Tumor Necrosis Factor-α-Induced Matrix Proteolytic Enzyme Production and Basement Membrane Remodeling by Human Ovarian Surface Epithelial Cells: Molecular Basis Linking Ovulation and Cancer Risk

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ABSTRACT

The majority of cancer is of surface/cyst epithelial origin. The ovarian surface epithelial cells are organized by a sheet of basement membrane composed mainly of collagen IV and laminin, and it is believed that the basement membrane greatly influences the physiological properties of ovarian surface epithelial cells. Previous studies in our laboratories indicated that loss of the basement membrane, an obligated step in ovulation, is also a critical step during the morphological transformation and tumor initiation of the ovarian surface epithelium. It is speculated that the loss of basement membrane in ovarian surface epithelial transformation may have similar biological mechanism to the loss of surface epithelial basement membrane in ovulation. However, the mechanisms involved in the ovarian surface epithelial basement membrane removal during ovulation are still not completely understood. In the current study, cultured human ovarian surface epithelial (HOSE) cells were examined for their abilities to produce matrix hydrolyzing enzymes and degrade basement membrane in response to a number of potential local mediators in ovulation. Among the candidate-stimulating factors tested, tumor necrosis factor (TNF)-α and IL-1β (to a lesser extent) were found to drastically increase urokinase type plasminogen activator (uPA) and matrix metalloproteinase (MMP)-9 activities secreted from HOSE cells. MMP-2, the other major HOSE cell-secreted gelatinase, is constitutively produced but not regulated. As demonstrated by immunofluorenscence staining and Western blot analysis, TNF-α treatment caused the degradation and structural reorganization of collagen IV and laminin secreted and deposited by HOSE cells in culture. Amiloride, an uPA inhibitor, not only inhibited the activity of uPA but was also able to suppress TNF-α-stimulated MMP-9 activity and prevented the TNF-α-stimulated remodeling of the basement membrane extracellular matrix, suggesting the contribution of uPA-mediated proteolytic cascade in this process. This study implicates the potential roles of TNF-α, uPA, and MMP-9 in ovarian surface epithelial basement membrane degradation and remodeling, which are processes during ovulation and may contribute to epithelial transformation. The findings may underscore the importance of TNF-α, uPA, and MMP-9 in ovarian surface epithelial basement membrane remodeling and may provide a molecular mechanism linking ovulation and ovarian cancer risk.

INTRODUCTION

The human ovarian surface is covered with a dynamic epithelium, which undergoes dramatic changes associated with ovulatory function and neoplastic transformation (1–4). The ovarian surface epithelium is a single layer of flat to columnar epithelium and separated from the underlying ovarian structures by a basement membrane. The major components of the basement membrane include collagen IV, laminin, entactin, and heparin sulfate proteoglycans (5, 6). In addition to serving as a mechanical barrier and organizer of the tissue structure,

the basement membrane also provides cues for cellular adhesion, growth, differentiation, and embryonic development (7–10).

Ovulation, triggered by luteinizing hormone (LH), is a complex process involving reciprocal paracrine interactions between surface epithelial cells and multiple stromal cell types (4, 7, 11, 12), resulting in the degradation of the basement membranes of the follicular wall and surface epithelium at a definite surface location (apex) followed by the expulsion of the oocyte. Proteolytic degradation of the basement membrane and connective tissue of the ovarian wall during follicular rupture is similar to an inflammatory process (11, 13–15). An influx of immune cells and increased concentration of cytokines and proteases occur during the LH peak. TNF-α is one of the main cytokines involved in this process and has emerged as a putative mediator of ovulation (13–15), synergizing with LH to induce ovulation. Other potential autocrine- and paracrine-mediating factors involved in ovulation include interleukin (IL)-1β (16), platelet-activating factor (PAF; Ref. 17), and prostaglandins (11, 12).

Previous studies have provided circumstantial evidence that the ovarian surface epithelium actively participates in ovulation by secreting proteolytic enzymes (4, 11, 12). This proteolytic degradation begins at the basal surface of the ovarian surface epithelium and advances inward toward the follicle. In cell culture, Kruk *et al.* (10) have demonstrated the capacity of human ovarian surface epithelial (HOSE) cells to secrete serine proteases and matrix metalloproteinases (MMPs), as well as to lyse Matrigel, although how these activities are regulated has not been explored. Several lines of evidence have implicated that both the MMP and plasminogen activator system contribute to the proteolytic activity needed at the time of ovarian rupture during ovulation (11, 12), *e.g.,* an increase in plasminogen activator and MMP activities has been detected during the preovulatory period (18–20), and synthetic MMP inhibitors suppressed ovulation in perfused rat ovaries (21, 22).

It has been proposed that the loss of basement membrane is a critical early step in the neoplastic transformation of ovarian surface epithelial cells (23–25). The cellular mechanisms that lead to basement membrane breakdown during neoplastic transformation and ovulation have been speculated to be similar (25). In the present study, we sought to evaluate the roles of HOSE cells and a number of potential local mediators of ovulation in the production of basement membrane hydrolyzing proteases and degradation of the basement membrane. Results from the current study may reveal factors and mechanisms involved in the loss of ovarian surface epithelial basement membrane in ovulation and neoplastic morphological transformation.

MATERIALS AND METHODS

Reagents. TNF-α, IL-1β, follicle-stimulating hormone (FSH), and LH were purchased from Calibiochem (La Jolla, CA). Prostaglandin factor (PGF)$_2$α was purchased from Cayman Chemical (Ann Arbor, MI). Carbamyl (c)-PAF, a nonhydrolyzable PAF, was purchased from Biomol (Plymouth Meeting, PA). All reagents, unless otherwise specified, were purchased from Sigma (St. Louis, MO).

Cell Culture. Primary HOSE cells were prepared from prophylactic oophorectomies, and the cells were transfected with SV40 large T antigen to prolong life span (24). Although these cells were prepared from disease-free "normal" ovaries, the cells likely contain BRCA1 or BRCA2 mutations (25) and may be highly abnormal in terms of growth properties and tumor-prone phenotypes. Three "immortalized" HOSE cell lines, HIO-80, HIO-114, and HIO-105, were cultured in medium 199 and MCDB 105 (1:1) supplemented with 5% fetal bovine serum, 0.25 unit/ml insulin, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

For Western blot analysis and gel zymography, confluent cells cultured in 60-mm plates were washed twice with PBS and switched to serum-free medium immediately before treatment with either TNF-α (10 ng/ml), IL-1β (5 ng/ml), PGF$_2\alpha$ (50 nM), c-PAF (10 nM), or LH (10 nM). To prepare lysates, cells were rinsed twice with PBS and lysed in 1 × SDS sample buffer [62.5 mM Tris (pH 6.8), 10% Glycerol, 1% SDS, and 0.1% Bromphenol Blue]. Conditioned medium was collected at the time indicated and concentrated 4-fold using a Centricon 10 concentrator (Amicon, Beverly, MA) before analysis. All experiments were done using all three HIO lines, and the results show quantitative variation between the lines, but the main conclusions from each treatment remain the same.

Western Blot Analysis. Total cell lysates were used for analyzing the expression of MMP-14, collagen IV, or laminin; concentrated conditioned medium mixed with sample buffer [0.25 M Tris, (pH 6.8), 40% glycerol, 4% SDS, and 0.4% bromphenol blue] at 3:1 ratio was used for MMP-2, MMP-9, MMP-14, MMP-19, tissue inhibitor of MMP (TIMP)-1, TIMP-2, or urokinase type plasminogen activator (uPA) analysis. Samples were boiled for 5 min and then loaded on 10% SDS polyacrylamide gel under reducing conditions (10 mM DTT). After electrophoresis, the proteins were electrotransferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), and nonspecific binding sites were blocked by incubating the membranes overnight at 4°C in 10 mM Tris (pH 7.5), containing 0.15 M NaCl, 0.05% Triton X-100, and 5% nonfat dry milk. The membrane was incubated with primary antibodies raised against collagen IV, Laminin, MMP-2, MMP-9, MMP-14, MMP-19, TIMP-1, TIMP-2, or uPA (Oncogene, San Diego, CA) for 1 h. Membranes were then probed with peroxidase-labeled goat antimouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 45 min. The signal was revealed using a chemiluminescence detection system (Pierce, Rockford, IL).

Gelatin and Casein Zymography. Concentrated condition medium was analyzed in nonreducing conditions on SDS-PAGE gels copolymerized with 0.1% gelatin for gelatinase and collagenase activities and 0.1% casein for plasminogen activator activities. After electrophoresis, the gels were washed three times with 2.5% Triton X-100 for 15 min at room temperature to remove SDS. Gels were then incubated in the development buffer [50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35] at 37°C for 16 h. The enzyme activity was visualized by staining the gel with 0.1% (w/v) Coomassie brilliant blue R-250 in 40% (volume for volume) methanol/10% (volume for volume) acetic acid for 2 h and destained in 10% (volume for volume) methanol with 7% acetic acid until bands became clear.

Immunofluorescence Microscopy. Human "immortalized" ovarian surface epithelial cells were grown to confluence on chamber slides and maintained for an additional 4 days with daily addition of 50 μg/ml ascorbate to promote collagen secretion. Cells were then treated as indicated for various times. At the end of treatment, the cultures were permeabilized in PBS containing 0.5% Triton X-100 and 5% sucrose for 3 min and then fixed in PBS containing 4% paraformaldehyde and 5% sucrose for 20 min, followed by washing with PBS. After blocking in 20% donkey serum (Jackson Immuno-Research Laboratories, West Grove, PA), the cultures were incubated with collagen IV monoclonal antibody (1:500; Sigma) for 1 h. After a 30-min PBS wash, the cultures were stained with FITC-conjugated donkey antimouse IgG (1:400; Jackson ImmunoResearch Laboratories) for 45 min. Propidium iodide (Molecular Probes, Eugene, OR) nuclear staining was then performed according to the manufacturer's instruction. Samples were mounted using the Prolong Antifade Kit (Molecular Probes) and examined using confocal microscopy.

RESULTS

Stimulation of uPA Secretion from HOSE Cells by TNF-α. Loss of basement membrane, a critical early step in the neoplastic transformation of ovarian surface epithelial cells (23, 24), also occurs during ovulation, an inflammatory-like response (11, 12). A number of known mediators of inflammation, such as TNF-α, IL-1β, PGF$_2\alpha$, and PAF, have also been implicated as potential mediators of ovulation (11, 12). Functional analyses indicate that uPA is involved in tissue degradation, whereas tissue-type plasminogen activator is involved in thrombolysis (26, 27). To further investigate the role of ovarian surface epithelial cells in basement membrane degradation, we therefore sought to determine whether these potential mediators affect uPA production from HOSE cells. LH, which has been shown to induce uPA secretion from sheep OSE, was also tested in the study. Human nontumorigenic ovarian surface epithelial HIO-80 cells were treated with either vehicle control, TNF-α (10 ng/ml), IL-1β (5 ng/ml), PGF$_2\alpha$ (50 nM), c-PAF (10 nM), or LH (10 nM). At 4, 7, and 18 h after treatment, conditioned media were collected and analyzed by Western blot for the presence of uPA. As shown in Fig. 1A, marked induction of uPA protein (M_r 50,000) occurs 4 and 7 h after TNF-α or IL-1β treatment, whereas PGF$_2\alpha$, LH, or c-PAF treatment had negligible effect. At 18 h, a significant increase in this endogenous uPA proteins (M_r 50,000) was observed compared with earlier time points in all experimental conditions, indicating that HOSE cells constitutively secrete this M_r 50,000 precursor form (single chain) of uPA. This precursor form can be activated to the two-chain form to become active high molecular weight uPA (HMW-uPA, M_r 100,000). The HMW-uPA can be further processed by removal of an NH$_2$-terminal fragment to produce an active low molecular weight form (LMW-uPA, M_r 33,000; Refs. 26 and 27). Induction of the HMW-uPA (M_r 100,000) and LMW-uPA (M_r 33,000) were detected exclusively in response to TNF-α treatment. The generality of TNF-α-induced uPA production in HOSE cells was tested using additional HOSE cell
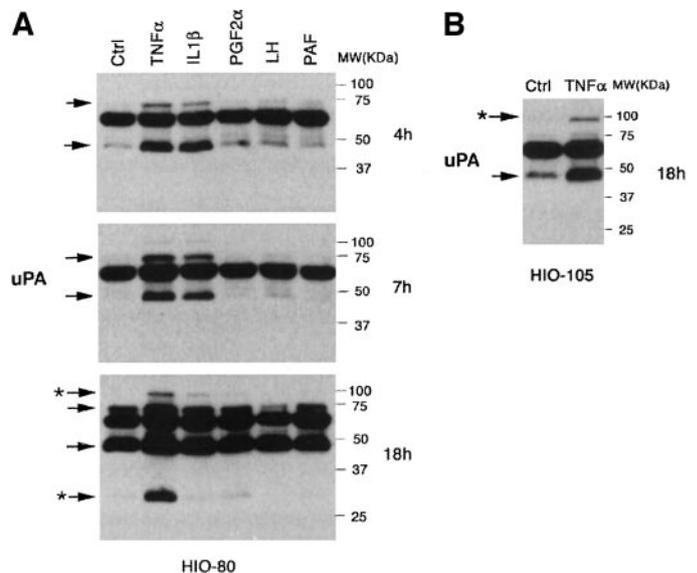

Fig. 1. Tumor necrosis factor (*TNF*)-α increases urokinase type plasminogen activator (*uPA*) protein production from human ovarian surface epithelial cells. In *A*, HIO-80 cells were treated with either vehicle as a control (*Ctrl*), TNF-α (10 ng/ml), interleukin (*IL*)-1β (5 ng/ml), prostaglandin factor (*PGF*)$_2\alpha$ (50 nM), luteinizing hormone (*LH*; 10 nM), or c-platelet-activating factor (*PAF*; 10 nM). Conditioned medium was collected at the time indicated and analyzed for uPA (M_r 33,000; 50,000; 75,000; and 100,000) by Western blotting. In *B*, HIO-105 cells were treated with vehicle as a control or TNF-α (10 ng/ml). Conditioned medium was isolated 18 h after treatment and assayed for uPA protein expression by Western blotting. The active species, M_r 33,000 and 100,000 proteins, are indicated by *.
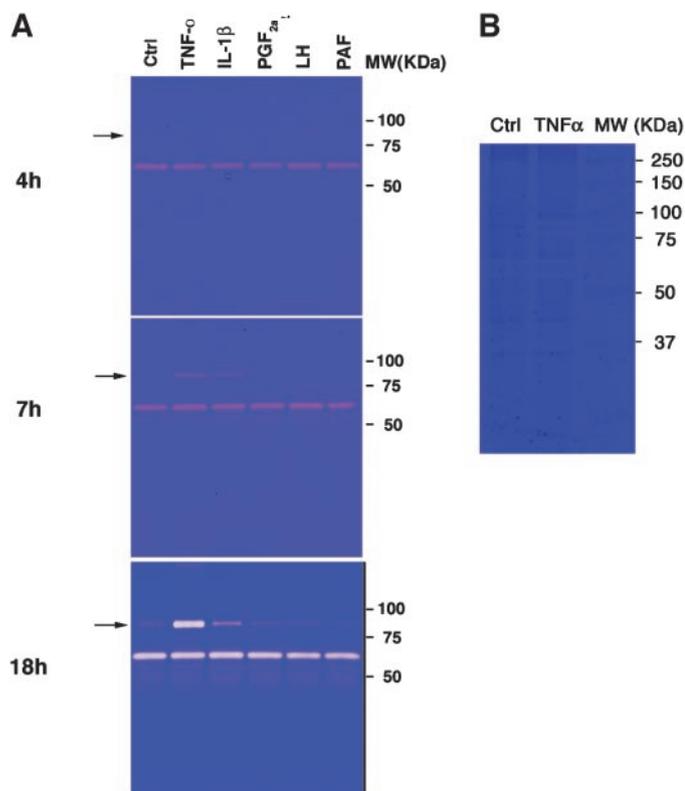
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Fig. 2. Tumor necrosis factor (*TNF*)α increases secretion of an M_r 88,000 gelatinase from human ovarian surface epithelial cells. In *A,* HIO-80 cells were treated with either vehicle as a control (*Ctrl*), TNF-α (10 ng/ml), interleukin (*IL*)-1β (5 ng/ml), prostaglandin factor (*PGF*)$_2$α (50 nM), luteinizing hormone (*LH*; 10 nM), or c-platelet-activating factor (*PAF*; 10 nM). Conditioned medium was collected at the time indicated, and its gelatinase activity was determined by gelatin zymography. In *B,* cell lysates were also used to analyze the activity of the cell-associated matrix metalloproteinases by zymography.

preparations. As shown in Fig. 1*B,* induction of M_r 50,000; 75,000; and 100,000 uPA was observed 18 h after TNF-α treatment in HIO-105 cells.

Secretion of Gelatinase/Collagenase Activities from HOSE Cells in Culture. The roles and expression of several MMPs in ovarian cancer and epithelial cells have been investigated (10, 27, 28). The presence of gelatinase/collagenase activities and their regulation by ovulatory hormones in HOSE cells and conditioned media was investigated by gelatin zymography. As shown in Fig. 2*A,* two major gelatinase species, M_r 68,000 and 88,000, are present in conditioned medium, and both activities accumulated with time. However, the M_r 68,000 activity was not affected by the treatments administered in the current study, including TNF-α, IL-1β, PGF$_2$α, c-PAF, and LH. Both TNF-α and IL-1β caused a time-dependent increase in the M_r 88,000 gelatinase activity (Fig. 2*A*), although the effect of IL-1β was much weaker than TNF-α at any given time point. PGF$_2$α, c-PAF, and LH had no effect on gelatinase activity under our experimental conditions.

Gelatinase activity was also determined in cell lysates isolated from TNF-α-treated HOSE cells. As shown in Fig. 2*B,* gelatinase activities in both control and TNF-α-treated cell lysates were negligible and much weaker than that from conditioned media, indicating that the majority of collagenase activity in HOSE cells is secreted, and cell-associated MMP is negligible. In addition, TNF-α appears to affect the activity of secreted, rather than cell-associated MMPs.

TNF-α-Stimulated Secretion of MMP-9 from HOSE Cells. On the basis of the molecular weight (10, 27, 28), it is possible that the M_r 68,000 collagenase in the conditioned medium is MMP-2 and the M_r 88,000 activity is MMP-9. The identity of TNF-α-stimulated M_r 88,000 gelatinase activity was furthered confirmed by Western blot

analysis. Similar to the stimulation pattern observed from gelatin zymography, a time-dependent increase of the M_r 88,000 protein recognized by MMP-9-specific antibody was observed in response to either TNF-α or IL-1β treatment (Fig. 3*A*). Again, much stronger induction was elicited by TNF-α compared with IL-1β. Stimulation of MMP-9 production by TNF-α was also observed in other HOSE cells: (*a*) immunoblotting in HIO-114 cells (Fig. 3*B*); and (*b*) gelatin zymography in HIO-105 cells (Fig. 3*C*). Quantitative but not qualitative variations of MMP-9 production in response to TNF-α were observed in different preparations of HIO cells.

We also examined the effect of TNF-α on the expression of other MMP proteins and the TIMP. Consistent with the results shown on zymographs, TNF-α did not alter the level of the M_r 68,000 MMP-2 protein, nor did it alter MMP-14, TIMP-1, and TIMP-2 protein expression (Fig. 4*A*).

MMP-19 is a novel member of the MMP family (29). Although little is known about its substrate specificity, up-regulation of MMP-19 during gonadotropin-induced ovulation suggests it may be involved in the tissue degradation that occurs during follicular rupture (29). MMP-19 protein expression was therefore analyzed in conditioned media obtained from TNF-α-, IL-1β-, PGF$_2$α-, c-PAF-, and LH-treated HOSE cells. TNF-α elicited a small increase in MMP-19 protein with the appearance of M_r 45–47,000; 37,000; and 28,000 forms (Fig. 4*B*). Additionally, the secreted MMP-19 from HOSE cells did not exhibit any significant gelatinase activity compared with MMP-2 and MMP-9 as analyzed by zymography. Nevertheless, on the basis of its regulation by TNF-α and expression during ovulation,

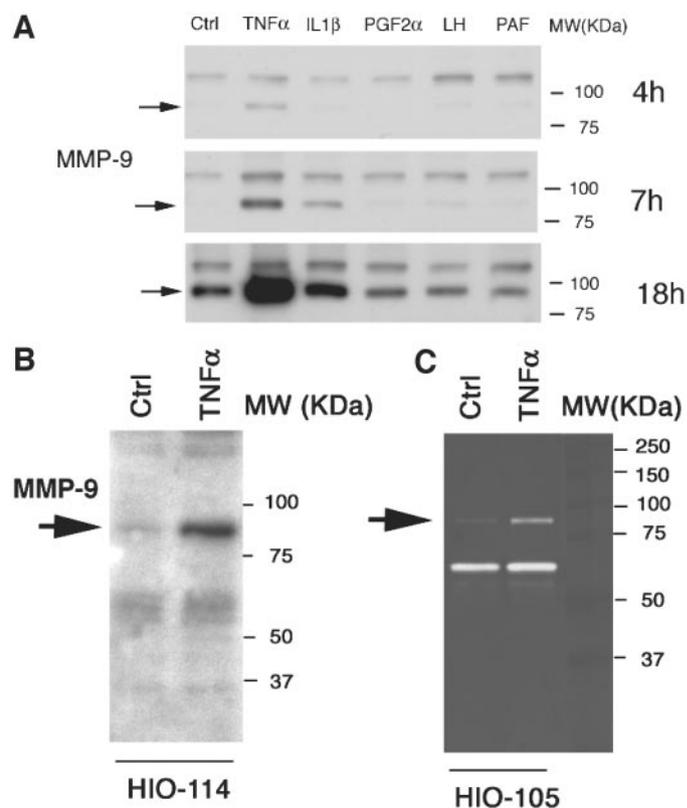Fig. 3. Tumor necrosis factor (*TNF*)-α stimulates matrix metalloproteinase (*MMP*)-9 protein secretion from human ovarian surface epithelial cells. In *A,* HIO-80 cells were treated with either vehicle as a control, TNF-α (10 ng/ml), interleukin (*IL*)-1β (5 ng/ml), prostaglandin factor (*PGF*)$_2$α (50 nM), luteinizing hormone (*LH*; 10 nM), or c-platelet-activating factor (*PAF*; 10 nM) for 4, 7, or 18 h. Conditioned medium was collected at the time indicated and analyzed by Western blotting. In *B,* conditioned medium from HIO-114 cells was analyzed for MMP-9 expression 18 h after TNF-α treatment by Western blotting. In *C,* conditioned medium from HIO-105 cells was analyzed for MMP activity 18 h after TNF-α treatment by gelatin zymography.
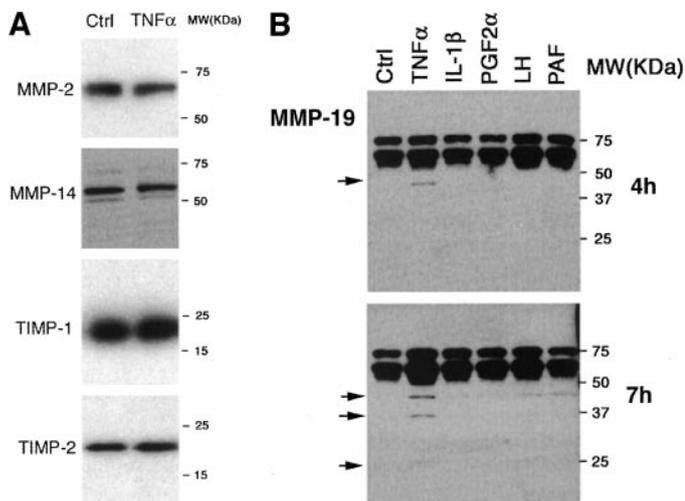
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Fig. 4. Effect of tumor necrosis factor (*TNF*)-α on production of matrix metalloproteinases (*MMP*) and tissue inhibitor of metalloproteinase (*TIMP*) by human ovarian surface epithelial cells. In *A*, HIO-80 cells were treated with either vehicle as a control or TNF-α (10 ng/ml). At 18 h after treatment, conditioned medium was collected and analyzed for expression of MMP-2, TIMP-1, and TIMP-2 by Western blotting. Cell lysates were isolated for analysis of MMP-14 expression by Western blotting. In *B*, HIO-80 cells were treated with either vehicle as a control (*Ctrl*), TNF-α (10 ng/ml), interleukin (*IL*)-1β (5 ng/ml), prostaglandin factor (*PGF*)$_2\alpha$ (50 nM), luteinizing hormone (*LH*; 10 nM), or c-platelet-activating factor (*PAF*; 10 nM). Conditioned medium was collected at 4 and 7 h after treatment and analyzed for MMP-19 by Western blotting.

MMP-19 may have a regulatory role, such as the activator of a protease cascade in tissue remodeling during ovulation.

Remodeling of HOSE Cell-Secreted Basement Membrane Matrix by TNF-α. The induction of uPA, MMP-9, and MMP-19 by TNF-α prompted us to investigate the effect of TNF-α on the integrity of the basement membrane reflected by the structure of extracellular type IV collagen. On culturing on tissue culture chamber slides for 4 days, confluent HOSE cells secrete a basement membrane-like extracellular matrix. This fine plexus of wispy strands can be maintained for ≥ 1 week. TNF-α was added to the culture after HOSE cells had secreted and deposited a layer of basement membrane matrix. As shown by immunofluorescence microscopy, the type IV collagen in the control culture appeared to be a delicate, foamy fine lacework surrounding the HOSE cells (Fig. 5). TNF-α treatment for 22 h caused condensation of type IV into much heavier branched beams separated by expanses with negligible amount of collagen (Fig. 5). Longer TNF-α treatment (60 h) resulted in a substantial reduction in the density and branching of collagen IV accompanied by thickening of remaining strands. To determine whether TNF-α treatment caused a quantitative difference on the level of basement membrane components, extracellular matrix was isolated together with cell lysate using SDS sample buffer 22 h after TNF-α treatment. The levels of collagen IV and laminin protein were determined by Western blot analysis. As shown in Fig. 6, a significant decrease in collagen IV (2.5-fold) and laminin (3.8-fold) protein was observed after TNF-α treatment. Results from these experiments indicate that TNF-α treatment of the cells is capable of inducing the degradation, reducing the density, and altering the structural organization of type IV collagen *in vitro*.

Inhibition of TNF-α-Stimulated uPA and MMP-9 Activities and Collagen IV Remodeling by Amiloride. Previous studies have shown that MMP activity can be regulated by uPA-dependent plasminogen plasmin generation (30). To dissect the cellular signaling pathways involved in TNF-α-induced MMP-9 activity, cells were pretreated with either amiloride (200 μM), a competitive uPA inhibitor (31); celecoxib (5 μM), a COX-2-specific inhibitor (32); or MK886 (20 nM), a specific inhibitor of 5-lipoxygenase-activating protein (33)

for 1 h before TNF-α treatment. Medium was collected 18 h after treatment and assayed for uPA and MMP activities by casein and gelatin zymography, respectively. As expected, amiloride blocked TNF-α-induced uPA activity (M_r 50,000 and 100,000), whereas the other inhibitors had no effect (Fig. 7A). Inhibition of uPA activity by amiloride was also accompanied by 90% reduction of TNF-α-induced MMP-9 activity without any observable effect on MMP-2 activity (Fig. 7B). Celecoxib and MK886 exerted no effect on TNF-α-stimulated MMP-9 activity (Fig. 7B), indicating that the arachidonic pathway is not involved in TNF-α-stimulated MMP-9 production. When Western blotting was performed using the same set of conditioned medium, we observed a substantial reduction in TNF-α-stimulated
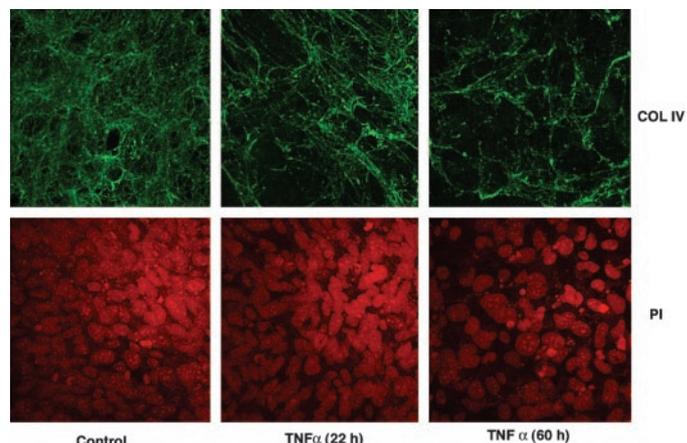

Fig. 5. Remodeling of human ovarian surface epithelial cell-secreted type IV collagen extracellular matrix induced by tumor necrosis factor (*TNF*)-α. HIO-80 cells were first cultured on chamber slides for 4 days to allow the cells to produce extracellular matrix basement membrane. TNF-α (10 ng/ml) or vehicle control was then added into the cultures. At 22 and 60 h after treatment, immunofluorescence staining using collagen IV antibodies and FITC-conjugated secondary antibodies was performed as described in "Materials and Methods." Nuclei were stained by propidium iodide (*PI*). The image was visualized by confocal microscopy of both FITC (*green*) and PI (*red*) staining. Similar staining patterns were observed in repeated experiments ($n = 3$). A representative image is shown.

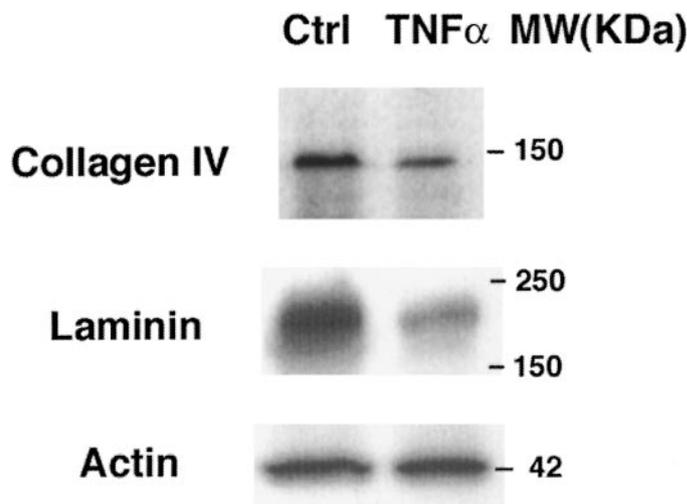Fig. 6. Tumor necrosis factor (*TNF*)-α treatment decreases collagen IV and laminin protein level in extracellular matrix. HIO-80 cells were first cultured on chamber slides for 4 days to allow the cells to produce extracellular matrix basement membrane. TNF-α (10 ng/ml) or vehicle control was then added into the cultures. At 24 h after treatment, cells and extracellular matrix materials on culture slides were collected, separated on a 7.5% SDS-acrylamide gel, and analyzed for collagen IV or laminin expression by Western blotting. Actin protein level was also analyzed as a loading control. The strengths of the signals were determined by NIH Imaging graphic program, and the fold changes were calculated.
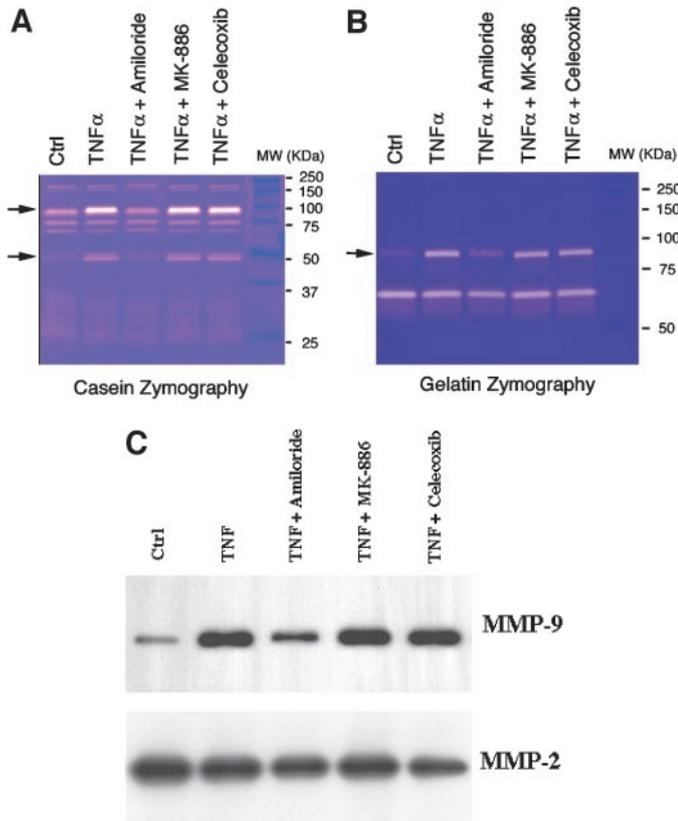
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Fig. 7. Inhibition of tumor necrosis factor (*TNF*)-α-induced urokinase type plasminogen activator and matrix metalloproteinase (*MMP*)-9 activity by amiloride. HIO-80 cells were treated with either amiloride (200 μM), MK-886 (20 nM), or celecoxib (5 μM) 1 h before TNF-α (10 ng/ml) treatment. Conditioned medium was collected 18 h after treatment and analyzed for urokinase type plasminogen activator activity by casein zymography (*A*) or MMP activity by gelatin zymography (*B*). In *C,* the conditioned medium was also analyzed for MMP-9 and MMP-2 by Western blotting using specific antibodies.

MMP-9 level but not MMP-2 protein expression by amiloride (Fig. 7*C*). Results from this study demonstrate that amiloride is capable of inhibiting TNF-α-stimulated uPA and MMP-9 activity.

To evaluate the role of MMP-9- and uPA-mediated activity in TNF-α-induced structural change of type IV collagen, the effect of amiloride on TNF-α-induced type IV collagen remodel was monitored. After culture of HOSE cells for 4 days to produce and deposit a layer of basement membrane matrix, the cells were incubated in the presence or absence of amiloride (200 μM) 1 h before the addition of TNF-α. Immunofluorescence staining was performed 22 h after TNF-α treatment. Collagen IV immunofluorescence microscopy in Fig. 8 illustrates that amiloride prevented TNF-α-induced structural change of type IV collagen. In the presence of amiloride, the condensation of fine collagen IV fibers into coarse beams and empty areas after TNF-α treatment was prevented. Because amiloride inhibits the activities of MMP-9 and uPA, results from this study suggest that uPA and MMP-9 are involved in TNF-α-induced remodeling of type IV collagen basement membrane matrix.

DISCUSSION

The critical roles of proteolytic cascade in ovarian function and pathology have long been recognized (10, 26–28). Most studies have focused on proteolytic enzyme produced from ganulosa cells. To our knowledge, this is the first report demonstrating secretion of uPA, MMP-9, and MMP-19 from HOSE cells in response to a proinflammatory cytokine, TNF-α. This increase in proteolytic activity was associated with degradation and remodeling of type IV collagen, the major component of basement membrane. Amiloride, a uPA inhibitor, shown to inhibit both uPA and MMP-9 activity in the present study, is also able to alleviate TNF-α-induced structural change of type IV collagen, suggesting the involvement of uPA/MMP-9 cascade in TNF-α-stimulated structural reorganization of type IV collagen. Additional studies are needed to define whether MMP-19 is necessary in this degradation process and its contribution to the overall proteolytic activity. All of the experiments described in this study have been carried out in all three cell lines: (*a*) HIO-80; (*b*) HIO-105; and (*c*) HIO-114. The conclusions are essentially identical in all three lines, with quantitative variations. It was observed that HIO-80 is the most and HIO-114 is the least responsive to TNF-α stimulation.

TNF-α, a pleiotropic polypeptide cytokine, is secreted from activated macrophages, as well as from several nonimmune cells in the ovary, such as granulosa and thecal cells (13–15). An increase in the concentration of TNF-α has been observed as ovulation occurs. TNF-α might access the HOSE by way of either the pelvic cavity (peritoneal fluid) or ovarian vasculature. Cells in close proximity to the preovulatory follicle (*i.e.,* within a limited diffusion radius) are probably exposed preferentially to the increased concentration of TNF-α, because of an acute increase in permeability of thecal vascular wreath. Mechanisms regulating TNF-α expression and release from resident ovarian cells remain controversial. It has been speculated that increased secretion of uPA by ovarian surface epithelial cells contiguous with the preovulatory follicles elicits a localized increase in tissue plasmin, which will release TNF-α from its anchor along the thecal endothelium (13–15).

The initiation of ovulation is induced by LH and FSH (11, 12). In the current study, we have not observed the induction of protease activities by LH and/or FSH in all three HOSE cell lines. A significant increase of uPA and MMP-9 secretion from HOSE cells was detected in response to TNF-α but not LH. Thus, we believe that LH and FSH do not induce the expression of uPA and MMPs directly in HOSE cells. TNF-α produced from other ovarian cell types that is induced by LH and FSH is thus a likely mediator for the induction of proteolytic activities from HOSE cells. Such an ovarian paracrine regulation may be required for the determination of a selective numbers of follicles in each ovulatory gonadotropin stimulation.

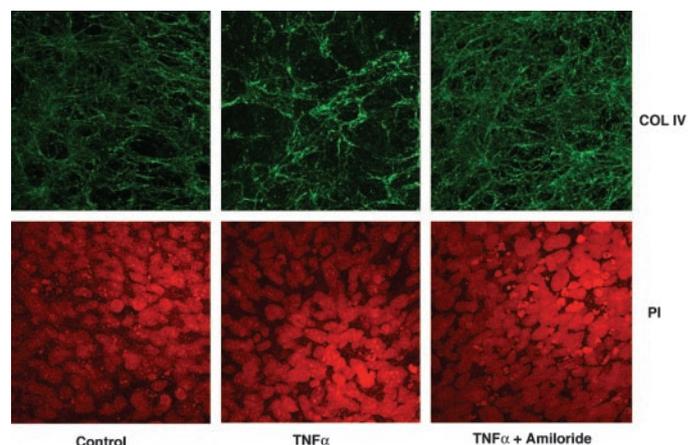Previous studies from Kruk *et al.* (10) demonstrated the expression

Fig. 8. Inhibition of tumor necrosis factor (*TNF*)-α-induced remodeling of type IV collagen by amiloride. HIO-80 cells were allowed to grow on chamber slides for 4 days to reach confluence density and produce extracellular matrix/basement membrane. The cultures were then treated with or without amiloride 1 h before TNF-α (10 ng/ml) addition. Immunofluorescence staining of collagen IV was performed 22 h after TNF-α treatment. Nuclei were stained with propidium iodide (*PI*). The images were visualized by confocal microscopy of both FITC (*green*) and PI (*red*) staining. Similar staining patterns were observed in repeated experiments (*n* = 3). A representative image is shown.

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of MMP-1 (collagenase), MMP-2 (gelatinase), and MMP-3 (stromelysin) RNA from OSE cells. Here, we further demonstrate that MMP-9 is the TNF-α-induced collagenase activity.

Amiloride is a potassium-sparing diuretic agent used clinically in the treatment of hypertension and has been shown to competitively inhibit the catalytic activity of uPA but not tissue-type plasminogen activator (31). In the present study, we have observed that inhibition of uPA activity by amiloride is associated with a 90% inhibition of MMP-9 activity, as well as a substantial decrease in MMP-9 protein expression. In fact, uPA-dependent plasminogen plasmin system has been described to cleave proMMP, which results in its activation (26, 27, 30). The mechanisms accounting for amiloride-mediated inhibition of MMP-9 protein secretion are not clear. Accumulating evidence demonstrates that the transcription factors, AP-1 and nuclear factor-κB, are necessary for induction of MMP-9 expression (34, 35). Amiloride has been shown to inhibit nuclear translocation and activation of nuclear factor-κB (35). It is therefore possible that inhibition of TNF-α-induced MMP-9 protein secretion by amiloride is mediated at the transcriptional level. Further study is needed to address this possibility.

Collagen IV turnover and remodeling are ubiquitous features of normal basement membrane matrix metabolism and reflects a balance between synthetic events, including biosynthesis, secretion, and assembly, and degradative events mediated by serine proteases and MMPs. In the present study, we observed that TNF-α treatment caused degradation and branching of collagen IV associated with thickening of the remaining strands. A reduction in collagen IV surrounding HOSE after TNF-α treatment may reflect one or the combination of the following: (*a*) a reduction in the biosynthesis or assembly of new collagen IV; (*b*) an increase in collagen IV degradation; and (*c*) differential changes in both parameters (the rate of degradation exceeds the rate of synthesis). Given the fact that the MMP-9- and uPA-mediated cascade is capable of degrading type IV collagen and a uPA inhibitor prevents the structural change in collagen IV matrix, it is plausible that TNF-α accelerated the rate of collagen IV degradation over the rate of its synthesis. Because MMPs are also required for collagen remodeling, the associated thickening of the remaining strands suggests TNF-α-induced MMP-9 may also affect the machinery of type IV collagen assembly. Such a condensation, with an associated increase in uncovered spaces seen *in vitro*, may be analogous to the loss of basement membrane seen *in situ* during the process of ovulation and tumorigenesis.

A previous study in our laboratory has shown that the basement membrane is often absent from preneoplastic ovarian surface epithelia located immediately adjacent to a morphologically neoplastic lesion, suggesting that the loss of the basement membrane is an early step toward the ovarian tumorigenicity (23). Thus, frequent loss of basement membrane in repeated ovulation, which is induced by gonadotropins and mediated by TNF-α, may increase the chance of ovarian surface epithelial cell transformation. The etiological link between ovarian cancer risk to incessant ovulation (36) or gonadotropin stimulation (37) is well established. Thus, TNF-α-stimulated basement membrane loss provides a mechanistic explanation for the incessant ovulation hypothesis. Additionally, prolonged exposure to TNF-α during pathophysiological conditions, such as inflammation and chronic gonadotropin stimulation, may promote ovarian carcinogenesis. An increase of proinflammatory cytokines, including TNF-α, occurs after natural and surgical menopause (38). The link between TNF-α exposure and basement membrane loss may provide an explanation that an increase in circulating TNF-α may be a contributing factor for a higher risk of ovarian cancer in postmenopausal women.

In conclusion, this study suggests that factors involved in ovulation, such as TNF-α, uPA, and MMP-9, may modulate ovarian cancer risk

by their roles in the degradation of basement membrane. Because ovulation-like loss of basement membrane is a possible etiological mechanism in ovulation-associated ovarian cancer risk (25), this study underscores the importance of TNF-α, uPA, and MMP-9 in ovarian surface epithelial basement membrane remodeling and may provide a molecular mechanism linking ovulation and ovarian cancer risk. Thus, these factors are also possible targets for chemopreventive intervention of ovarian cancer.

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Tumor Necrosis Factor-α-Induced Matrix Proteolytic Enzyme Production and Basement Membrane Remodeling by Human Ovarian Surface Epithelial Cells: Molecular Basis Linking Ovulation and Cancer Risk

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