detected using streptavidin-horseradish peroxidase (1:500; Dako Cytomation A/S, Glostrup, Denmark) for 45 minutes or an ABC Elite Kit (Vector Laboratories, Inc., Burlingame, CA) for F4/80 and diaminobenzidine (Invitrogen Corp.). The sections were counterstained with hematoxylin (Sigma-Aldrich) and mounted in DPX medium, and a coverslip was applied.

Morphometric Analyses

All morphometric analyses were performed by an assessor (M.Z.J.) blinded to genotype. H&E staining was performed on every tenth section from each lesion, and five sections randomly selected for morphometric analysis were then imaged using NanoZoomer software (Hamamatsu Photonics KK, Hamamatsu City, Japan). The glandular area fraction was determined by measuring the total area of the glands (epithelium and lumen) and dividing by the total area of the tissue in these five sections from each lesion.

The abundance of stained cells was quantified in five randomly chosen fields of two sections from each lesion at 10 $\times$ magnification. Only cells with a visible nucleus were included in the analysis. Macrophage density was determined by manually counting F4/80-positive cells in tissue within a 100- μ m depth from the surface of the lesion. The macrophage density from these five areas was averaged for each lesion. Images for α -SMA and vWF staining were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Each field was converted to eight-bit gray images and adjusting binary images using the threshold option. The area comprised of horseradish peroxidase-positive cells in the entire stromal tissue region was measured and was divided by the total stromal area to obtain the percentage area that was positive for α -SMA or vWF. Individual blood vessels were traced to obtain the area of vessels per field divided by the total stromal area in each field to calculate the area of vessels per micrometer squared. BrdU-positive cells were manually counted in five randomly selected fields within the epithelial and stromal compartments, and were expressed as a percentage of the total number of cells in these compartments per microscope field.

Statistical Analysis

Data from histologic and immunohistologic analyses were averaged for each lesion to provide a single result for each mouse. In the weight and morphometric analyses, median and ranges were calculated. The nonparametric Mann-Whitney *U* test (PRISM 5; GraphPad Software, Inc., San Diego, CA) was used to evaluate the effect of host genotype (*Tgfb1*^{+/+} versus *Tgfb1*^{-/-}), with significance inferred at $P < 0.05$.

Results

Endometriosis-like lesions were evident in comparable percentages of recipient mice irrespective of genotype, including 13 of 19 *Tgfb1*^{+/+} mice (Figure 1A; 68%), 4 of 4 *Tgfb1*^{+/-} mice (100%), and 5 of 8 *Tgfb1*^{-/-} mutant mice (Figure 1B; 63%). In each experiment, there was some variation in the number of mice that developed lesions. However, in both the control *Tgfb1*^{+/+} ($n = 1$ to 3) and *Tgfb1*^{-/-} ($n = 0$ or 1) groups, the incidence of lesion development was comparable across the experiments.

The median (range) weight of lesions that developed in *Tgfb1*^{-/-} mice was reduced by 11-fold compared with lesions that developed in *Tgfb1*^{+/+} mice: 2.8 (2.4 to 15.6) mg and 31.3 (1.3 to 122.0) mg, respectively ($P = 0.0305$). The lesion weight in the *Tgfb1*^{-/-} mice demonstrated a sixfold reduction compared with lesions that developed in *Tgfb1*^{+/-} mice: 16.2 (12.7 to 19.2) mg (Figure 1C). A twofold decrease in median lesion weight in *Tgfb1*^{+/-} mice compared with *Tgfb1*^{+/+} mice was also observed. These results suggest a gene dose-response effect, although the small sample size in the *Tgfb1*^{+/-} group precluded statistical analysis.

Sections cut from endometriosis-like lesions from *Tgfb1*^{+/+} ($n = 13$) and *Tgfb1*^{-/-} ($n = 5$) mice were

Figure 1. Lesion development in *Tgfb1*^{-/-} mice. Xenografted human eutopic epithelium formed endometriotic lesions on the peritoneal wall of *Tgfb1*^{+/+} (A) and *Tgfb1*^{-/-} (B) immunodeficient mice (arrows). The weight of the lesions was compared in tissue grafted into *Tgfb1*^{+/+} (*n* = 13), *Tgfb1*^{+/-} (*n* = 4), and *Tgfb1*^{-/-} (*n* = 5) (C) mice. Data were analyzed using the Mann-Whitney *U* test (**P* < 0.05). Each symbol represents data from one lesion, with the median weight shown as a bar.

examined morphometrically (Figure 2, A–D). Endometrial glands were observed within a surrounding stroma in sections stained with H&E. In *Tgfb1*^{+/+} mice, the mean (range) percentage of glandular and stromal areas each comprised approximately half of the total endometriotic lesion area: 52.2% (30.5% to 66.7%) and 47.8% (33.3% to 69.5%), respectively (Figure 2E). In contrast, the median glandular area in lesions from *Tgfb1*^{-/-} mice was reduced to 35.4% (14.1% to 64.0%), with a corresponding increase in the stromal area to 64.6% (36.0% to 86.0%) (*P* < 0.05).

Mouse-derived macrophages present in the human endometriosis-like lesions were examined immunohistochemically in *Tgfb1*^{+/+} and *Tgfb1*^{-/-} mice (Figure 3, A and B, respectively). In both groups, murine macrophages were observed predominantly in the peripheral regions of the lesions, and were sparsely distributed within the central part of the tissue. Median (range) macrophage abundance up to a 100-μm depth into the lesion from the periphery was reduced by 66% in lesions from *Tgfb1*^{-/-} mice compared with *Tgfb1*^{+/+} mice: 613 (148 to 1532) cells/mm² and 1801 (528 to 3072) cells/mm², respectively (*P* < 0.05) (Figure 3C). F4/80 antibody detects macrophages of mouse origin only, as confirmed by positive staining in mouse uterine tissue (Figure 3G) and the absence of staining in human endometrium (Figure 3H). Nonspecific rat IgG antibodies did not produce specific staining in xenograft tissue (Figure 3I).

Immunostaining with α-SMA antibody enabled examination of the abundance of myofibroblasts in the stromal

compartment of endometriosis-like lesions from *Tgfb1*^{+/+} and *Tgfb1*^{-/-} mice (Figure 3, D and E, respectively). The median (range) area comprised by myofibroblasts throughout the lesion was reduced by 47% in lesions from *Tgfb1*^{-/-} mice compared with *Tgfb1*^{+/+} mice: 15.0% (4.3% to 28.1%) positivity in *Tgfb1* wild-type mice compared with 8.0% (0.7% to 14.9%) positivity in *Tgfb1*^{-/-} mice (*P* < 0.05) (Figure 3F). Staining did not occur with mouse IgG negative control antibodies in xenograft tissue (Figure 3J).

The incorporation of a synthetic nucleoside, BrdU, was used to assess the proliferative activity of the endometriosis-like lesions. Proliferating cells were evident in both the stromal and epithelial compartments in lesions from *Tgfb1*^{+/+} and *Tgfb1*^{-/-} mice (Figure 4, A and B, respectively). The mean (range) number of BrdU-positive epithelial cells [7.4% (0.5% to 17.1%) for *Tgfb1*^{+/+} mice, and 5.6% (0.6% to 15.9%) for *Tgfb1*^{-/-} mice] and stromal cells [3.4% (0.3% to 9.4%) for *Tgfb1*^{+/+} mice, and 2.1% (0.6% to 7.8%) for *Tgfb1*^{-/-} mice] was not altered by genotype (Figure 4C). No positive BrdU staining was identified in either mouse (Figure 4H) or human negative tissue controls.

Blood vessel distribution in endometriosis-like lesions was examined via vWF immunostaining. Blood vessels were observed throughout lesions from *Tgfb1*^{+/+} and

Figure 2. Representative histologic findings of lesions from *Tgfb1*^{+/+} (*n* = 13) (A and B) and *Tgfb1*^{-/-} (*n* = 5) (C and D) mice. Original magnification: ×10 (A and C); ×40 (B and D). The percentage of lesions comprised of gland and stroma was quantified (E) and analyzed using the Mann-Whitney *U* test (**P* < 0.05). Boxes represent the 25th to 75th percentile, with median line; error bars represent 5th and 95th percentiles.

Figure 3. Macrophage and smooth muscle actin abundance representative of lesions from *Tgfb1*^{+/+} (*n* = 13) (A and D) and *Tgfb1*^{-/-} (*n* = 5) (B and E) mice. A and B, F4/80-stained mouse macrophages were observed entering into the lesion (lesion edge indicated by a dotted line). Macrophage abundance was quantified in the first 100 μm from the edge of the tissue (C). Smooth muscle actin was observed in the stroma of the lesions (D and E), and was quantified (F) at 10× magnification. F4/80-antibody staining on mouse uterine (G) and human endometrial (H) sections and nonspecific rat IgG negative control for F4/80 on xenograft tissue. F4/80 antibody negative control (I) and α-SMA-negative mouse IgG control (J) in ectopic endometrial lesion. Data were analyzed using the Mann-Whitney *U* test (**P* < 0.05). Boxes represent the 25th to 75th percentile, with median line; error bars represent 5th and 95th percentiles.

Tgfb1^{-/-} mice (Figure 4, D and E, respectively), and neither the median (range) area of the vessels [398 (158 to 1202) μm² and 333 (243 to 471) μm², respectively] (Figure 4F) or the percent positive staining with the lesions [0.9% (0.3% to 2.1%) and 0.9% (0.1% to 1.2%, respectively)] (Figure 4G) were affected by genotype. vWF antibody detection of both mouse and human vessels can be observed in mouse uterine tissues (Figure 4I) and human endometrium tissues (Figure 4J) but not in control tissues (Figure 4H). Specific staining of xenograft tissue was not identified using polyclonal rabbit IgG (Figure 4K).

Discussion

Findings of the present study demonstrate that host TGFB1 is a key determinant of development of human endometriosis-like lesions in an immune-deficient mouse model. In addition to an 11-fold decrease in lesion weight, TGFB1 deficiency resulted in a significant reduction in glandular area in the lesions recovered from *Tgfb1*^{-/-} mice. The combined result is a dramatic reduction in glandular epithelium within endometrial xenografts in the absence of host-derived TGFB1.

Three mammalian isoforms of TGFB (TGFB1, TGFB2, and TGFB3) exert biological effects through a common

receptor signaling pathway.¹⁵ Because of their high degree of gene and sequence homology, there is 100% cross-species bioactivity between mouse and human TGFB proteins.¹⁶ Studies in mice carrying a null mutation in the *Tgfb1* gene have revealed vital and nonredundant roles for TGFB1 in regulation of many reproduction-associated processes.^{13,17–19} Although reproductive function is severely compromised in these mice, uterine structure is overtly normal, with luminal and glandular epithelium present in the expected architecture and abundance.¹³ Therefore, TGFB1 is not critical for development of murine eutopic endometrium, and the observed effects of TGFB1 deficiency on human ectopic endometriotic tissue must reflect either dysfunction in tissue remodeling at an ectopic site or different species-specific requirements of the human tissue.

The importance of TGFB1 in endometriotic lesion development is supported by several studies that have identified high levels of *Tgfb1* mRNA and TGFB1 protein in peritoneal fluid, ectopic lesions, and serum in women with endometriosis.^{9–11} However, in a nude mouse model, TGFB1 promoted progesterone-mediated suppression of endometriosis-like lesion development when preincubated with the endometrial tissue before xenografting. TGFB1 did not influence lesion development when preincubated with estradiol alone, which implies

Figure 4. Proliferation and blood vessel density representative of lesions from *Tgfb1*^{+/+} ($n = 13$) (A and D) and *Tgfb1*^{-/-} ($n = 5$) (B and E) mice. Proliferating cells, identified by BrdU incorporation, were observed in both the epithelial and stromal compartments (A and B), and were quantified (C). Blood vessels, identified by vWF immunostaining, were observed throughout the lesions (D and E), and average vessel area (F) and the abundance of vWF-positive staining (G) was quantified at 10× magnification. Mouse uterus negative tissue control for BrdU (H). Positive endothelial cell staining with rabbit anti-vWF antibodies in mouse uterus (I) and human endometrium (J) and nonspecific rabbit IgG staining in a xenograft (K). Data were analyzed using the Mann-Whitney *U*-test ($P < 0.05$). Box represents the 25th to 75th percentile, with median line; error bars represent the 5th and 95th percentiles.

that TGFB1 does not promote early lesion development (attachment and initial survival).²⁰ This is consistent with our observation of no effect of TGFB1 deficiency on lesion attachment, inasmuch as both *Tgfb1*^{-/-} and *Tgfb1*^{+/+} mice exhibited a similar frequency of lesion formation. The effect of TGFB1 on tissue remodeling in later stages of ectopic endometrial development was not assessed in the study by Bruner-Tran et al²⁰ because only human explants were subject to alterations in TGFB1 exposure, and the host response was minimally affected.

We found that in a host TGFB1-deficient environment, decreased lesion weight is associated with reduced infiltration of host-derived macrophages and myofibroblasts at day 10 of lesion development. This suggests that the most important phase of endometriotic lesion development affected by TGFB1 deficiency is a later phase, during glandular and stromal remodeling.

TGFB1-Regulated Remodeling of Ectopic Endometrium

Decreased host macrophages and myofibroblasts within the xenografts of *Tgfb1*^{-/-} mice suggest that these cells perform critical functions during ectopic endometrial gland development and that the function of these host cells and/or their recruitment into the lesions is TGFB1-

dependent. An effect on gland development seems difficult to reconcile with the lack of effect of TGFB1 deficiency on epithelial cell proliferation. However, it is possible that early-phase development is more dependent on TGFB1 than on turnover of cells in established glands. Alternatively, apoptosis could have been more active in lesions from TGFB1-deficient mice. Both of these possibilities could be tested by examining proliferation, apoptosis, and percentage of epithelium and stroma at other time points of ectopic endometrial lesion development in *Tgfb1*^{-/-} mice.

TGFB1 induces epithelial-mesenchymal transition,²¹ and TGFB1 deficiency could act to reduce epithelial differentiation of mesenchymal cells without altering cell numbers through proliferation. This explanation would also account for the reduction in the epithelial area fraction in the absence of reduced proliferation in TGFB1-deficient lesions.

In human endometriotic lesions, abundant TGFB1 is evident in inflammatory cells in the endometrial stroma, and is also observed in the glandular epithelium.^{22,23} This is consistent with the localization of TGFB1 to the glandular epithelium and inflammatory macrophage cells in the periphery of ectopic endometrial lesions in a rat model of endometriosis.²⁴ TGFB types I and II receptors²³ and components of the SMAD family²⁵ are present in similar locations as TGFB1 in distinct stromal cell pop-

ulations and in the glandular epithelium of human eutopic and ectopic endometrium.

Together, these studies suggest that there are two TGFB1 secreting compartments in ectopic endometrial lesions: one in the glandular epithelium, and the other in inflammatory cells in the stroma. Inasmuch as our model does not manipulate TGFB1 expression in the xenotransplanted tissue, it is most likely that the observed reduction in lesion weight is the result of impaired function of infiltrating host macrophages.

Macrophages are highly versatile cells that mediate immune system responses, tissue remodeling, and homeostasis.²⁶ Bacci et al²⁷ recently demonstrated that M1-type macrophages, which are associated with inflammation, suppress endometriotic lesion development, whereas M2-type macrophages, associated with wound healing and tissue remodeling, enhance lesion development. There is increasing evidence that the microenvironment has a substantial influence on the relatively plastic phenotype of macrophages and that TGFB1 promotes "development-associated," or M2-type macrophage activity.²⁸

Macrophages are key producers of TGFB1 in healing tissues,²⁶ and are commonly observed in close proximity to collagen-producing myofibroblasts, as was noted in the present study. Macrophages induce trans-differentiation of fibroblasts to myofibroblasts, as evidenced by actin stress fibers via TGFB1-mediated pathways.²⁹ Extracellular matrix turnover is controlled by macrophages through both TGFB1-mediated stimulation of myofibroblast production of collagen³⁰ and TGFB1-mediated regulation of the balance of proteases and protease inhibitors.³¹ Macrophages also regulate fibrogenesis by secreting chemokines that recruit fibroblasts and other inflammatory cells.²⁶

α -SMA-positive stromal cells are a feature of human ectopic endometrial tissues,^{32,33} and species-specific immunohistochemical staining in xenografts from the nude mouse model of endometriosis indicated that these α -SMA-positive myofibroblasts are host-derived.⁵ TGFB1 promotes fibroblast production of extracellular matrix molecules such as type 1 collagen and osteopontin, which are present in high levels in transcriptomes from ectopic endometrial lesions from human tissues and mouse models.^{5,34} In the host TGFB1-deficient environment, myofibroblast abundance was reduced. The absence of myofibroblasts likely leads to reduced production of extracellular matrix, which provides the scaffolding for proliferating cells in remodeling tissues. This also is likely to contribute to the reduced weight of lesions in *Tgfb1*^{-/-} mice.

Other features of tissue remodeling are cell proliferation and angiogenesis. These processes did not seem to be altered by host TGFB1 deficiency. TGFB1 produced by the human grafts may act to alleviate any proliferative or angiogenic deficiency in *Tgfb1*^{-/-} mice. A study of wound healing also suggests that TGFB2 and TGFB3 can promote tissue remodeling in *Tgfb1*^{-/-} mice.³⁵ The overlap in function between TGFB isoforms and other factors, such as interferon- γ , that influence TGFB1 activity³⁶ may explain our observation that TGFB1 was not a prerequisite for cellular proliferation and blood vessel formation. A

further study is being performed to directly assess TGFB1 and its isoforms using frozen sections from *Tgfb1*^{-/-} and control mice. In the future, we hope to also assess lesions from this model at various times in lesion development.

Clinical Implications for Treatment of Endometriosis

The complexity of cellular interactions in the developing ectopic endometrial lesion necessitates development of *in vivo* models. The baboon model is most representative of human endometriotic disease.^{37,38} However, that model, like other syngeneic rodent models,³⁹ cannot be used to test the hypothesis that TGFB1 deficiency in only one tissue compartment alters endometriotic lesion development. Furthermore, genetic modification in primates is not possible. The model of endometriosis used in the present study provides the only opportunity to study human lesion formation in a genetically modified system. Human eutopic endometrium is xenografted into mice with severe combined immunodeficiency to prevent a rejective immune response to foreign tissue because the adaptive immune system in these mice is not functional. However, their innate immune response is normal, and macrophages are recruited to sites of inflammation and tissue remodeling.^{35,14} Potential cross-species interactions between proteins derived from human endometrium and murine host are a limitation of this model for replicating human disease.

Through xenografting of human eutopic endometrium subcutaneously into TGFB1-deficient and TGFB1-replete mice, we have demonstrated that TGFB1 deficiency suppresses lesion development by influencing both the stromal and epithelial cell compartments. This model manipulates TGFB1 abundance only in the host mouse and not in the human endometrial fragment. Inasmuch as suppression of development can be achieved by reducing TGFB1 levels in tissues that support the endometrial graft, the opportunity exists to develop therapeutic strategies that target the TGFB1 signaling pathway or downstream effector mechanisms in the peritoneal support tissues to prevent or diminish recurrence of endometriosis.

Conclusions

Findings of the present study provide proof of principle that endometriotic lesion development depends on bioavailability of TGFB1 in peritoneal support tissues. This indicates that lesion development could reasonably be suppressed by targeting the TGFB1 pathway in host tissues in women with endometriosis. Moreover, suppression of TGFB1 activity in specific target cell lineages, including circulating monocytes or myofibroblast progenitors, could be an accessible, tailored, and targeted treatment for endometriosis.

Acknowledgments

We thank the patients at Women's and Children's Hospital Adelaide who kindly provided endometrial tissue for this study.

References

1. Viganò P, Parazzini F, Somigliana E, Vercellini P: Endometriosis: epidemiology and aetiological factors. *Best Pract Res Clin Obstet Gynaecol* 2004, 18:177–200
2. Giudice LC: Clinical practice. Endometriosis. *N Engl J Med* 2010, 362:2389–2398
3. Sampson JA: Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynaecol* 1927, 14:442–469
4. Flores I, Rivera E, Mousses S, Chen Y, Rozenblum E: Identification of molecular markers for endometriosis in blood lymphocytes by using deoxyribonucleic acid microarrays. *Fertil Steril* 2006, 85:1676–1683
5. Hull ML, Escareno CR, Goddard JM, Doig JR, Johnson CM, Phillips SC, Smith SK, Tavaré S, Print CG, Charnock-Jones DS: Endometrial-peritoneal interactions during endometriotic lesion establishment. *Am J Pathol* 2008, 173:700–715
6. Jakowlew SB: Transforming growth factor-beta in cancer and metastasis. *Cancer Metastasis Rev* 2006, 25:435–457
7. Massague J, Blain SW, Lo RS: TGF-beta signaling in growth control, cancer, and heritable disorders. *Cell* 2000, 103:295–309
8. Omwandho CO, Konrad L, Halis G, Oehmke F, Tinneberg HR: Role of TGF-beta in normal human endometrium and endometriosis. *Hum Reprod* 2010, 25:101–109
9. Oosterlynck DJ, Meuleman C, Waer M, Koninckx PR: Transforming growth factor-beta activity is increased in peritoneal fluid from women with endometriosis. *Obstet Gynecol* 1994, 83:287–292
10. Loverro G, Maiorano E, Napoli A, Selvaggi L, Marra E, Perlino E: Transforming growth factor-beta 1 and insulin-like growth factor-1 expression in ovarian endometriotic cysts: a preliminary study. *Int J Mol Med* 2001, 7:423–429
11. Pizzo A, Salmeri FM, Ardia FV, Sofo V, Tripepi M, Marsico S: Behaviour of cytokine levels in serum and peritoneal fluid of women with endometriosis. *Gynecol Obstet Invest* 2002, 54:82–87
12. Noyes RW, Hertig AT, Rock J: Dating the endometrial biopsy. *Am J Obstet Gynecol* 1975, 122:262–263
13. Ingman WV, Robker RL, Woitkiez K, Robertson SA: Null mutation in transforming growth factor beta1 disrupts ovarian function and causes oocyte incompetence and early embryo arrest. *Endocrinology* 2006, 147:835–845
14. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, et al: Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992, 359:693–699
15. Dunker N, Kriegstein K: Targeted mutations of transforming growth factor-beta genes reveal important roles in mouse development and adult homeostasis. *Eur J Biochem* 2000, 267:6982–6988
16. Cheifetz S, Weatherbee JA, Tsang ML, Anderson JK, Mole JE, Lucas R, Massague J: The transforming growth factor-beta system, a complex pattern of cross-reactive ligands and receptors. *Cell* 1987, 48:409–415
17. Ingman WV, Robertson SA: The essential roles of TGFB1 in reproduction. *Cytokine Growth Factor Rev* 2009, 20:233–239
18. Ingman WV, Robertson SA: Mammary gland development in transforming growth factor beta1 null mutant mice: systemic and epithelial effects. *Biol Reprod* 2008, 79:711–717
19. Ingman WV, Robertson SA: Transforming growth factor-beta1 null mutation causes infertility in male mice associated with testosterone deficiency and sexual dysfunction. *Endocrinology* 2007, 148:4032–4043
20. Bruner-Tran KL, Eisenberg E, Yeaman GR, Anderson TA, McBean J, Osteen KG: Steroid and cytokine regulation of matrix metalloproteinase expression in endometriosis and the establishment of experimental endometriosis in nude mice. *J Clin Endocrinol Metab* 2002, 87:4782–4791
21. Pakala SB, Singh K, Reddy SD, Ohshiro K, Li DQ, Mishra L, Kumar R: TGF-beta1 signaling targets metastasis-associated protein 1, a new effector in epithelial cells. *Oncogene* 2011, 30:2230–2241
22. Komiyama S, Aoki D, Komiyama M, Nozawa S: Local activation of TGF-beta1 at endometriosis sites. *J Reprod Med* 2007, 52:306–312
23. Tamura M, Fukaya T, Enomoto A, Murakami T, Uehara S, Yajima A: Transforming growth factor-beta isoforms and receptors in endometriotic cysts of the human ovary. *Am J Reprod Immunol* 1999, 42:160–167
24. Chegini N, Gold LI, Williams RS: Localization of transforming growth factor beta isoforms TGF-beta 1, TGF-beta 2, and TGF-beta 3 in surgically induced endometriosis in the rat. *Obstet Gynecol* 1994, 83:455–461
25. Luo X, Xu J, Chegini N: The expression of SMADs in human endometrium and regulation and induction in endometrial epithelial and stromal cells by transforming growth factor-beta. *J Clin Endocrinol Metab* 2003, 88:4967–4976
26. Wynn TJ, Barron L: Macrophages: master regulators of inflammation and fibrosis. *Semin Liver Dis* 2010, 30:245–257
27. Bacci M, Capobianco A, Monno A, Cottone L, Di Puppo F, Camisa B, Mariani M, Brignole C, Ponzoni M, Ferrari S, Panina-Bordignon P, Manfredi AA, Rovere-Querini P: Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. *Am J Pathol* 2009, 175:547–556
28. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J: Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* 2005, 175:342–349
29. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA: Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002, 3:349–363
30. Gabbiani G: The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 2003, 200:500–503
31. Wahl SM, McCartney-Francis N, Allen JB, Dougherty EB, Dougherty SF: Macrophage production of TGF-beta and regulation by TGF-beta. *Ann NY Acad Sci* 1990, 593:188–196
32. van Kaam KJ, Schouten JP, Nap AW, Dunselman GA, Groothuis PG: Fibromuscular differentiation in deeply infiltrating endometriosis is a reaction of resident fibroblasts to the presence of ectopic endometrium. *Hum Reprod* 2008, 23:2692–2700
33. Anaf V, Simon P, Fayt I, Noel J: Smooth muscles are frequent components of endometriotic lesions. *Hum Reprod* 2000, 15:767–771
34. Arimoto T, Katagiri T, Oda K, Tsunoda T, Yasugi T, Osuga Y, Yoshikawa H, Nishii O, Yano T, Taketani Y, Nakamura Y: Genome-wide cDNA microarray analysis of gene-expression profiles involved in ovarian endometriosis. *Int J Oncol* 2003, 22:551–560
35. Crowe MJ, Doetschman T, Greenhalgh DG: Delayed wound healing in immunodeficient TGF-beta 1 knockout mice. *J Invest Dermatol* 2000, 115:3–11
36. Delvig AA, Lee JJ, Chrzanowska-Lightowlers ZM, Robinson JH: TGF-beta1 and IFN-gamma cross-regulate antigen presentation to CD4 T cells by macrophages. *J Leukoc Biol* 2002, 72:163–166
37. D'Hooghe TM, Bamber CS, Raeymaekers BM, Koninckx PR: Development of spontaneous endometriosis in baboons. *Obstet Gynecol* 1996, 88:462–466
38. Hastings JM, Fazleabas AT: A baboon model for endometriosis: implications for fertility. *Reprod Biol Endocrinol* 2006, 4(Suppl 1):S7
39. Sharpe-Timms KL: Using rats as a research model for the study of endometriosis. *Ann NY Acad Sci* 2002, 955:318–327; discussion 340–312, 396–406