Macrophage Migration Inhibitory Factor Receptor, CD74, is Overexpressed in Human and Baboon (*Papio Anubis*) Endometriotic Lesions and Modulates Endometriotic Epithelial Cell Survival and Interleukin 8 Expression

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Abstract

CD74 is the primary receptor for macrophage migration inhibitory factor (MIF). Although expression of MIF has been described in endometriotic lesions, the cellular localization and function of the MIF receptor, CD74, are poorly understood. To further explore the role of CD74 in the pathophysiology of endometriosis, we utilized specimens from women with diagnostically confirmed endometriosis, women with no signs or symptoms of endometriosis (controls), and 8 baboons with experimentally induced endometriosis. Compared to eutopic endometrium from women with endometriosis, CD74 transcript expression was significantly increased in endometriotic lesion tissue. Similarly, cellular expression of CD74 was significantly greater in ectopic lesion tissue compared to paired eutopic endometrium, which both expressed greater CD74 expression compared to eutopic endometrium from control patients. Localization of CD74 was predominant to epithelial cells of ectopic and matched eutopic endometrium and was not influenced by the stage of the menstrual cycle. Eutopic endometrium from control patients did not express detectable levels of CD74 protein by immunohistochemistry. This pattern of expression and CD74 protein localization could be recapitulated in endometriotic lesion tissue from baboons with experimentally induced disease. Transfection of the endometriotic epithelial cell lines, I2Z with CD74 short hairpin RNA (shRNA), resulted in a significant decrease in CD74 protein expression, which was associated with a significant reduction in cellular proliferation as well as the expression of the prosurvival cytokine interleukin 8. Together, these data support the hypothesis that CD74 is elevated in endometriotic lesion tissue and may contribute to the pathogenesis of endometriosis by promoting cell survival.

Keywords

endometriosis, women, baboon, CD74

Introduction

Endometriosis is a significant disease in which endometrial stromal and glandular tissue grow in ectopic locations, primarily within the pelvic cavity. Endometriosis affects 10% to 15% of reproductive-age women and is characterized by pelvic pain, increased anxiety and stress as well as infertility.¹⁻⁶ The cost of endometriosis diagnosis and treatment was estimated at an annual cost of over US\$22 billion in the United States alone nearly a decade ago.⁷ One of the underlying factors associated with this high cost stems from the inadequacy of current treatment options. Current medical treatment approaches are centered on reducing endogenous estrogen production and/or

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action. Shortcomings to this approach include lack of effectiveness in a subset of patients, unwanted/intolerable side effects in those women who do achieve pain relief, and potential adverse effects on bone, cardiovascular, and neuronal health. Thus, there is a clear need for the identification of novel targets for endometriosis treatment, which would minimize off-target effects and be more acceptable to users.

We and others have demonstrated that targeting the cytokine macrophage migration inhibitory factor (MIF) may be an alternative option to traditional antiestrogen-based approaches. The MIF is a cytokine that is secreted by endometriotic cells *in vitro* and exhibits mitogenic activity by promoting the growth of endothelial cells⁸ as well as the ability to stimulate prostaglandin E2, cyclooxygenase-2,⁹ vascular endothelial growth factor, interleukin 8 (IL-8), and monocyte chemotactic protein-1.¹⁰ These factors are all associated with a proliferative and angiogenic phenotype conducive to endometriotic establishment and/or growth.

Further, *in vivo* evidence suggests that MIF is predominantly expressed in glandular epithelial cells of both eutopic endometrium^{11,12} and robustly expressed in epithelium of active and early/stage I endometriotic lesions¹³ with focal stromal staining in both tissue types. The expression level of MIF in human endometriotic lesion tissue is also inversely related to the expression of proliferative factors such as cyclin E1.¹⁴ The MIF levels are also elevated in the peritoneal fluid¹⁵ and peripheral blood¹⁶ of women with endometriosis, and MIF secretion is enhanced in peritoneal macrophages from women with the disease.¹⁷ Using mouse experimental models for endometriosis, we¹⁸ and others¹⁹ have demonstrated that the MIF antagonist, 4,5-Dihydro-3-(4-hydroxyphenyl)-5-isoxazoleacetic acid methyl ester (ISO-1), reduces endometriotic lesion size, and this appears to occur independent of circulating estrogen levels/estrous cyclicity. More recently, additional proof of principal was provided using an ovariectomized, estrogen-supplemented mouse model for endometriosis that incorporated MIF-deficient mice as both tissue recipient and tissue donor.²⁰ Although these data strongly suggest that MIF is associated with the pathogenesis of endometriosis, it still remains unclear how this ligand may mediate lesion survival *in vivo*.

In 2003, CD74 (also referred to as invariant chain) was identified by an expression cloning approach as the first receptor for MIF.²¹ Cell surface-expressed CD74 can form either homodimers or heterodimers with other cell surface receptors, namely, CD44 or Chemokine receptor-2/Chemokine receptor-4 (CXCR2/CXCR), to form a molecular complex capable of eliciting cellular signaling responses.^{22,23} Although Veillat and colleagues¹⁰ have demonstrated using *in vitro* stromal cell cultures that CD74 (along with CD44) mediates MIF induction of factors proposed to contribute to the pathophysiology of endometriosis, there is little to no knowledge on the expression of CD74 in human endometriotic lesion tissue. As such, the objective of this study was to examine CD74 expression in endometriotic lesion tissue and to incorporate *in vivo* and *in vitro* models to further

Table 1. Patient Demographics.

Study Group/Age Range	Diagnosis/Stage of Menstrual Cycle	Lesion Type (N) ^a
Endometriosis (N = 57) 21-45 years	Leiomyomas	
	Proliferative (N = 7)	
	Secretory (N = 8)	
	Endometrial polyps	
	Proliferative (N = 2)	
	qRT-PCR studies (N = 33)	Lesion type (N = 68)
	Stages I/II endometriosis (N = 12)	
	Menstrual (N = 1)	P (1)
	Proliferative (N = 4)	P (3), O (1), F (1)
	Secretory (N = 7)	P (4), O (2), F (1), CDS (1)
	Stages III/IV endometriosis (N = 21)	
	Menstrual (N = 2)	P (2)
IHC studies (N = 24)	Proliferative (N = 8)	P (8), F (1)
	Secretory (N = 11)	P (12), O (4), F (2), CDS (3)
	Stages I/II endometriosis	
	Proliferative (N = 2)	P (1), O (1)
	Secretory (N = 3)	P (1), O (2)
	Stages III/IV endometriosis	
	Proliferative (N = 10)	P (8), O (2)
	Secretory (N = 9)	P (6), O (3)

Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction; P, peritoneal biopsy; O, ovarian endometrioma; F, fibrotic lesion; CDS, cul-de sac lesion; IHC, immunohistochemical.

^aN indicates the number of each lesion type within group.

understand the role of this MIF receptor in endometriosis pathophysiology.

Materials and Methods

Human Patients and Tissue Acquisition

The study was approved by the institutional review boards of both the University of Kansas Medical Center and Cleveland Clinic. Written informed consent was obtained prior to surgical removal of endometriotic lesion tissue and endometrial biopsies. Samples were obtained from patients aged between 21 and 45 who presented with pelvic pain due to failed previous endometriosis treatment and were undergoing surgical removal of endometriotic lesion tissue. A total of 33 patients were enrolled (n = 3 in the menstrual stage of the menstrual cycle, n = 12 in the proliferative stage of the menstrual cycle, and n = 18 in the secretory stage of the menstrual cycle) and included women with stages I/II (N = 11) and stages III/IV (N = 22) endometriosis (Table 1). No patients had taken gonadotropin-releasing hormone analogs or hormonal therapy within 3 months prior to surgery. A total of 33 endometrial biopsies (eutopic endometrium) and 41 matched (same patient) endometriotic lesions were collected. All specimens were collected by the same surgeon (TF) at Cleveland Clinic with emphasis on minimizing

sample contamination from underlying/surrounding nonendometriotic lesion tissue. To do so, endometriotic lesions were excised and sent to pathology for confirmation of endometriosis, which was defined as the presence of endometrial glands and stroma. Tissue was excised using sharp scissors with no energy. During the excision, the underlying tissue was separated from the lesion tissue. A portion of the same sample lesion that was sent for endometriosis confirmation by a pathologist was utilized for research. Research samples were immediately snap-frozen, stored at -80°C , and then shipped to the University of Kansas Medical Center. The stage of the menstrual cycle was determined from the patient's medical records, with day 1 defined as the onset of menses.

Tissue samples used for immunohistochemical (IHC) analysis were obtained from the University of Kansas Medical Center, Department of Pathology and Laboratory Medicine (Table 1). Endometrial tissue in the control groups consisted of women with uterine leiomyomas ($N = 12$) or endometrial polyps ($N = 6$) of which 9 samples were obtained during the proliferative stage of the menstrual cycle ($N = 9$) and 9 from the secretory ($N = 9$) stage of the menstrual cycle. Eutopic and matched ectopic endometriotic lesion tissues (ectopic) were obtained from women with endometriosis during the proliferative ($N = 12$) and secretory ($N = 12$) stages of the menstrual cycle.

Samples were subjected to RNA extraction followed by quantitative real-time polymerase chain reaction (qRT-PCR) analysis as described below. As no difference in *CD74* transcript expression was noted among stages of the menstrual cycle, stages of endometriosis, or influenced by medications, data were collapsed and analyzed as ectopic versus eutopic tissues for *CD74* expression for the 3 variants, *CD74a*, *CD74b*, and *CD74c*.

Baboon Model of Endometriosis and Sample Collection

All animal studies were approved by the Animal Care and Use Committee of the University of Illinois at Chicago and Michigan State University. Experimental endometriosis was induced in baboons ($N = 8$) as previously described.²⁴ Briefly, baboons received intraperitoneal inoculation with menstrual endometrium on day 2 of 2 consecutive menstrual cycles (day 1 = the first day of menses). Eutopic and endometriotic lesion specimens were collected at the indicated time points, processed, and mounted on glass slides. Slides were shipped to the University of Kansas Medical Center for *CD74* IHC localization ($n = 8$ for each time point except 3 months where $n = 6$). The progression of disease was monitored and endometriosis was confirmed and staged by laparotomy in each animal by consecutive laparoscopies and video recording at each of the time points following inoculation.

RNA Isolation and qRT-PCR Analysis

The qRT-PCR was performed as described previously.^{14,18} Briefly, total RNA was isolated using Tri-Reagent (Sigma Chemical Co, St Louis, Missouri) according to recommendations of the manufacturer. Total RNA ($1\text{ }\mu\text{g}$ in $20\text{ }\mu\text{L}$) was

reverse transcribed using reverse transcription kits (Applied Biosystems, Foster City, California) following the manufacturer's protocol. Primers for *CD74* isoforms a (*CD74a*), b (*CD74b*), and c (*CD74c*) were designed using Primer-Blast and synthesized by Integrated DNA Technology (Coralville, Iowa). Sequences for the human *CD74a* (NM_001025159) were forward: 5'-ACGCTCCACCGAAAGTACTGA-3' and reverse: 5'-GGTGGACAGCAGGGATGTG-3'; *CD74b* (NM_004355) forward: 5'-GAGACACCTTAAGAACAC-CATGGA-3' and reverse: 5'-AACAGGAGCCAATGGTG-CAT-3'; *CD74c* (NM_001025158) forward: 5'-ATCAGAA GCCAGTCATGGATGA-3' and reverse: 5'-AGGATG-GAAAAGCCTGTGTACAG-3'; *IL-8/CXCL8* (NM_000584) forward: 5'-ACCGGAAGGAACCATCTCAC-3' and reverse: 5'-GGCAAACTGCACCTTCACAC-3'; and *MIF* (NM_002415) forward: 5'-GCGCCTGCGCATCAG-3' and reverse: 5'-CGCGTTCATGTCGTAATAGTTGA-3'. To verify that detected changes in *CD74* transcript expression were not a result of differences in endometriotic epithelial cell content between eutopic and ectopic samples, we analyzed expression of the luminal and glandular epithelial marker cytokeratin 18 (*KRT18* variant 1) by qRT-PCR using the following primers: *KRT18v1* (NM_000224): forward, 5'-GAGGGCTCAGATCTTCGCAA-3' and reverse, 5'-AGCCCATGGATGTCGTCTC-3'. Resulting material was then used for independent qRT-PCR which was carried out on an Applied Biosystems HT7900 sequence detector. To account for differences in starting material, human 18S primers (ThermoFisher, Carlsbad, California) were used, and values were expressed as fold change as indicated. All samples were run in triplicate, and the average value was used in subsequent calculations. The $2^{\Delta\Delta\text{CT}}$ method was used to calculate the fold-change values among samples as described previously by our group.^{14,18} All data are displayed as the mean $\pm$ standard error of the mean (SEM) normalized to 18S expression.

Immunohistochemistry

The IHC analysis was performed on tissue fixed with 10% neutral-buffered formalin following the protocol supplied by Vector Laboratories Inc (Burlingame, California). Briefly, endometrial and endometriotic lesion tissue specimens from both species were fixed in 10% neutral-buffered formalin and embedded in paraffin wax ($5\text{ }\mu\text{m}$ sections) followed by deparaffinization with xylene and rehydration with a graded series of ethanol. Antigen retrieval was conducted using antigen-unmasking solution (H-3300; Vector Laboratories Inc). Sections were incubated in 3% H_2O_2 in water for 6 minutes for quenching of endogenous peroxidase activity. For human tissue, *CD74* IHC localization was conducted using a mouse monoclonal antibody (ab9514; Abcam) at a 1:25 dilution, while baboon IHC localization was conducted using a rabbit monoclonal antibody (Abcam, Cambridge, Massachusetts; ab64772) at a 1:250 dilution following the VECTASTAIN elite ABC kit protocols for mouse (PK6102; Vector Laboratories Inc) and rabbit (PK6101; Vector Laboratories Inc),

respectively. Slides were then stained with 3,3'-Diaminobenzidine substrate (SK-4100; Vector Laboratories Inc) and briefly counterstained with hematoxylin. Positive IHC reactions were defined as dark brown reaction cytoplasmic staining for CD74 and level of expression was assessed using the semiquantitative H-score index using the formula: $H - \text{Score} = \text{level of signal} \times \text{percentage of cells expressing signal at that level}$, with levels of 0 representing no staining, 1 weak staining, 2 moderate staining, and 3 strong staining using an internal, independent standard reference for each level of staining.

Western Blot Analysis

Total protein was extracted from cell culture samples using Radioimmunoprecipitation assay (RIPA) buffer (1X RIPA, catalog #9806, Cell Signaling Technologies, Danvers, Massachusetts). Protein concentration in each sample was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, California). The same amount of protein (30 μg) was subjected to 12% bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (w/v) gel electrophoresis and electroblotted onto nitrocellulose membranes (GE Healthcare, Pittsburgh, Pennsylvania). Rabbit anti-CD74 (1:250; Abcam Inc, Cambridge, Massachusetts) and goat anti-rabbit secondary antibody (1:5000; GE Healthcare) were used. Stripping and reprobing for β -actin (ab8227; Abcam; 1:10 000 dilution) were conducted to normalize CD74 protein expression levels. Immunodetection was carried out using an enhanced chemiluminescence kit (Thermo Scientific, Waltham, Massachusetts).

Cell Culture and Transfections

The human endometriotic epithelial cell line, 12Z (originally described by Zeitvogel et al²⁵), was provided by Dr Joe Arosh, Texas A&M University.²⁶ Cells were separately cultured in phenol red-free Dulbecco minimum essential medium (DMEM)/Ham F12 (Fisher Scientific, Pittsburgh, Pennsylvania) + 10% charcoal-stripped fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, Georgia) + Pen-Strep (Life Technologies, Carlsbad, California) in T75 flasks and seeded at 1×10^6 cells/mL of media until approximately 90% confluency. Cells were then passed and plated in 6- or 24-well plates at a density of 1×10^5 cells/mL in DMEM/Ham F12 media lacking FBS and Pen-Strep. The next day, cells were transfected as described below for each specific experiment.

To assess the impact of CD74 on cell proliferation, 12Z cells were reverse transfected with 50 nmol/L of CD74 small-interfering RNA (siRNA). Briefly, 12Z cells were cultured in phenol red-free DMEM:F12 supplemented with 10% charcoal-stripped FBS, penicillin, and streptomycin. Cells were switched to media lacking antibiotics and FBS at 50% confluency. Reverse transfection was accomplished using siPORT NeoFX transfection agent according to recommendations of the manufacturer (Applied Biosystems) using siRNAs specific for human CD74 transcripts (Dharmacon RNAi Technologies, Lafayette, Colorado) or control, nontargeting sequences

(Dharmacon). Twenty-four to forty-eight hours after transfection, CD74 levels were assessed by Western blotting.

Statistical Analysis

Data were analyzed using GraphPad InStat 3 (GraphPad Software, Inc, La Jolla, California). All data from human eutopic endometrium and endometriotic lesion samples were expressed as fold change from matched eutopic endometrial controls (ectopic/eutopic). Matched eutopic control values were expressed as the mean $\pm$ SEM, which was determined by averaging baseline expression of each eutopic endometrial sample. Once the mean values were calculated for eutopic and ectopic endpoints, data were then subjected to normality tests, and those which were not considered normally distributed were analyzed using nonparametric tests. Specific data analysis methods are provided in each figure legend for lesion and eutopic endometrium (unpaired *t* test or Mann-Whitney *U* test). When an *F* test indicated statistical significance, post hoc analysis was made using the Tukey honest significant difference procedure. For all statistical analysis, significance was set at $P < .05$ for all comparisons.

Results

Expression of CD74 was not affected by stage of menstrual cycle, stage of endometriosis (I/II vs III/IV), or type of lesion ($P > .05$; data not shown). As such, we pooled data across stages of menstrual cycle, disease severity, and type of lesion and analyzed the data as eutopic versus ectopic lesions. CD74 transcript expression was significantly higher for each transcript, CD74a, CD74b, and CD74c, in endometriotic lesion tissue compared to paired eutopic endometrium (Figure 1A-C, respectively). The increased levels of CD74 transcript expression (a, b, or c variant) in lesion compared to eutopic endometrium were not attributed to an enrichment in epithelial cells in lesion tissue, as there were no significant differences in expression levels of the endometrial epithelial marker *KRT18v1* between tissue types (eutopic = 0.99 ± 0.026 ; ectopic = 1.37 ± 0.28 ; fold change from eutopic; $P > .05$).

We next evaluated CD74 protein localization by immunohistochemistry. Localization studies revealed that CD74 was not expressed in eutopic endometrium from women without visible signs or symptoms of endometriosis in either the proliferative or the secretory stage of the menstrual cycle (Figure 2). In contrast, CD74 was expressed in eutopic endometrium from women with endometriosis, but this expression was not influenced by the stage of the menstrual cycle (Figure 2). Localization was predominantly in glandular epithelium with a graded expression pattern from most abundant in the basalis layer with expression decreasing to undetectable levels in the functionalis layer (Figure 2). In corresponding, paired endometriotic (ectopic) lesion tissue, expression was again predominantly detected in the glandular epithelial cells, and this expression was also not influenced by the stage of the menstrual cycle (Figure 2).

Figure 1. CD74 mRNA is increased in human endometriotic lesion tissue compared to matched eutopic endometrium. Matched endometriotic lesion and corresponding eutopic endometrial tissue was processed for RNA isolation, and CD74a (A), CD74b (B), and CD74c (C) mRNA variant expression was determined by qRT-PCR as described in “Materials and Methods” section. All data are displayed as the mean $\pm$ standard error of the mean (SEM), and *P* values are indicated for each assessment for eutopic endometrial samples (*N* = 33) and corresponding endometriotic lesion samples (*N* = 46) for CD74a, CD74b, and CD74c mRNA. Data did not pass normality testing and were, therefore, analyzed using the nonparametric Mann-Whitney *U* test. ***P* < .001 compared to matched eutopic endometrium. qRT-PCR indicates quantitative real-time polymerase chain reaction; mRNA, messenger RNA.

Semiquantitative assessment using the H-SCORE system revealed that the level of expression for CD74 protein was significantly greater in eutopic endometrial glands compared to stromal cells in both stages of the menstrual cycle (Figure 2) and that a similar pattern (epithelial $\gg$ stroma) was also detected in ectopic lesion tissue (Figure 2). Comparison in the level of CD74 expression between eutopic and ectopic endometrial epithelial revealed that the level of lesion expression was significantly greater and that this increase was similar between stages of the menstrual cycle (Figure 2). Finally, stromal expression of CD74 was absent in eutopic endometrium of both controls and patients with endometriosis having minimal expression in the endometriotic lesion tissue during both stages of the menstrual cycle.

To evaluate whether in vivo CD74 expression was associated with potential functionality of CD74, we evaluated *IL-8* expression in the same lesion samples in which we assessed CD74 expression. As depicted in Figure 3, ectopic endometrial lesion tissue expressed approximately 16-fold higher levels of *IL-8* transcript expression (*P* < .001) compared to paired eutopic tissue.

To examine whether this pattern of expression in the eutopic endometrium results from the presence of ectopic lesions (endometriosis), we utilized the well-characterized baboon model for the disease. As depicted in Figure 4, much like that in patients without endometriosis, CD74 expression was undetectable in eutopic endometrium from baboons prior to endometriosis induction (month 0). Low and sporadic, glandular expression of CD74 was detected as early as 6 months postinduction (Figure 4, green arrow). At 15 months postinduction, CD74 was strongly expressed in the glandular epithelium within the basalis layer with decreasing expression toward the functionalis layer. In ectopic lesions, CD74 expression was again predominant in the glandular

epithelium, similar to that in human lesion tissue (Figure 2). Unfortunately, due to limitations within the experimental design, we could not assess whether CD74 was expressed in the basalis layer prior to the 15-month time point, which was the terminal time point in our studies.

We next wished to determine the functional significance of CD74 expression using an in vitro cell culture model. As CD74 was predominantly expressed by endometriotic epithelial cells (Figures 2 and 4), we utilized the well-characterized endometriotic epithelial cell line, 12Z,^{25,26} as our experimental model. CD74 transcript variants and protein (assessed by Western blots) were expressed by 12Z cells (data not shown). We assessed cell proliferation, which we had previously demonstrated to be modulated by MIF in an epithelial-like cell line,¹⁴ as well as *IL-8* expression¹⁰ which has been proposed as a mediator for endometriotic cell survival²⁷ via MIF–CD74 interactions.¹⁰ As depicted in Figure 5, transfection of 12Z cells with CD74 short hairpin RNA (shRNA) reduced CD74 protein expression at 24 and 48 hours compared to nontargeting shRNA transfected cells (Figure 5A). Associated with this decrease in CD74 protein expression was a significant reduction in 5-bromo-2'-deoxyuridine incorporation at 48 hours posttransfection (Figure 5B). Similarly, knockdown of CD74 expression in 12Z cells was associated with a significant reduction in *IL-8* transcript expression compared to nontargeting-transfected control cells at 24 and 48 hours posttransfection (Figure 5C).

Discussion

We¹⁸ and others^{10,19,20} have demonstrated that MIF may be a viable therapeutic target for endometriosis treatment based upon in vivo experimental animal studies. Despite these initial studies, a thorough description of the in vivo expression of MIF

Figure 2. Localization of CD74 in eutopic and ectopic endometrial tissue from women with endometriosis and in eutopic endometrium from women without the disease. Immunohistochemical localization for CD74 was performed as described in “Materials and Methods” section. Eutopic endometrium was obtained from women without endometriosis (control) during the proliferative (N = 9) or secretory (N = 9) stage of the menstrual cycle, while both eutopic and matched ectopic endometriotic lesion tissues (ectopic) were obtained from women with endometriosis during the proliferative (N = 12) and secretory (N = 12) stages of the menstrual cycle. Black arrows indicate lack of staining within endometrial glands, yellow arrows indicate punctate staining of epithelial cells within glands, and red arrows indicate uniform, strong staining of epithelial cells within glands. White scale bar represents 100 μ m. Top panel depicts representative IHC staining and the bottom panel depicts semiquantitative assessment of CD74 staining using the H-score system with data being displayed as the mean $\pm$ SEM. Asterisks (* P < .05; ** P < .01) indicate statistically significant different means between stroma and glands CD74 expression within tissue type, while “#” and “+” indicate statistically significant different means (P < .05) in gland CD74 level of expression among the 3 types of tissues (control eutopic, endometriosis eutopic, and endometriosis ectopic). C-eutopic indicates eutopic endometrium from control (nonendometriosis) patients; E-eutopic indicates eutopic endometrium from patients with endometriosis; and E-ectopic indicates matched ectopic/endometriotic lesion tissue from women with endometriosis. IHC indicates immunohistochemical; M, myometrium.

receptors has been lacking. We began our assessment with CD74, which was first described as the putative MIF receptor²¹ that forms homodimers with the ligand to induce cell signaling as well as heterodimers with CD44^{28,29} and possibly CXCR2 and CXCR4.^{22,23} We first assessed CD74 transcript expression (isoforms a, b, and c) and found that compared to matched eutopic tissue, endometriotic lesion CD74 expression was

significantly greater. This observation is in agreement with a recent report by Mahdian and colleagues³⁰ who found that CD74 (isoform that was evaluated was not specified) transcript expression was greater in ovarian endometriomas compared to matched eutopic endometrial samples. Although this group was also able to detect differences between the proliferative and secretory stages of the cycle, we did not observe

Figure 3. *IL-8* mRNA is increased in human endometriotic lesion tissue compared to matched eutopic endometrium. Matched endometriotic lesion and corresponding eutopic endometrial tissue were processed for RNA isolation, and *IL-8* mRNA expression was determined by qRT-PCR as described in “Materials and Methods” section. All data are displayed as the mean $\pm$ standard error of the mean (SEM), and *P* values are indicated for eutopic endometrial samples (*N* = 33) and corresponding endometriotic lesion samples (*N* = 46). Data did not pass normality testing and were, therefore, analyzed using the non-parametric Mann-Whitney *U* test. * *P* < .001. qRT-PCR indicates quantitative real-time polymerase chain reaction; *IL-8*, interleukin 8; mRNA, messenger RNA.

stage-specific differences in either eutopic or ectopic endometrium from women with endometriosis. Differences between studies may be attributed to the fact that our sample population was composed primarily of peritoneal lesions compared to the study by Mahdian and colleagues³⁰ in which the study group consisted of ovarian endometriomas. Second, the majority of our samples were obtained during the secretory stage, while three-quarters of the population in their study was composed of patients in the proliferative stage of their menstrual cycle.

In addition to *CD74* transcript expression, we also assessed *CD74* protein localization in eutopic endometrium from women without visible signs of endometriosis in comparison to eutopic and ectopic endometrium from women with endometriosis. Eutopic endometrium from women without signs of endometriosis (controls) lacked *CD74* protein expression. In contrast, *CD74* protein was detected in both eutopic and ectopic lesions from women with endometriosis. Cellular localization of *CD74* was endometrial epithelial cell specific if not predominant, with stromal cells showing little to no *CD74* expression. Further, the intensity of staining in the eutopic endometrium was greater in glands of the basalis region with the level of glandular expression decreasing in the functionalis layer.

Valentijn and colleagues³¹ proposed that the basalis contributes to ectopic lesion formation in endometriosis following retrograde menstruation. In their study, glandular expression of β -catenin was evident in glands of the basalis layer, suggestive of an active Wnt pathway. The MIF/MIF-*CD74* signaling has been reported to increase β -catenin signaling in neural stem/progenitor cells and promote cell survival.^{32,33} Further, Xia and colleagues³⁴ recently demonstrated that the regenerative

functions of MIF are mediated through *CD74*-dependent signaling in mesenchymal stem cells. Thus, it may be plausible that *CD74* expression in glandular epithelium within the basalis compartment plays a similar role in endometrial regeneration, cell survival, and/or contribution to the development of ectopic lesions/endometriosis.

In the current study, we were unable to verify stromal cell expression of *CD74* in vivo or in vitro. This is in stark contrast to previous reports which have demonstrated its expression in vivo⁹ and in vitro.^{9,10} Differences in stromal expression patterns between our studies and those by Carli et al⁹ and Veilat et al¹⁰ may be related to differences in antibodies employed, method of immunohistochemistry, and/or patient population characteristics. Localization studies have reported that *CD74* is expressed within the epithelial compartment of the colon,³⁵ lung,³⁶ and stomach,³⁷ where it has been proposed to function as a cell survival, cell repair mediator, and mucosal immunity, respectively. Stromal localization has been limited to localization within breast carcinoma.³⁸

To begin to decipher why and how *CD74* was expressed in both eutopic and ectopic endometrial tissues, we utilized the well-characterized baboon model of endometriosis.²⁴ We evaluated *CD74* protein localization in eutopic endometrium prior to induction of endometriosis and found, similar to that in eutopic endometrium from women without endometriosis symptoms, no *CD74* expression by IHC localization. *CD74* expression became robust 15 months post endometriosis induction in both ectopic and eutopic endometrial tissues localizing primarily to glandular epithelium in both tissue types. Remarkably, the pattern of eutopic endometrial *CD74* expression (basalis greater than functionalis) as well as that in ectopic tissue observed in women with endometriosis was recapitulated in baboons with induced disease. These observations suggest 2 important points with respect to *CD74* expression. First, data obtained using the baboon data would indicate that *CD74* expression in ectopic lesion tissue is a result of the disease and not due to inherent *CD74* expression in eutopic endometrium which may become ectopic (via reverse menstruation). Second, the presence of the ectopic lesion tissue appears to modulate eutopic (especially basalis expression) *CD74* expression. Unfortunately, our experimental design did not allow us to sample ectopic lesion tissue at various time points after induction to determine the temporal pattern of *CD74* expression nor did it allow for procurement of endometrial sampling to evaluate basalis expression of *CD74* at the earlier time points after induction of disease.

Our demonstration that in vitro knockdown of *CD74* resulted in reduced cell survival and *IL-8* expression is in agreement with that of others. The MIF has been shown to stimulate *IL-8* expression via *CD74* in epithelial cell lines,³⁹ and *IL-8* is known to stimulate endometriotic cell survival in vitro.²⁷ Much like *CD74*, IHC localization of *IL-8* has demonstrated its expression in both stromal and epithelial cells of eutopic and ectopic tissues, with glandular epithelium expression being predominant.⁴⁰ In endometriotic stromal cells,

Figure 4. Localization of CD74 in eutopic endometrial tissue from baboons with experimentally induced endometriosis. Immunohistochemical localization for CD74 was performed as described in “Materials and Methods” section. Eutopic endometrial specimens were obtained from baboons prior to induction of endometriosis (month 0) and then again at 3, 6, and 9 months after induction of disease by endometriectomy. At 15 months, baboons were necropsied, and eutopic endometrium obtained. Data are representative for N = 8 baboons. Black arrows indicate lack of minimal staining within endometrial glands, yellow areas indicate punctuate staining of epithelial cells within glands, and red arrows indicate uniform, strong staining of epithelial cells within glands. Green arrows indicate signal in the endothelium of blood vessels. White scale bar represents 100 μ m. The level of CD74 expression detected by immunohistochemistry from all samples was quantified using the semi-quantitative H-SCORE system as described in “Materials and Methods” section. Asterisk (* $P < .05$) indicates statistical significance among means between only the 15-month eutopic and ectopic (endometriosis) endometrium. Data are displayed as the mean $\pm$ standard error of the mean (SEM).

knockdown of CD74 expression was associated with a reduced ability of MIF to stimulate the prosurvival factor, IL-8.¹⁰ In the current study using 12Z cells, reduction in CD74 expression was associated with significantly lower levels of IL-8 transcript expression. These observations coupled with our observed reduction in cell proliferation in cells transfected with CD74 siRNA lead us to conclude that CD74 plays a functional role in events conducive to endometriotic epithelial 12Z cell survival, and we postulate a similar role in vivo. In addition to MIF, we are aware that endometrial epithelial IL-8 expression has been demonstrated to be regulated by several factors produced by

endometrial epithelial cells, including prostaglandin E2⁴¹, IL-1 β ,⁴² and leptin,⁴³ all of which induce IL-8 expression independent of CD74 expression.

In summary, we demonstrate that CD74 transcript and protein are elevated in endometriotic lesion tissue and that this expression is predominantly localized to endometriotic epithelial cells. Further, eutopic endometrium from women with endometriosis expresses CD74 protein that localizes to glandular epithelium with glands of the basalis expressing robust levels. These patterns of expression were recapitulated in an experimental baboon model of endometriosis, which suggests that inherent alterations in eutopic endometrium may not contribute to disease

Figure 5. Suppression of CD74 expression decreases endometriotic epithelial cell survival and *IL-8* mRNA expression in vitro. Endometriotic epithelial 12Z cells were cultured and transfected with siRNA to CD74 or a nontargeting (NT) sequence as described in “Materials and Methods” section. A portion of these cells were subjected to protein isolation and subsequent Western blot analysis to verify knockdown of CD74 protein (A). Additionally, transfected cells were subjected to BrdU incorporations as outlined in “Materials and Methods” section to assess cell proliferation. Reduction in cell survival and knockdown of CD74 expression was also associated with reduction in transcript levels of the endometriotic prosurvival cytokine, *IL-8* (C). Data are displayed as the mean $\pm$ standard error of the mean (SEM) and are representative of 3 separate experiments (N = 3). * $P < .05$ compared to respective NT siRNA within each time point. IL-8 indicates interleukin 8; mRNA, messenger RNA; siRNA, small-interfering RNA.

progression. Finally, disruption in CD74 expression in vitro was associated with reduced cellular proliferation and IL-8 expression. Taken together, our results suggest that elevated levels of receptor (CD74) and ligand (MIF) may contribute to the pathophysiology of endometriosis and may represent a novel, nonhormonal therapeutic target for endometriosis treatment.

Declaration of Conflicting Interests

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