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Tumor Necrosis Factor-α Regulates Inflammatory and Mesenchymal Responses via Mitogen-Activated Protein Kinase Kinase, p38, and Nuclear Factor κB in Human Endometriotic Epithelial Cells

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ABSTRACT

Tumor necrosis factor (TNF)-α is central to the endometriotic disease process. TNF-α receptor signaling regulates epithelial cell secretion of inflammation and invasion mediators. Because epithelial cells are a disease-inducing component of the endometriotic lesion, we explored the response of 12Z immortalized human epithelial endometriotic cells to TNF-α. This report reveals the impact of disruption of established TNF-α-induced signaling cascades on the expression of biomarkers of inflammation and epithelial-mesenchymal transition (EMT) from endometriotic epithelial cells. Note that we show the molecular potential of soluble TNF-R1 [TNF binding protein (TBP)] and a panel of small molecule kinase inhibitors to block endometriotic gene expression directly. The TNF-α receptor is demonstrated to signal through IκB kinase complex (IKK) 2 $>$ IκB $>$ nuclear factor κB, extracellular signal-regulated kinase $>$ mitogen-activated protein kinase kinase (MEK), p38, and phosphatidylinositol 3-kinase (PI3K) $>$ Akt1/2. TNF-α induces the expression of transcripts for inflammatory mediators interleukin (IL)-6, IL-8, regulated on activation normal T cell expressed and secreted, TNF-α, granulocyte macrophage–colony-stimulating factor (GM-CSF), and monocyte chemoattractant protein (MCP)-1 and also invasion mediators matrix metalloproteinase (MMP)-7, MMP-9, and intracellular adhesion molecule-1. Indeed, TBP inhibits the TNF-α-induced expression of all the above endometriotic genes in 12Z endometriotic epithelial cells. The secretion of IL-6, IL-8, GMCSF, and MCP-1 by TNF-α is blocked by TBP. Interestingly, MEK, p38, and IKK inhibitors block TNF-α-induced IL-8, IL-6, and GM-CSF secretion and 12z invasion, whereas the PI3K inhibitors do not. The only inhibitor to block MCP-1 expression is the p38 inhibitor. Last, TBP, MEK inhibitor, or p38 inhibitor also block cell surface expression of N-cadherin, a marker of mesenchymal cells. Taken together, these results demonstrate that interruption of TNF-α-induced signaling pathways in human endometriotic epithelial cells results in decreased expression and secretion of biomarkers for inflammation, EMT, and disease progression.

Endometriosis is a female disease presenting with painful and persistent lesions within the peritoneum. Retrograde menstruation of the endometrial cells into the peritoneal cavity causes endometriosis in 10% of all women (Sampson, 1927). For the majority of women, the immune response in the peritoneum that follows is sufficient to suppress attachment and transformation of refluxed tissue. In those women that develop the disease, endometrial cells become attached to the mesothelial cell layer lining the peritoneal cavity and initiate a cascade of events that includes localized invasion and transformation of cells within the lesion (Sampson, 1927).

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ABBREVIATIONS: TNF, tumor necrosis factor; IL, interleukin; GM-CSF, granulocyte macrophage–colony-stimulating factor; MCP, monocyte chemoattractant protein; EMT, epithelial-mesenchymal transition; MMP, matrix metalloproteinase; TBP, TNF binding protein; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MEK, mitogen-activated protein kinase kinase; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; PI3K, phosphatidylinositol 3-kinase; IKK, IκB kinase complex; Erk, extracellular signal-regulated kinase; NF, nuclear factor; TBST, Tris-buffered saline/Tween 20; MSD, Meso Scale Discovery; PCR, polymerase chain reaction; C_T, threshold cycle; HPRT, hypoxanthine phosphoribosyltransferase; ANOVA, analysis of variance; BSA, bovine serum albumin; qPCR, quantitative polymerase chain reaction; RANTES, regulated on activation, normal T-cell-expressed and secreted; ICAM, intracellular adhesion molecule; AS602868, 1-(4-{4-[4-(4-chloro-phenyl)-pyrimidin-2-ylamino]-benzoyl}-piperazin-1-yl)-ethanone.

In endometriosis, activated peritoneal leukocytes responding to ectopic menstrual effluent secrete TNF-α, which elicits inflammatory and necrotic gene expression from epithelial cells (Braun et al., 2002; Szyllo et al., 2003). TNF-α mRNA is up-regulated in both the endometrium and peritoneum of women with endometriosis compared with unaffected women (Kyama et al., 2006). It is noteworthy that TNF-α receptors are expressed by endometrial cells in women with endometriosis (Kharfi et al., 2003). Therefore, both TNF-α and its receptor are expressed in the epithelial endometriotic cells.

Experimental evidence supporting a critical regulatory role of inflammation, particularly TNF-α, in endometriosis is strong (Berkkanoglu and Arici, 2003). A targeted approach to controlling TNF-α driven inflammation in endometriosis was achieved in the rat model with administration of TNF-α-soluble receptor (D'Antonio et al., 2000). Two independent results of use of soluble TNF-α receptor in baboon models of endometriosis indicate that therapies targeting the TNF-α mediated inflammatory cascade have the potential to treat endometriosis (Barrier et al., 2004; D'Hooghe et al., 2006). These in vivo efficacy studies in animal models of endometriosis further validate the importance of inhibiting TNF-α receptor signaling in the treatment of endometriosis.

TNF-α mediates inflammation in the surrounding tissue, in part, by inducing the expression of IL-6, IL-8, GM-CSF, and MCP-1. Epithelial cells are a major source of these cytokines in the human endometrium (Giacomini et al., 1995; Fahey et al., 2005; Meter et al., 2005). IL-6 is a chemoattractant for monocytes, whereas IL-8 activates angiogenesis and neutrophil migration and differentiation. GM-CSF stimulates granulocyte and monocyte differentiation from hematopoietic stem cells (Hamilton and Anderson, 2004). MCP-1 mediates both acute and chronic inflammation through recruitment of mast cells, eosinophils, and macrophages to the site of inflammation (Conti and DiGioacchino, 2001). These inflammatory innate immune cells induce inflammatory and epithelial-mesenchymal transition (EMT) responses in surrounding epithelial cells.

Endometriotic epithelial cells have also been shown to express mesenchymal markers (N-cadherin) amid diminishing levels of epithelial markers (E-cadherin), further supporting that a population of endometriotic cells undergo the dedifferentiation process of EMT (Gaetje et al., 1997). Endometriotic lesions express aberrant or elevated levels of matrix metalloproteinases (MMPs) (Osteen et al., 2003). TNF-α induces the expression of MMP-9 from endometrial cells (Curry and Osteen, 2003). Interestingly, active MMP-9 is increased in the eutopic and ectopic endometrium of women with endometriosis compared with normal endometrium (Liu et al., 2002).

Absence of good cellular models for endometriosis has hindered development of therapies that target lesions preferentially over eutopic endometrium. Recently, a set of immortalized endometriotic epithelial cells and stromal cells have been developed and shown to exhibit many characteristics of primary epithelial and stromal endometriotic cells (Zeitvogel et al., 2001). The availability of these cells has enabled investigation of direct pharmacologic responses of isolated endometriotic epithelial and stromal cells. The remarkable change that occurs in epithelial endometriotic cells compared with stromal endometriotic cells suggests that a major contribution to the disease occurs within epithelial cells (Banu et al., 2007b).

We report here the ability of TNF-α binding protein (TBP; STNF-R1) and kinase inhibitors to reverse TNF-α-induced and/or TNF-α-independent effects on 12Z endometriotic epithelial cells. TBP competes with TNF-α receptor for TNF-α binding to the receptor and so is used as a positive control to attribute the direct inhibitory effects on specific signaling pathways within endometriotic epithelial cells. TNF-α is shown to stimulate production of endometriotic biomarkers from 12Z cells similar to those within the peritoneal cavity of women with endometriosis. TBP and novel kinase inhibitors reverse the effects of TNF-α-induced expression of cellular adhesion markers and MMPs that define mesenchymal endometriotic cells. Last, kinase inhibitors also cause reversion of the invasive phenotype. These studies clearly link TNF-α with maintenance of the inflammatory and mesenchymal endometriotic properties. These results could lead to development of drugs for endometriosis that target signaling pathways uniquely modified in ectopic lesions over eutopic endometrium.

Materials and Methods

Cell Culture, Cytokines, and Inhibitors. The simian virus 40 T-antigen-transformed human ectopic endometrial epithelial cell line 12Z was maintained in DMEM/Ham's F12 medium supplemented with 10% fetal calf serum and penicillin-streptomycin at 37°C and 5% CO_2 (Zeitvogel et al., 2001). Cells were passaged at 75% confluence in T-150 culture flasks to a 1:50 dilution. For stimulation experiments, cells (20,000/well) were seeded in 96-well plates; the following day, cells were washed once with PBS, and serum-free media were added. Stimulation with various factors was carried out in serum-free media for 24 h. Culture supernatant was stored at −80°C until used for determination of cytokine levels. For blocking signaling pathways, cells were incubated with inhibitors for 30 min before the addition of TNF-α. Culture media, antibiotics, and serum were obtained from Invitrogen (Carlsbad, CA). TNF-α was purchased from R&D Systems (Minneapolis, MN). The MEK inhibitor PD98059, the p38 inhibitor SB203580, and the PI3K inhibitor Wortmannin were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). The IKK2 inhibitor (AS602868) was synthesized at Serono (Frelin et al., 2003). TBP (Onercept) was produced at Serono and was used at 100 μg/ml (D'Antonio et al., 2000; McKenna et al., 2007).

Antibodies. Phospho-Akt, Erk, IκB, NFκB, p38, and unphosphorylated Erk and NFκB were purchased from Cell Signaling Technology Inc. (Danvers, MA). Akt1/2, IκB-a, p38, and immunofluorescence antibodies for E-cadherin and N-cadherin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-human IgG (γ)-horseradish peroxidase conjugate used as secondary antibody was obtained from Bio-Rad Laboratories (Hercules, CA).

Western Blot. For production of cell lysate, 10^6 treated cells were lysed in 1 ml of radioimmunoprecipitation assay buffer with 250 nM NaOVan with 1/10 of a tablet of Protease Inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN). Protein concentration was determined by BCA assay according to the manufacturer's protocol (Bio-Rad Laboratories). Thirty micrograms of protein was loaded per well of a 10% Tris-Bis gel in an Xcell Sure Lock System (Invitrogen). Gels were run at 120 V until the dye front was at the bottom. Proteins were transferred from gels to Immobilon membrane (Millipore, Billerica, MA) at 25 V for 1.5 h. The membranes were then incubated overnight at 4°C with primary antibody in 5% milk/TBST/0.02% NaAz at the manufacturer's recommended dilution while rocking in a square Petri dish. Membranes were washed in TBST three times for 5 min each and then incubated while rocking slowly at room temperature for 1 h in a 1:5000 dilution of horseradish peroxidase-

conjugated secondary antibody in 10 ml of TBST. The membranes were washed, as above, and were placed on glass and semidried by gentle blotting with a Kimwipe. One milliliter of ECL/Luminol (Santa Cruz Biotechnology, Inc.) was added to the membrane, left for 1 min, and then removed. Membranes were semidried and covered with plastic wrap to prevent complete drying. The plastic covered semidry membranes were exposed to film in the darkroom for 10 s to 1 min and developed. The resultant film was scanned for images.

Quantification of Phosphoproteins and Secreted Cytokines by Electrochemiluminescence. Electrochemiluminescence assays were performed on biological triplicate samples using capture antibody precoated 96-well multispot plates from Meso Scale Discovery (MSD; Gaithersburg, MD). Twenty-five microliters of supernatant or calibrator was added to each well and incubated with shaking for 1 h at room temperature. Specific protein levels were quantitated by adding 25 μl of 1 μg/ml specific detection antibody labeled with MSD SULFO-TAG reagent to each well and incubated with shaking for 1 h at room temperature. The plate was then washed three times with PBS/0.05% Tween 20 and 150 μl of 2× read buffer was added to each well. Plates were immediately read using the SECTOR Imager 6000, and data were quantitated using Discovery Workbench and SOFTmax PRO 4.0 software.

Quantitative Real-Time PCR. RNA was isolated from 12Z cells on a 60-mm cell culture dish using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was treated with DNase I (QIAGEN, Valencia, CA), and purity of RNA was increased using an RNeasy kit (QIAGEN) before cDNA synthesis. One microgram of total RNA was reverse transcribed using oligo(dT) priming and the Superscript III First-strand cDNA synthesis kit (Invitrogen). RT-minus samples served as a control to exclude the possibility that the amplified product was derived from contaminating undigested genomic DNA. cDNA corresponding to 100 ng of input RNA was amplified in duplicate with the TaqMan Universal PCR Master Mix on a custom TaqMan Low Density Array (Applied Biosystems, Foster City CA). Real-time PCR was conducted with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Differential target gene expression was calculated according to the $2^{-\Delta\Delta C_T}$ method using HPRT as an endogenous control (Fleige et al., 2006). Interarray reproducibility was proven by repeated measurements of control cDNA samples on three different arrays. C_T values were approximately normally distributed. Target gene C_T values were normalized to the mean of HPRT values. *P* values were computed by nonparametric one-way ANOVA with a 95% confidence interval and Tukey post-test using SigmaStat software (Systat Software, Inc., San Jose, CA).

Immunofluorescence. After treatment, monolayers of 12Z cells in 96-well plates were washed with PBS, and 30 μl of 10% formalin/PBS was added and incubated for 1 h at room temperature. After incubation, the cells were washed three times in PBS. To permeabilize and block, the cells were treated overnight at 4°C in 0.2% Tween 20/0.1% BSA/PBS. Permeabilization and blocking solution was aspirated, replaced with a 1:100 dilution of primary antibody in 0.2% Tween 20/0.1% BSA/PBS, and incubated overnight at 4°C. Cells were then washed five times in PBS. A 1:1000 dilution of secondary antibody in 0.2% Tween 20/0.1% BSA/PBS was added and incubated for 1 h at room temperature. After incubation, cells were washed, and fluorescence was visualized on a Nikon Eclipse TE 2000-S (Nikon, Tokyo, Japan).

Invasion Assay. Cells were cultured at not greater than 70% confluence. Cells were serum starved in 0% serum media for 5 h. At 2.5 h of serum starvation, the 24-well invasion plate (BD Biosciences, San Jose, CA) was removed from −20°C storage and allowed to come to room temperature for 30 min. Once the plate was warmed, 500 μl of 37°C DMEM was added to each apical chamber. The plate was allowed to rehydrate for 2 h at 37°C in a 5% CO_2 environment. After 5 h of serum starvation, cells were trypsinized, and the cell concentration was adjusted to $4e^4$/500 μl in serum-free media. The DMEM was carefully removed from apical chambers of rehydrated plates without disturbing the layer of BD Matrigel Matrix on the membrane. Media containing chemoattractant (10% serum) or no chemoattractant (no serum) were added to each basal chamber. Membranes were inserted into wells making sure no air bubbles were trapped under the membrane. Five hundred microliters of cell suspension ($4e^4$ cells) was added to the apical chambers. The BD BioCoat Tumor Invasion System was then incubated for 24 h at 37°C, 5% CO_2. After incubation, medium was carefully removed from the apical and basal chambers by inverting the plates and gently dabbing plates on absorbent paper. All noninvading cells were removed from the Matrigel membrane using a cotton swab. The invasive cells on the lower surface were stained using a Diff-Quick kit (Dade Behring, Inc., Deerfield, IL). Five hundred microliters of each of the three Diff-Quick solutions was added in succession to each well, and membranes were incubated for 2 min with each solution, aspirating between each solution. The membranes were then washed two times with water. The number of invasive cells (purple nuclei, pink cytoplasm) in each membrane was counted with a dissecting microscope.

Statistics. Data were analyzed by ANOVA followed by Tukey tests (Systat Software, Inc.). Differences between groups were considered significant when $P < 0.05$.

Results

TNF-α Signaled through the IκB/NFκB, MEK/Erk, p38, and PI3K/Akt Kinase Cascade in Epithelial Endometriotic Cells. In this first set of experiments 12Z cells were validated as a model system to study TNF-α receptor regulation of kinase signaling in endometriotic cells in vitro. The 12Z cell line is immortalized, inherently endometriotic, and invasive (Zeitvogel et al., 2001). To measure the ability of TNF-α to signal through various kinase cascades recombinant TNF-α receptor (TBP) was used to block the binding of endogenous or exogenous TNF-α to the TNF-α receptor. Specific intracellular kinase signaling pathways downstream of the receptor were blocked by the use of small molecule inhibitors. The Western blot in Fig. 1A shows that TNF-α receptor phosphorylates (Ser32/36) and degrades the IκB (NFκB inhibitor), and subsequently phosphorylates NFκB (Ser536), whereas TBP and an IKK2 inhibitor block this change. TNF-α also induces kinase signaling through MEK to Erk (Thr202/Tyr204) and through p38 (Thr180/Thyr 182). Indeed, a MEK inhibitor blocks the TNF-α-induced phosphorylation of MEK target Erk and not p38 phosphorylation (Fig. 1B).

Multiplexed electrochemoluminescent assays were used to further confirm and expand on the TNF-α-induced kinase signaling pathways in 12Z epithelial endometriotic cells seen

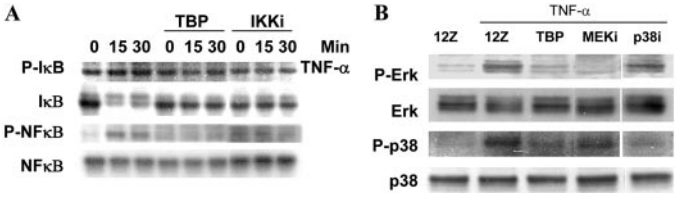

Fig. 1. Pharmacological Inhibition of TNF-α mediated kinase signaling in endometriotic epithelial cells. A, 12Z cells were treated with 15 ng/ml TNF-α for 0, 15, or 30 min with or without 100 μg/ml TBP, 1 μM, or 10 μM IKK2 inhibitor, or treated with carrier control for a 30-min pretreatment. P-IκB (Ser 32/36) and P-NFκB (Ser 536) levels were determined by Western blot analysis, and unphosphorylated forms were used as a loading control. B, 12Z cells were treated with 15 ng/ml TNF-α for 15 min with or without 100 μg/ml TBP, 10 μM PD98059, or 50 μM SB203580 or treated with carrier control for a 30-min pretreatment. P-Erk (Thr202/Tyr204) and P-p38 (Thr180/Tyr182) levels were determined by Western blot analysis, and unphosphorylated forms were used as a loading control. Representative results of at least three experiments are shown.

by Western blot. TBP specifically blocks TNF-α-induced phosphorylation of p38 (Thr421/Tyr182), Akt (Ser473), NFκB (Ser468), and Erk (Thr202/Tyr204 and Tyr185/Thr187) (Fig. 2, A–D). TBP completely inhibits the phosphorylation of NFκB by the peak of activity at 5 min (Fig. 2C). In comparison with TBP, the PI3K inhibitor Wortmannin completely inhibits the TNF-α-induced phosphorylation of Akt, whereas TBP reduces the phosphorylation after the initial burst in P-Akt (Fig. 2B). The MEK inhibitor PD98059 and TBP significantly reduce the phosphorylation of Erk at 15 min after treatment (Fig. 2D). These data demonstrate a direct effect of TNF-α on 12Z endometriotic cells and antagonism of TNF-α receptor signaling in epithelial endometriotic cells by the TNF-α binding protein. Small-molecule kinase inhibitors specifically block their enzymatic targets in 12Z cells. Taken together, these data show that the TNF-α receptor signals through a known array of kinase pathways.

TBP and Kinase Inhibitors Blocked Transcription of Inflammatory Cytokines Involved in Endometriosis. The next aim was to determine whether 12Z cells respond to TNF-α in a manner consistent with its proposed proinflammatory role. In this set of experiments, the effects of TNF-α on inflammatory cytokine mRNA expression were evaluated by qPCR with and without kinase inhibitors or TBP. As shown in Fig. 3, A to F, IL-8, IL-6, MCP-1, GM-CSF, RANTES, and TNF-α mRNA expression was increased 40- to 900-fold upon addition of TNF-α. TBP blocked the induction of all six cytokines, whereas the PI3K inhibitor did not. Expression of IL-6, MCP-1, and GM-CSF mRNA was reduced to basal levels by MEK, p38, and IKK2 inhibitors (Fig. 3, B and D). Both RANTES and TNF-α mRNA were reduced by p38 and IKK2 but not by the MEK inhibitor (Fig. 3, E and F), whereas IL-8 mRNA was reduced by MEK and the IKK2 inhibitor but not by p38 inhibitor (Fig. 3A). Expression of mRNA for ICAM-1 by endometriotic (12Z) was increased by TNF-α (Fig. 3C), consistent with previous observations in primary endometriotic tissues (González-Ramos et al., 2007). Although TBP inhibited expression of TNF-stimulated ICAM-1 mRNA to untreated levels, the IKK2 inhibitor alone was capable of reducing ICAM-1 mRNA. This is consistent with previous studies that demonstrate TNF-α signaling through IKK2 and NFκB regulates ICAM-1 expression and monocyte adhesion (Chen et al., 2001).

TBP Inhibited TNF-α-Induced IL-8, IL-6, GM-CSF, and MCP-1 Secretion. To determine whether the increases in mRNA levels of IL-8, IL-6, GM-CSF, and MCP-1 also result in increased protein secretion, 12Z cells were treated with different doses of TNF-α (0.01–100 ng/ml) for 24 h. Cytokine secretion in the culture supernatant was evaluated by multiplex electrochemiluminescence assays. As shown in Fig. 4, A to D, TNF-α stimulated dose-dependent secretion of IL-6, IL-8, GM-CSF, and MCP-1 in 12Z culture supernatant. To evaluate the ability of TBP to reverse the effects of TNF-α on cytokine production, increasing concentrations of TBP were added to cultures containing 15 ng/ml TNF-α. After 24-h incubation in the presence of 15 ng/ml TNF-α, GM-CSF, IL-6, MCP-1, and IL-8 secreted by 12Z cells were elevated by 200-, 1000-, 900-, and 32-fold over basal, respectively (Fig. 4). Addition of TBP to cell cultures in the absence of added TNF-α did not cause a significant change on cytokine levels. As expected, TBP dose-dependently reduced TNFα-mediated increase in cytokines when added to TNF-α-treated 12Z cultures. At a concentration of 10 μg/ml, TBP reduced the secretion of IL-8, IL-6, and GM-CSF to the baseline, whereas to inhibit MCP-1 production, a 10-fold higher concentration of TBP was required (Fig. 4C). These results confirm that 12Z cells produce very little, if any, TNF-α in culture and that TBP effectively abolishes the direct effect of exogenous TNF-α on endometriotic cells.

Kinase Inhibitors Differentially Affected the Secretion of IL-8, IL-6, GM-CSF, and MCP-1. In an effort to develop orally bioavailable therapies for endometriosis, we have evaluated the effects of known and novel inhibitors of intracellular signaling pathways reported to be involved in TNF-α receptor function. In the experiments reported here,

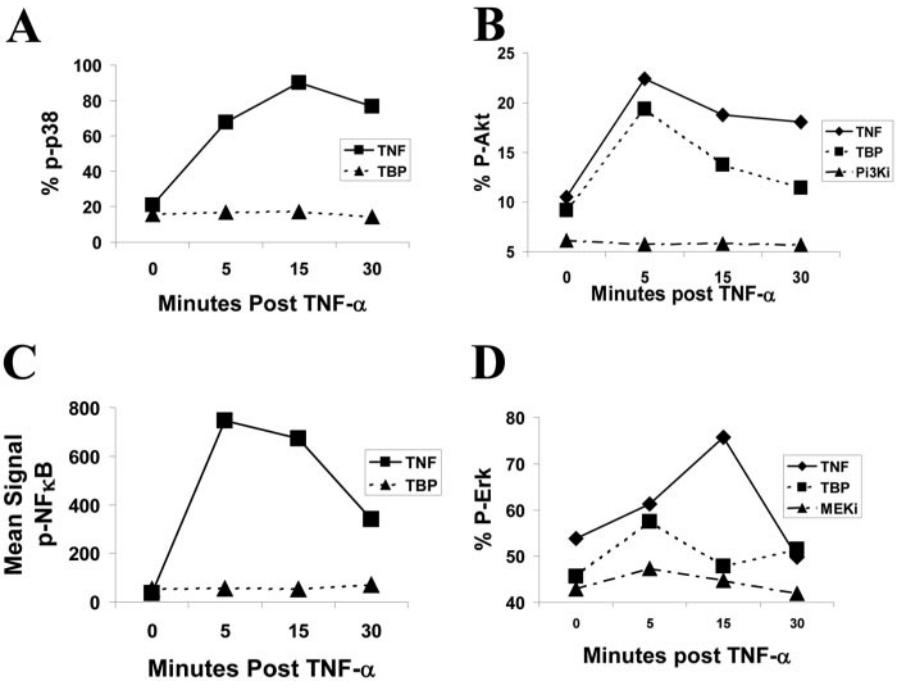

Fig. 2. TBP and kinase inhibitors block TNF-α signaling through multiple kinase pathways. A, 12Z cells were treated with 15 ng/ml TNF-α for 0, 5, 15, or 30 min with or without 100 μg/ml TBP or treated with carrier control for a 30-min pretreatment. P-p38 (Thr180/Tyr182) levels were determined by MSD analysis, and unphosphorylated forms were used as a control for percent phosphorylated determination. B, 12Z cells were treated with 15 ng/ml TNF-α for 0, 5, 15, or 30 min with or without 100 μg/ml TBP, 1 μM Wortmannin, or treated with carrier control for a 30-min pretreatment. P-Akt (Ser473) levels were determined by MSD analysis, and unphosphorylated forms were used as a control for percent phosphorylated determination. C, 12Z cells were treated with 15 ng/ml TNF-α for 0, 5, 15, or 30 min with or without 100 μg/ml TBP or treated with carrier control for a 30-min pretreatment. P-NFκB (Ser468) mean signal levels were determined by MSD analysis. D, 12Z cells were treated with 15 ng/ml TNF-α for 0, 5, 15, or 30 min with or without 100 μg/ml TBP or 10 μM PD98059 or treated with carrier control for a 30-min pretreatment. P-Erk (Thr202/Tyr204) levels were determined by MSD analysis, and unphosphorylated forms were used as a control for percent phosphorylated determination. Representative results of at least three experiments are shown.

we present the effects of kinase inhibitors on cytokine protein expression. As shown in Fig. 5, A to D, TNF-α stimulation increased IL-6, IL-8, GM-CSF, and MCP-1 secretion, and kinase inhibitors tested had specific effects on each of the cytokines. PI3K inhibitor did not block secretion of any of the cytokines. MEK, p38, and IKK2 inhibitors blocked GM-CSF and IL-6 secretion from endometriotic epithelial cells (Fig. 5, A and B), and only MEK and p38 inhibitors blocked IL-8 and MCP-1, respectively. These results suggest that the IKK, MEK, and p38 pathways and not the PI3K pathway regulate TNF-α-stimulated GM-CSF, IL-6, IL-8, and MCP-1 secretion in endometriotic epithelial cells. Interestingly, the TNF-α-induced levels of IL-8 or MCP-1 protein were not affected by p38 and IKK2 inhibitor or by MEK and IKK2 inhibitor, respectively, although the RNA levels of these cytokines were reduced by these inhibitors. The RNA levels were not induced in the presence of inhibitor and TNF-α, but the protein levels were. It is possible that protein degradation or translation rates are not blocked, but the RNA degradation or transcription rates are affected.

TBP and Kinase Inhibitors Blocked Transcription of Extracellular Matrix Remodeling Mediators Involved in Endometriosis. To validate the direct effects of TNF-α and inhibitors on expression of genes involved in EMT of endometriotic cells, 12Z cells were exposed to TNF-α in the presence or absence of TBP. TNF-α caused a dramatic increase in expression of MMP-7 and MMP-9 mRNA. Levels of MMP-7 and MMP-9 mRNA were reduced 6- and 300-fold, respectively, in the presence of TBP as measured by qPCR (Fig. 6A). MMP-7 mRNA levels were not significantly affected by the panel of kinase inhibitors, whereas MMP-9 mRNA levels were reduced by MEK, P38, and IKK2 but not PI3K inhibitor. Indeed, the expression of MMPs is indicative of the EMT process. These results validate that 12Z cells serve as an appropriate in vitro model to evaluate effects of inflammatory cytokines and their inhibitors on invasive and inflammatory gene expression in endometriotic cells.

Inhibition of MEK, p38, or IKK Reduced Invasion of Human Endometriotic Epithelial Cells (12Z). 12Z cells are inherently invasive as endometriotic epithelial cells

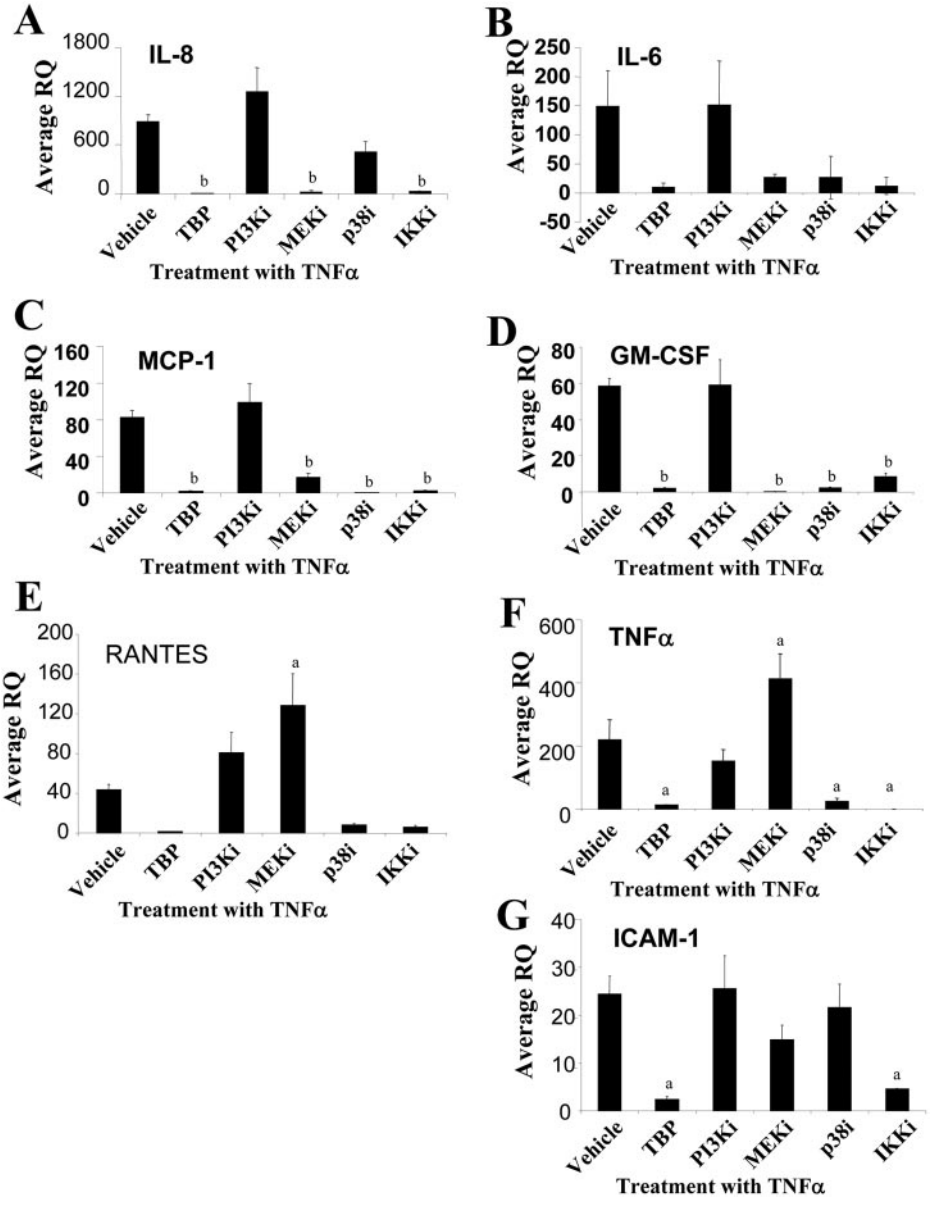

Fig. 3. TBP blocks expression of TNF-α-induced transcripts involved in inflammation and invasion. Total RNA was isolated from serum-starved 12Z cells or from cells after the addition of 100 μg/ml TBP, 1 μM Wortmannin, 10 μM PD98059, 50 μM SB203580, 1 μM IKK2 inhibitor, and 15 ng/ml TNFα or vehicle for 24 h. Inflammation or invasion associated transcript levels were determined by low-density array qPCR using HPRT as the endogenous control/reference. All treated groups were normalized to the untreated 12Z/calibrator. Data are the mean of three replicate. S.D. is shown. A, relative quantity of IL-8 transcript is shown for all groups. B, relative quantity of IL-6 transcript is shown for all groups. C, relative quantity of MCP-1 transcript is shown for all groups. D, relative quantity of GM-CSF transcript is shown for all groups. E, relative quantity of RANTES transcript is shown for all groups. F, relative quantity of TNF-α transcript is shown for all groups. G, relative quantity of ICAM-1 transcript is shown for all groups. Representative results of at least three experiments are shown. a, $P < 0.05$; b, $P < 0.01$; compared with TNF-α-treated cells, ANOVA followed by Tukey test.

(Zeitvogel et al., 2001). Addition of TNF-α alone (serum-free conditions) was unable to increase the inherent invasive activity of 12Z cells. Addition of TNF-α moderately increased serum-induced invasion, although neither the effect of TNF-α nor the addition of TBP significantly affected 12Z invasion. However, TNF-α- and serum-induced invasion was significantly reduced by inhibiting MEK, p38, and IKK signaling, but not by inhibiting PI3K signaling (Fig. 7). PI3K is involved in TNF-α-induced cell survival, whereas the IKK > IκB > NFκB, p38, and ERK > MEK pathways induce invasiveness of cells. Although TNF- α did not increase the number of invasive epithelial cells, it was considered that the invasive potential of 12Z cells that express elevated levels of MMP-2 and MMP-9 could be dependent on changes in cell-cell adhesion that are not observed in invasion assays. It was considered that TNF-α might be responsible for reducing cell-cell adhesion of 12Z cells and endometriotic cells by modifying N-cadherin expression.

TBP, MEK Inhibitor, and p38 Inhibitor Blocked TNF-α-Induced N-Cadherin Expression. A shift from expression of E-cadherin to N-cadherin is a proven marker for the cellular shift from epithelial to mesenchymal phenotype. Previously, 12Z cells were characterized to express N-cadherin over E-cadherin. To investigate whether TNF-α receptor signaling altered their cellular phenotype, 12Z cells were immunostained for N-cadherin, E-cadherin, and cytokeratin after treatment with TNF-α with and without TBP or kinase inhibitors. Nuclear staining with Hoechst was used to monitor changes in cell numbers. E-cadherin was not detected in 12Z culture but was detected in cell line controls (data not shown). As demonstrated earlier, 12Z cells normally express

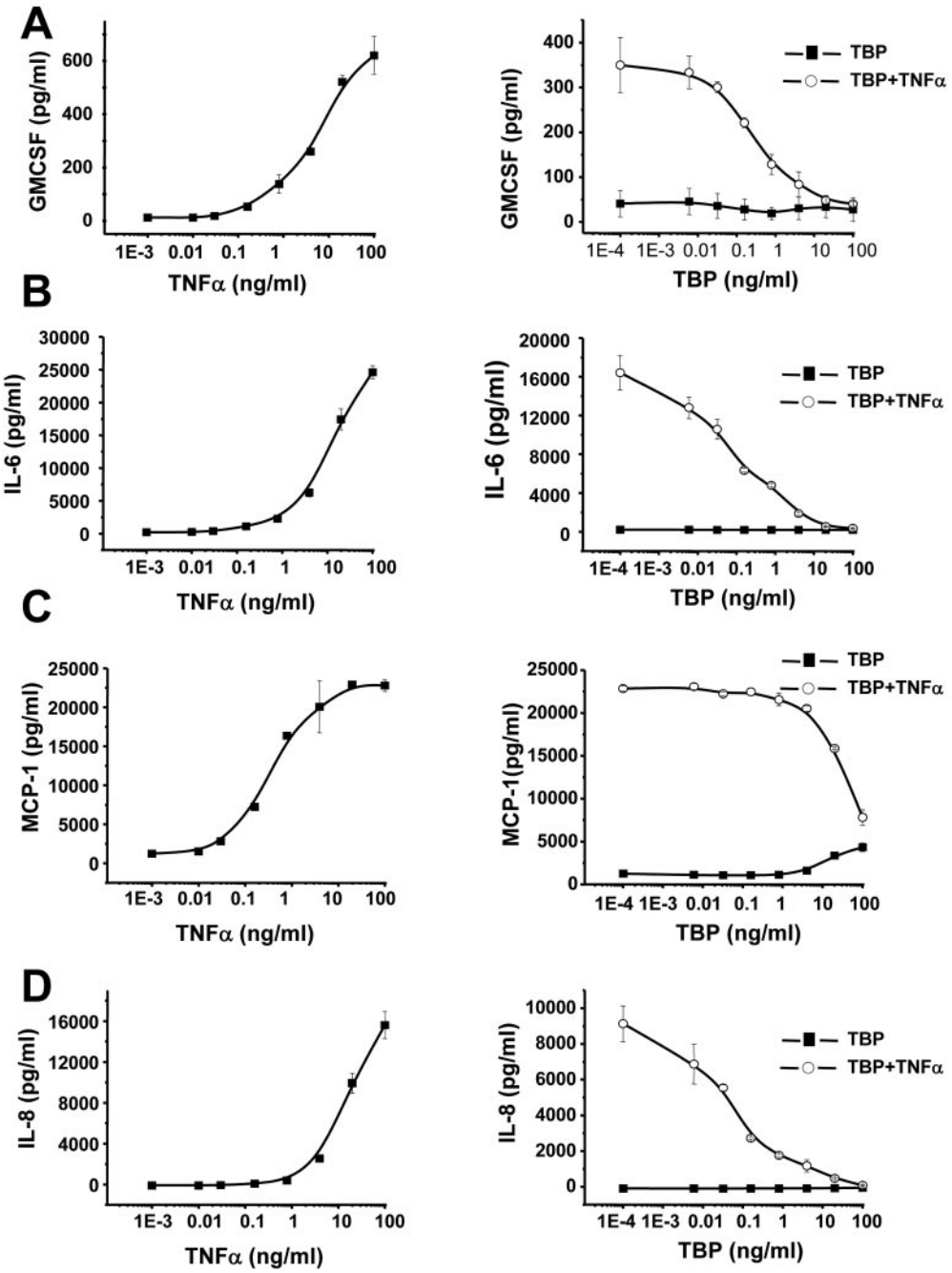

Fig. 4. TBP blocks TNF-α-induced inflammatory cyto-/chemokine secretion. 12Z cells were treated with various concentrations, 100 μg/ml TBP or TNF-α or TBP in the presence of 15 ng/ml TNF-α, for 24 h. Cyto-/chemokines were measured in the supernatant using the MSD multiplex assay as described under *Materials and Methods*. A, TNF-α dose response-induced GM-CSF kinetics graph and TBP blocking TNF-α dose response-induced GM-CSF kinetics graph are shown. B, TNF-α dose response-induced IL-6 kinetics and TBP blocking TNF-α dose response-induced IL-6 kinetics graph are shown. C, TNF-α dose response-induced MCP-1 kinetics and TBP blocking TNF-α dose response-induced MCP-1 kinetics graph are shown. D, TNF-α dose response-induced IL-8 kinetics and TBP blocking TNF-α dose response-induced IL-8 kinetics graph are shown. Data are the mean of three replicates. S.D. is shown.

low levels of N-cadherin at cellular junctions and in the cytoplasm (Fig. 8A). 12Z cells express significantly higher levels of N-cadherin at cellular junctions and in the cytoplasm after 24 h of treatment with TNF-α (Fig. 8B). Addition of 100 μg/ml TBP with TNF-α (15 ng/ml) significantly reduced N-cadherin expression (Fig. 8C). Interestingly, exogenous MEK or p38 inhibitor significantly blocked TNF-α-induced N-cadherin expression (Fig. 8, D and E), whereas PI3K and IKK2 inhibitors did not reduce N-cadherin staining (data not shown). E-cadherin expression was still undetectable with any treatment (data not shown). Taken together, these data indicate that TNF-α promotes invasive potential of endometriotic cells, whereas treatment of endometriotic epithelial cells with TBP, MEK inhibitor, or p38 inhibitor lowers the expression of mesenchymal markers of the endometriotic phenotype and reverts the cells to an expected phenotype for normal endometrial epithelial cells.

Discussion

The findings presented here indicate that MEK > Erk, p38, and IKK2 > IκB > NFκB phosphorylation of downstream targets of TNF-α receptor results in the regulation of inflammation and invasion mediators independent of PI3K > AKT. Indeed, microarray analysis of eutopic endometrium identified up-regulation of several genes in two important signaling pathways: RAS/RAF/mitogen-activated protein kinase and PI3K in patients with endometriosis versus controls (Matsuzaki et al., 2005). Our data show that interruption on PI3K signaling does not significantly affect the presented

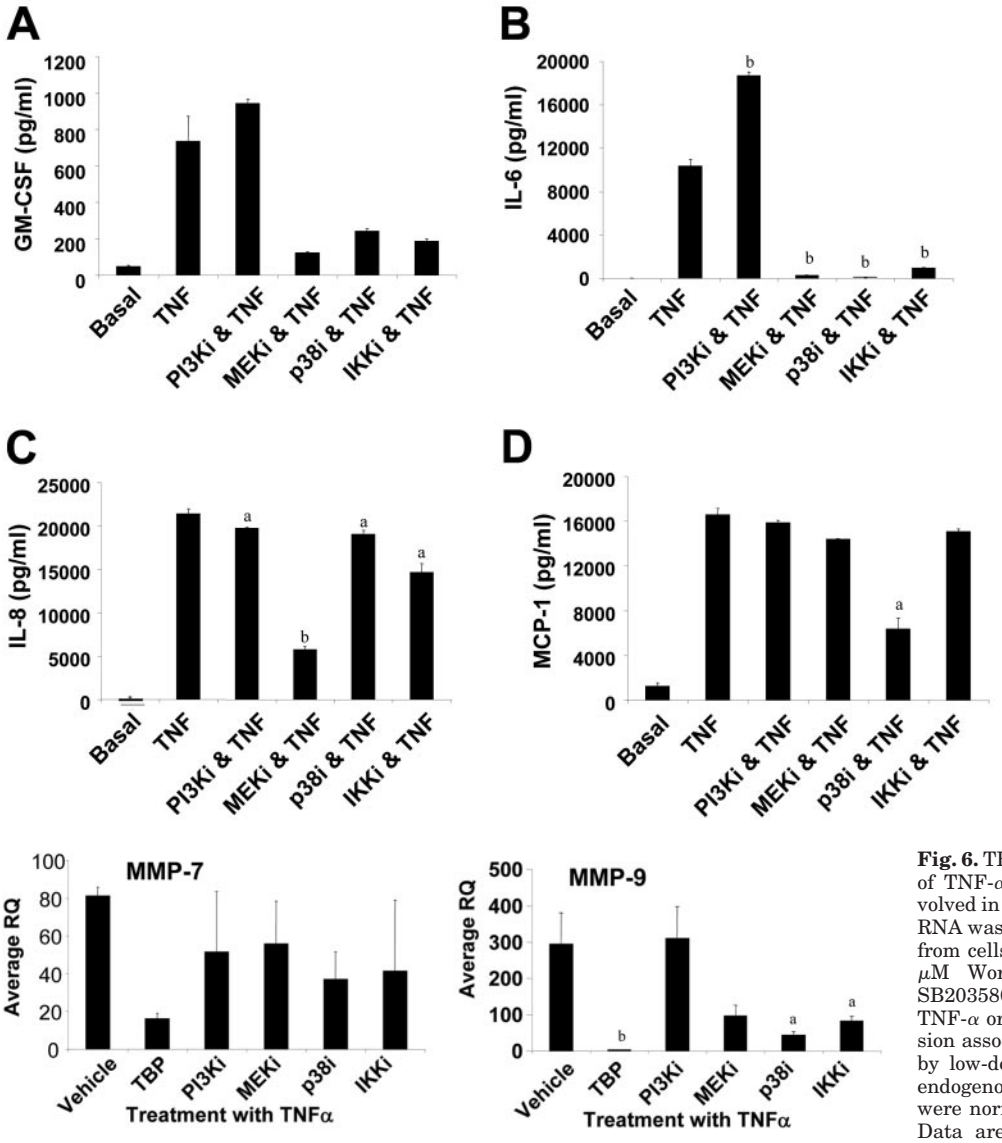

Fig. 5. Effect of inhibitors on TNF-α-induced cytokine secretion. 12Z cells were treated with 1 μM wortmannin, 10 μM PD98059, 50 μM SB203580, 1 μM AS602868 (IKK2 inhibitor), or vehicle, with or without 15 ng/ml TNF-α for 24 h in the absence of serum. Concentrations of secreted proteins were measured by MSD. A, inhibitors blocking TNF-α-induced GM-CSF kinetics graph is shown. B, inhibitors blocking TNF-α-induced IL-6 kinetics graph is shown. C, inhibitors blocking TNF-α-induced IL-8 kinetics graph is shown. D, inhibitors blocking TNF-α-induced MCP-1 kinetics graph is shown. Data are the mean of three replicate. S.D. is shown. a, $P < 0.05$; b, $P < 0.01$; compared with TNF-α-treated cells, ANOVA followed by Tukey test.

Fig. 6. TBP and kinase inhibitors block expression of TNF-α-induced matrix metalloproteinases involved in epithelial mesenchymal transition. Total RNA was isolated from serum-starved 12Z cells or from cells after the addition of 100 μg/ml TBP, 1 μM Wortmannin, 10 μM PD98059, 50 μM SB203580, 1 μM IKK2 inhibitor, and 15 ng/ml TNF-α or vehicle for 24 h. Inflammation or invasion associated transcript levels were determined by low-density array qPCR using HPRT as the endogenous control/reference. All treated groups were normalized to the untreated 12Z/calibrator. Data are the mean of three replicate. S.D. is shown. A, relative quantity of MMP-7 transcript is shown for all groups. B, relative quantity of MMP-9 transcript is shown for all groups. Representative results of at least three experiments are shown. a, $P < 0.05$; b, $P < 0.01$; compared with TNF-α-treated cells, ANOVA followed by Tukey test.

biomarkers of the epithelial endometriotic phenotype. Although the design of these studies was limited to the use of kinase inhibitors, which restricts the breadth of interpretations of these results, the concentration of these inhibitors used was selected to minimize the effect of the compound on off-target pathways. Nevertheless, the clinical benefit of TBP, etanercept, and infliximab on experimental endometriosis in baboons confirms the relevance of interrupting TNF-α signaling as a means of disease treatment. The model in Fig. 9 depicts a simplified diagram of TNF-α signaling through kinase pathways and inducing the expression of proteins involved in inflammation and invasion in endometriosis.

Evaluation of novel therapies for treatment of endometriosis has been difficult because of the difficulty of the cellular and animal models for this disease. Since their initial description several years ago, 12Z cells have been increasingly used by investigators to model cellular pathophysiology of endometriosis because in many ways they recapitulate the physiology of endometriotic epithelial cells. 12Z cells demonstrate some similar attributes as primary endometriotic cells (Zeitvogel et al., 2001) and also properties similar to fibroblast-derived endometrial stromal cells (Banu et al., 2007b). 12Z cells and endometriotic cells express high levels of COX-2 and PGE2 that are associated with the pain of endometriosis (Banu et al., 2007a). Moreover, TNF-α caused methylation of the progesterone receptor promoter in 12Z cells in a manner that resembles primary endometriotic cells (Wu et al., 2006, 2007a). 12z cells were prepared from red lesions of American Fertility Society stage I to II endometriosis. Early stage I and II endometriotic lesions have also been described as the more invasive cell type compared with cells from later stage III to IV endometriotic lesions. Inhibiting TNF signaling at stage I or II has been proposed to be potentially more effective strategy than at later stages of disease (American Fertility Society stage III–IV). Previous studies demonstrated that 12Z cells possessed similar invasive properties as primary endometriotic cells and that this invasiveness correlated with expression of N-cadherin in greater amounts than E-cadherin (Zeitvogel et al., 2001). These previous studies suggested that 12Z cells offered a unique opportunity to investigate processes of endometriosis disease progression with a specific focus on endometriotic epithelial cells and how the molecular endometriotic disease phenotype can be pharmacologically treated.

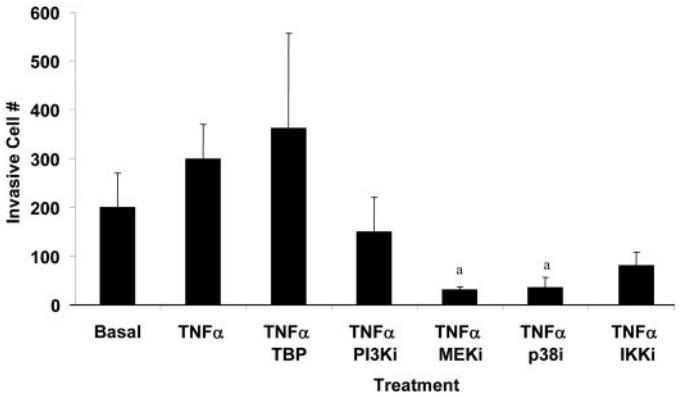

Fig. 7. MEKi, IKKi, and p38i inhibit 12Z invasion into Matrigel. 12Z cells were treated with vehicle, 100 μg/ml TBP, 1 μM Wortmannin, 10 μM PD98059, 50 μM SB203580, or 1 μM AS602868 (IKK2 inhibitor), with or without 15 ng/ml TNF-α for 30 h in the absence of serum. Treated 12Z cells were allowed to invade Matrigel for 24 h as described under *Materials and Methods*. Invasive 12Z cells were stained and counted at 24 h after treatment. a, $P < 0.05$; compared with TNF-α-treated cells, ANOVA followed by Tukey test.

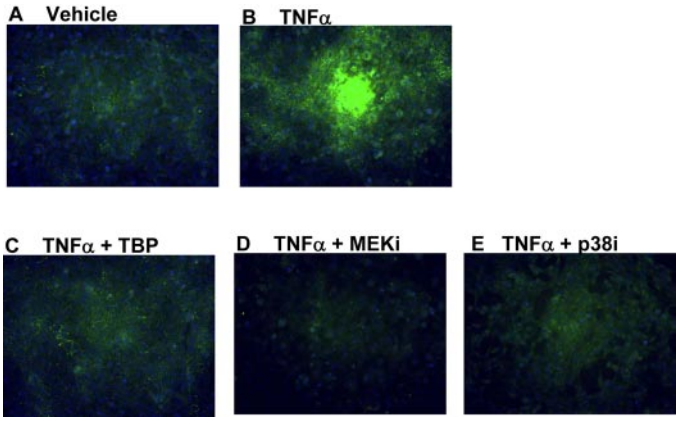

Fig. 8. TBP, MEK inhibitor, and p38 inhibitor reduce the expression of N-cadherin induced by TNF-α in 12Z cells. Serum-starved 12Z cells were stimulated with various agents for 24 h and fixed with paraformaldehyde. Fixed cells were stained with primary antibody against N-cadherin and Hoechst as described under *Materials and Methods*. Fluorescence images were captured at a specific wavelength for each dye. Images were merged to obtain cell surface expression of N-cadherin. A, vehicle-treated 12Z cells show basal levels of N-cadherin expression. B, TNF-α (15 ng/ml)-treated 12Z cells show elevated levels of N-cadherin expression. C, TNF-α + TBP (100 μg/ml) show normalized levels of N-cadherin expression. D, TNF-α + PD98059 reduce levels of N-cadherin expression below steady-state levels. E, TNF-α + SB203580 also reduced levels of N-cadherin expression below steady-state levels. Representative results of at least three experiments are shown.

Peritoneal fluid from women with endometriosis has been found to contain elevated levels of IL-6, IL-8, and TNF-α. Primary cultures of endometriotic epithelial cells have been shown to produce IL-6, IL-8, and TNF-α (Bergqvist et al., 2000; Luk et al., 2005). MCP-1 and RANTES have also been found elevated in peritoneal fluid of women with endometriosis, and previously, their production has been reported to originate from stromal cells (Bersinger et al., 2006; Kalu et al., 2007). Results from our studies clearly demonstrate an effect of TNF-α, a primary effector of inflammatory responses, to increase production of IL-6, IL-8, MCP-1, and GM-CSF from 12Z epithelial endometriotic cells. Furthermore, our results confirm that neutralization of the effect of TNF-α with TBP suppresses production of these cytokines. Interestingly, various kinase inhibitors affect various biomarkers of endometriosis. TBP followed by MEKi and then p38i has the most signif-

icant normalization to a noninflammatory and nonmesenchymal molecular and cellular phenotype.

The studies presented here demonstrate that cytokines commonly found in the peritoneal cavity of endometriotic patients (IL-6, IL-8, GM-CSF, and MCP-1) were also secreted by 12Z cells stimulated with TNF-α. Indeed, TBP completely reversed the effects of TNF-α on IL-6, IL-8, and GM-CSF production from 12Z cells, whereas inhibition of the TNF-α response by compounds that block downstream kinase signaling from TNF-α receptor demonstrated pathway-specific patterns of inhibition. Expression of mRNAs for cytokines and cellular adhesion markers that are indicative of an inflammatory and mesenchymal phenotype was increased by the presence of TNF-α and differentially decreased in the presence of inhibitors. MEKi blocks IL-6, IL-8, and MCP-1, whereas p38i blocks IL-6, GM-CSF, and MCP-1. Inhibition of IKK2 inhibited all four cytokines, placing IKK2 $>$ IκB $>$ NFκB signaling as a key pathway of TNF-α signaling to inflammatory cytokine secretion from endometriotic epithelial cells.

Endometrial epithelial cells normally secrete MMPs during normal endometrial breakdown during menstruation. The pathogenesis of endometriosis is partially due to secretion of ectopic endometrial cells into the peritoneum. It has been well established in cellular and animal models that MMPs are expressed at higher levels in cycle-matched samples of ectopic endometrium than in eutopic endometrium (Zhou and Nothnick, 2005). TNF-α has been shown to induce epithelial-mesenchymal transformation of several cell types, and long-term exposure to TNF-α leads to unrecoverable transformation (Chaudhuri et al., 2007). In the present study, it is presumed that 12Z cells have already undergone some aspects of epithelial-mesenchymal transformation, and others have been induced experimentally by short-term exposure to TNF-α. TBP and inhibitors of TNF-α signaling caused reversion of the mesenchymal endometriotic phenotype of the cells as measured by changes in MMP and N-cadherin expression. In the case of MMP expression, MMP-7 and MMP-9 expression were induced by TNF-α. MMP-7 expression was reduced by TBP and marginally affected by p38i, whereas MMP-9 was also dramatically blocked by TBP, p38, and IKK2 inhibitors and to a lesser extent by the MEK inhibitor. The redundancy of the TNF-α-induced kinase signaling to MMP-9 indicates MMP-9 could be important for the pathogenesis of epithelial endometriotic cells.

Expression of cellular adhesion molecules cytokeratin, E-cadherin, and N-cadherin has previously been used to distinguish endometriotic epithelial cells from normal endometrial cells and as well 12Z endometriotic cells from eutopic

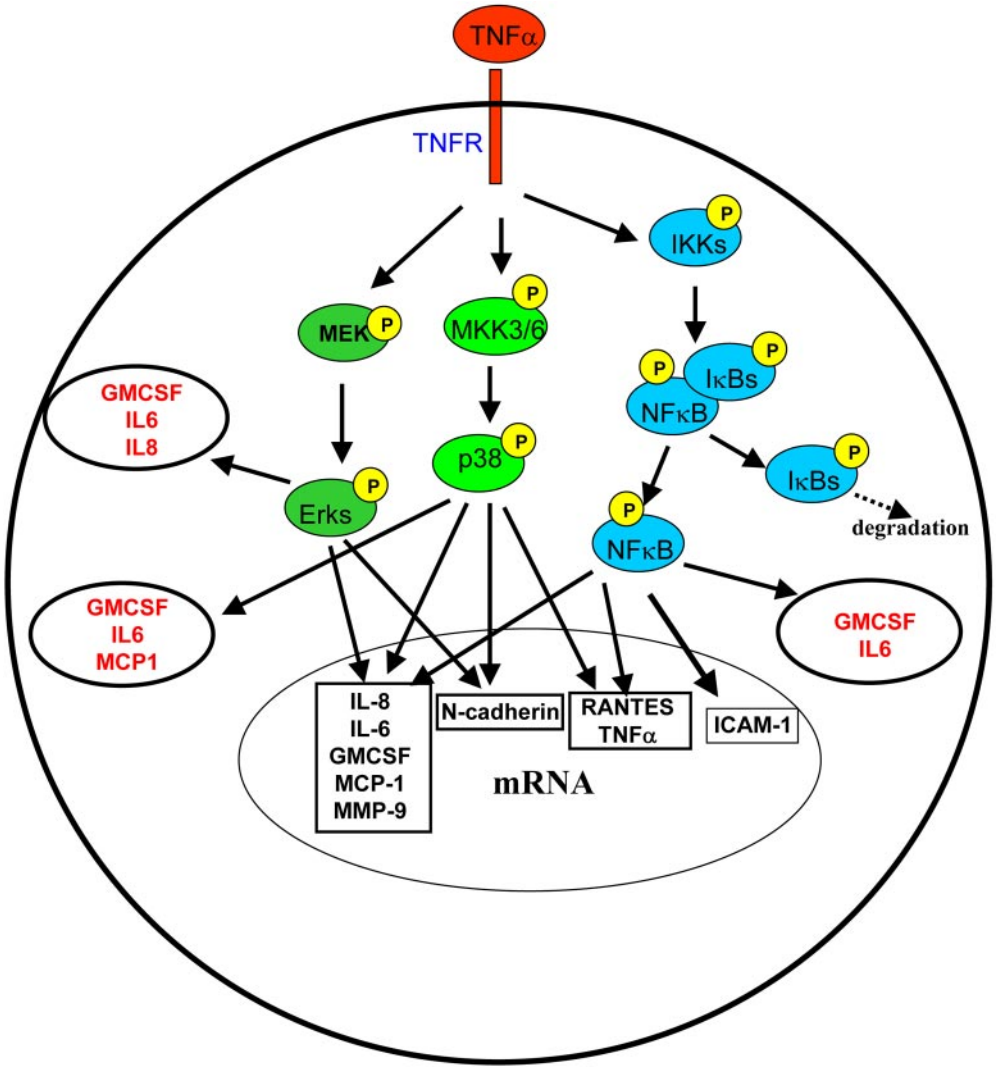

Fig. 9. Schematic representation of TNF-α-induced signaling in epithelial endometriotic cells. TNF-α signals through the TNF-α receptor. TNF-α activates pathways involved in regulating expression of inflammation and invasion mediators, including the PI3K, MEK, JNK, p38, and IKK pathways. The findings presented here indicate that TNF-α regulates components of inflammation and invasion through diverse signaling cascades in epithelial endometriotic cells.

endometrium (Gaetje et al., 1997). In the present experiments, we measured the ability of TNF-α to exacerbate the endometriotic phenotype of these cells. Addition of TNF-α caused elevation of N-cadherin. Neutralization of the TNF-α effect with TBP reversed the change in cadherin expression to a less mesenchymal phenotype (lower expression of N-cadherin) previously observed in endometrial cells rather than endometriotic cells. These results suggest there may be a direct beneficial effect of TBP or specific kinase inhibitors during treatment for endometriosis on reversion of an endometriotic phenotype.

The lack of E-cadherin and increased expression of N-cadherin marks an epithelial-mesenchymal transition with loss of adherin junctions. Decreased expression of E-cadherin in epithelial cells was found in peritoneal endometriosis as compared with normal endometrium (Poncelet et al., 2002). It has been proposed that E-cadherin-negative invasive endometriotic cells represent the cell population that causes endometriosis in vivo (Gaetje et al., 1997). These previous studies examining cadherin expression in eutopic and ectopic tissues support our findings with TNF-α signaling and N-cadherin expression. Consistent with our findings, additional invasion promoting factors shrew-1 and CD147 are found elevated in endometriotic cells and in 12Z cells relative to eutopic endometrium (Bharti et al., 2004; Schreiner et al., 2007). Treatment of the immortalized endometriotic cells with trichstatin A, a histone deacetylase inhibitor, reduces the invasiveness and reactivates E-cadherin expression in these cells (Wu et al., 2007b).

The present study highlights various signaling pathways modulating TNF-α-mediated effects in human endometriotic cell line. These results, although obtained from immortalized endometriotic cells but not primary cells, provide us evidence to explore the use of specific kinase inhibitors to treat endometriosis. Clinically, a preferred therapeutic would balance the pharmacologic consequences of broad TNF-α antagonism by neutralizing agents, with selective agents that target specifically sites of inflammation in endometriotic lesions. Future studies will focus on inhibition of preferred kinase targets in animal models of endometriosis to further validate these pathways for developing novel therapeutics in human.

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