

Activation of the Estrogen Receptor Contributes to the Progression of Pulmonary Lymphangiomyomatosis via Matrix Metalloproteinase-Induced Cell Invasiveness

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Context: The role of estrogens in the pathogenesis of lymphangiomyomatosis (LAM), an aggressive and destructive, eventually fatal lung disease of women, is poorly understood.

Objective: The study was conducted to test the hypothesis that the lung disease in LAM is estrogen mediated and to determine whether estrogens contribute to the invasiveness of LAM.

Design: *In vitro* cell culture of spindle-shaped LAM cells (LAMD-SM) were isolated and propagated from affected lungs. Estrogen receptor (ER)- α and ER β analyses were conducted by RT-PCR. ER α and ER β , tissue inhibitor of metalloproteinase-2, and matrix metalloproteinases (MMP)-2 had Western blot analysis for protein assessment. Activity assays were performed for MT1-MMP, MMP-2, and tissue inhibitor of metalloproteinase-2. Assessment of MMP-2 promoter function was done via transfection assays. Cell invasion chambers were used to determine and quantitate cell invasiveness.

Setting: The study was conducted at an academic medical center.

Patients: Tissue and cells were obtained from patients as outlined in approved institution review board protocol (97/007).

Intervention: LAMD-SM cells were treated with a specific MMP-2 antibody or a nonspecific inhibitor, doxycycline.

Main Outcome Measures: Activity of MMP-2 and invasiveness of LAMD-SM cells were measured.

Results: LAMD-SM cells express functional ERs (ER α and ER β), which undergo rapid intracellular turnover in their unbound state. 17 β -Estradiol (E₂) enhances the transcriptional ER activity. E₂-induced ER activation increases synthesis and activity of MMP-2 through posttranscriptional mechanisms in LAMD-SM. The E₂/ER-mediated increase of MMP-2 promotes LAMD-SM invasiveness, in assays *in vitro*, which can be inhibited by specific antibodies against MMP-2 or doxycycline, an inhibitor of MMPs.

Conclusion: The invasion and destruction of lung parenchyma in LAM is, at least partially, an estrogen-MMP-driven process, which has major implications for therapeutic interventions. (*J Clin Endocrinol Metab* 93: 1625–1633, 2008)

Lymphangiomyomatosis (LAM) is an aggressive interstitial lung disease that is found only in women. Slowly progressive dyspnea and increasing fatigue associated with recurrent

events of spontaneous pneumothorax are the usual, albeit nonspecific, clinical manifestations. Gross pathological findings in this sporadically occurring disease include hyperinflated lungs

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Abbreviations: DAPI, 4',6'-Diamidino-2-phenylindole; E₂, 17 β -estradiol; ER, estrogen receptor; ERE, estrogen-responsive element; HMB, human melanoma black; LAM, lymphangiomyomatosis; LAMD-SM, spindle-shaped LAM cells; MMP, matrix metalloproteinase; MT1, membrane type 1; TIMP, tissue inhibitor of metalloproteinase.

with loss of lung parenchyma accompanied by the development of cysts diffusely distributed throughout the lung leading to a Swiss cheese appearance in its end stage (1, 2).

The proliferation of immature-appearing smooth muscle-like cells called LAM cells has been proposed to play a central role in the destruction of lung parenchyma in LAM disease. LAM cells are found lining the walls of cysts, along pulmonary lymphatics, as well as in blood vessel walls, in which they grow in an infiltrative, disorganized, and nonconcentric pattern forming obstructive cell bundles. LAM cells are morphologically heterogeneous and present as large epithelioid cells with clear cytoplasm or as small- to medium-sized spindle-shaped cells (3). Epithelioid LAM cells show immunoreactivity to human melanoma black (HMB)-45, a mouse monoclonal antibody directed against a glycoprotein expressed in cells of melanocytic lineage, termed gp-100 (4), and are predominantly located in the periphery of LAM lesions. In contrast, spindle-shaped LAM cells are rapidly proliferating cells in the center of LAM nodules and are HMB-45 negative (4).

Estrogens early on have been implicated in the pathogenesis of LAM, a nonreproductive women's disease. In fact, initial studies of affected lung tissue using biochemical and immunohistochemical techniques revealed, although inconsistently, expression of estrogen receptors (ERs) and progesterone receptors in cells of LAM lesions (5–7). In contrast, various therapies aimed at preventing ER activation in LAM cells by lowering circulating estrogen levels through ovariectomy or GnRH administration, by trying to block ER activity with the partial ER antagonist/agonist tamoxifen or attempting to lower ER expression in LAM by treatment with progesterone failed to slow or stop progression of the disease in most of the affected women (1, 5, 8, 9). Additional questions about the validity of the estrogen hypothesis in LAM were raised by the study by Matsui *et al.* (10). These investigators demonstrated ER expression in the HMB-45-positive epithelioid but not the fast proliferating small spindle-shaped LAM cells.

Nevertheless, the HMB-45-negative, small spindle-shaped LAM cell type is more likely to be responsible for the destruction of connective lung tissue due to the release of matrix metalloproteinases (MMPs). MMPs are extracellular matrix-degrading enzymes that are involved in a variety of diseases ranging from glomerulosclerosis, rheumatoid arthritis, and cancer (11–13). Small spindle-shaped LAM cells show abundant staining, in particular, for MMP-2, MMP-9, and membrane type 1 (MT1)-MMP (10). However, the hypothesis, that estrogens through ER stimulation modulate MMP activity in LAM cells and promote invasiveness, remained to be tested.

In the past, the elucidation of the pathogenesis of LAM has been hampered by the lack of appropriate animal models. The current availability of LAM-affected lung tissue obtained from patients at the time of lung transplantation allowed us to isolate, propagate, and further characterize the small, HMB-45-negative, spindle-shaped LAM cell type (LAM-D-SM) (14). Our findings presented herein provide convincing evidence for the important role that the activation of ERs by estrogens plays in the destruction of lung tissue in women with LAM.

Subjects and Methods

Cell culture

LAMD-SM were isolated and propagated from lung tissue removed from LAM patients as described previously (14). Cells were also isolated from age-matched women undergoing lung resection for lung carcinoma as per institutional review board protocol 97/007 ($n = 7$). Cell lines between passage numbers five through eight were used for all studies. In experiments designed to examine 17β -estradiol (E_2) effects, cells were transferred into phenol red-free DMEM (Life Technologies, Bethesda, MD) supplemented with 100 μ g/ml of penicillin/streptomycin and glutamine containing 15% charcoal-stripped fetal bovine serum (Hyclone, Pittsburgh, PA). Proliferation was assessed in the presence of physiological concentrations of E_2 (0, 0.1, 1, and 10 nM). Cell number, determined at d 1 and 3 with a Coulter cell counter (Hialeah, FL), was not affected by E_2 .

Real-time PCR

mRNA was prepared from LAM and control patients as previously described and real-time PCR performed (15). Standards were prepared from the human ER α plasmid kindly provided by Tremblay *et al.* (16). The full-length ER α was excised by restriction digest and run on an agarose gel. The cDNA was extracted from the gel and quantified. Thereafter decreasing concentrations of purified ER α was subsequently run at the same time as unknown samples in a real-time PCR. A standard curve was generated and unknowns calculated from the curve. TaqMan ribosomal RNA control reagents to detect 18S ribosomal RNA served as an endogenous control to normalize for variations in the isolated RNA amount.

Western blot analysis

ER α , ER β , tissue inhibitor of metalloproteinase (TIMP)-2, steroid receptor coactivator-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and MMP-2 (Chemicon, Temecula, CA) protein expression was examined by Western blot as previously described (15). Briefly, 100,000 cells were plated (Nalge Nunc International, Rochester, NY) and treated in phenol red-free medium and 0.1% charcoal-stripped serum with either vehicle or increasing concentrations of E_2 (0.1–10 nM). In addition, tissue samples were cut in 2-mm pieces and homogenized in lysis buffer. Protein was extracted with lysis buffer and the concentration assessed using the Pierce BCA protein assay kit (Rockford, IL). For ER α , MMP-2, and TIMP-2, 10–50 μ g of protein lysate were fractionated on 10% polyacrylamide gels and transferred to nitrocellulose membranes as previously described. For immunoprecipitation experiments, 100 μ g of protein extract were incubated with ER β antibody or normal goat IgG for 1 h at 4 C, followed by the addition of protein G-agarose overnight. The resulting protein G-antibody conjugate was centrifuged at 4 C and washed four times with PBS (pH 7.4). The final pellet was resuspended in PBS, sample buffer added, and the mixture boiled for 3 min before analysis as described above. Immunoreactive bands were determined by exposing the nitrocellulose blots to a chemiluminescence solution (Santa Cruz Biotechnology) followed by exposure to X-Omat AR film (Eastman Kodak Co., Rochester, NY). The specificity of the signal was demonstrated by incubating blots with an excess of the corresponding specific immunizing peptide. Densitometry was performed using Image J 1.17 software (National Institutes of Health, Bethesda, MD) to determine relative amounts of protein. Experiments on individual cell lines were performed in duplicate using duplicate wells for treatment. Tissue experiments were performed in duplicate.

Degradation studies

To study ER α degradation, LAMD-SM and control SM cells were treated with the proteasome inhibitor MG132. (Sigma Chemical Co., St. Louis, MO). Briefly, cells were plated as for Western analysis or PCR and treated for 6 h with MG132 at a concentration of 1 μ M.

MMP-2 and TIMP-2 activity

Either 150,000 LAMD-SM cells or 250,000 control SM cells were plated in 6-well plates (Nalge Nunc International) and exposed to phenol red-free medium supplemented with 15% charcoal-stripped serum during treatment with E_2 (0.1–10 nM) or ICI (1 μ M), a complete ER antagonist (Tocris, Ellisville, MO) in the presence and absence of E_2 (10 nM). Cell supernatants were collected 24 h after treatment and the protein concentration determined. MMP-2 and MMP-9 activities were measured as described (17). Standards (Chemicon) were electrophoresed in parallel. Zymography gels (Invitrogen, Carlsbad, CA) were incubated for 24 and 48 h, respectively, for MMP-2 and MMP-9 in 50 mM Tris buffer, allowing determination of total proteolytic MMP activities with no interference from their associated tissue inhibitors. Densitometry, using Image J software (National Institutes of Health), was used to determine relative MMP-2 and MMP-9 activities. In addition, MMP-2 and TIMP-2 were measured using the human Biotrak activity assay that recognizes mouse MMP-2 or TIMP-2 activity, respectively, and normalized to cell number (Amersham Biosciences, Piscataway, NJ). Three independent experiments were performed in duplicate and results expressed as percentage of control (vehicle treated).

MT1-MMP activity

LAMD-SM and control SM cells were plated in 24-well plates and grown to confluence at which time cells were treated as above. After treatment the media were removed and replaced with 250 μ l of extraction buffer provided with the MT1-MMP Biotrak activity assay system (Amersham Biosciences) and incubated at 4 C for 15 min. The supernatant was assayed for MT1-MMP activity according to manufacturer's directions for lower endogenous MT1-MMP levels (assay range 0.125–4 ng/ml). Data were normalized to protein concentration for each sample.

Transfection and luciferase assays

LAMD-SM and control SM cells were plated in basal medium with 20% charcoal/dextran-treated fetal bovine serum (<5 pg/ml estrogens) in 24-well plates. Cells were transfected using GenePorter (Promega, Madison, WI) with either a 4ERE-TATA-Luc or the human MMP-2-promoter-luciferase reporter gene construct and the β -galactosidase gene pRSV- β gal (0.4 μ g/well) to control for transfection efficacy. Cells were subsequently treated with vehicle or E_2 ranging from 0.1 to 10 nM. After 48 h, cells were harvested and luciferase and β -galactosidase assays, were performed as previously described (15). Briefly, cells were washed two times in PBS and lysed with 100 μ l of reporter lysis buffer (Promega) at room temperature for 15 min. Wells were scraped and the lysate transferred to a Microfuge tube, vortexed, and microcentrifuged for 2 min at 4 C. The supernatant was collected and frozen at –70 C until assayed.

Cell invasion assays

The cell invasion assay was performed according to manufacturer's directions (Chemicon International). Briefly, chambers were equilibrated and rehydrated and a cell suspension (1×10^6 cells) was prepared in serum-free media. Three hundred microliters of the cell suspension were added to the inner chamber, and the outer chamber was filled with 20% fetal bovine serum containing media and allowed to incubate for 4 h before the addition 10 nM E_2 . For time-course experiments, the chambers were allowed to incubate for 8, 12, 16, or 24 h. In some experiments an antisera to MMP-2 (5, 50, 500) or doxycycline 10 mg/ml or ICI 182,780 (10^{-6} M) was added at the same time as E_2 . A nonspecific rabbit IgG was used as control. After a 24-h incubation, the media were removed and the chambers were cleaned and stained according to the provided protocol. Cell invasion chamber membranes were then excised from their supporting cylinders, placed on glass slides, and mounted with Vectashield mounting media with 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Images of bright-field and fluorescent visual fields were captured with an Olympus IX71 microscope (Center Valley, PA) and the MicroSuite Five software (Olympus). Quantification of the number of invading cells was performed with Image-Pro Plus

(version 6.0; Media Cybernetics, Inc., Silver Spring, MD). The Count/Size function was used after the Threshold was adjusted to properly detect the DAPI stain in the captured images. For images with high concentrations of cells, the Watershed Split command was used to obtain an accurate count of invading cells.

Statistical analysis

Statistical differences between experimental groups were determined by ANOVA and Tukey's multiple comparison *post hoc* test. In those experiments requiring analysis of two groups, Student *t* test was performed. Data are expressed as means \pm SEM (GraphPad Prism, San Diego, CA). Each experiment was performed at least twice on at least LAM patients and three controls (except where noted), and duplicate wells collected. In the case of transfections, triplicate wells were collected for each treatment.

Results

LAMD-SM cells maintain their *in vivo* phenotype

LAMD-SM and control SM cells expressed fibroblast antigen and smooth muscle α -actin and showed no immunoreactivity to HMB-45 *in vitro* (14). Cytogenetic analysis of G-banded metaphases from LAMD-SM and control SM cells demonstrated that LAM cells are diploid and have no signs of chromosome instability such as chromosomal breaks, rearrangements or telomeric associations (data not shown).

LAMD-SM cells express ER mRNA

To investigate the role estrogens play in LAM, we performed quantitative real-time RT-PCR on total RNA extracted from LAMD-SM and control SM cells. We found that LAMD-SM cells expressed 1175 ± 358 copies of ER α mRNA ($n = 5$), compared with 186 ± 70 copies of ER α mRNA in control SM cells ($n = 4$, $P < 0.05$) (Fig. 1A).

LAMD-SM cells express ER protein

The levels of ERs account, at least in part, for the sensitivity and response to estrogens in cells and tissues (15, 17, 18). We examined LAM lesions and the LAMD-SM cell lines derived from them to determine whether they, in fact, express ER α and ER β protein, a basic prerequisite for the validation of the estrogen hypothesis in LAM. Pulmonary tissue from unaffected women and SM age-matched cell lines served as controls. The cells in LAM lesions and LAMD-SM cell lines expressed ER α and ER β , which confirmed the findings of some of the previous immunohistochemical studies on LAM tissue (7). Control lung tissue and SM cells expressed the same ER subtypes (Fig. 1). Unexpectedly, Western analysis revealed that the amounts of ER α and ER β protein in cells of LAM tissue and LAMD-SM cell lines were lower than in those of control lung tissues and cell lines. The relative amount of ER α protein was $11.10 \pm 6.0\%$ in LAM tissue, compared with $99.97 \pm 14.1\%$ of control lung tissue ($n = 3$, $P < 0.005$, Fig. 1B). The levels of ER α protein were $57.3 \pm 8.4\%$ in the LAMD-SM and $99.67 \pm 0.3\%$ in the control SM cell lines ($n = 3$, $P < 0.05$, Fig. 1B). Similarly, the relative amounts of ER β were $73.2 \pm 6.2\%$ in LAM tissue, compared with $100.0 \pm 0.4\%$ in the lung parenchyma of controls ($n = 3$, $P < 0.05$) and $56.6 \pm 13.3\%$ in LAMD-SM *vs.* $107.2 \pm 11.0\%$ ($P <$

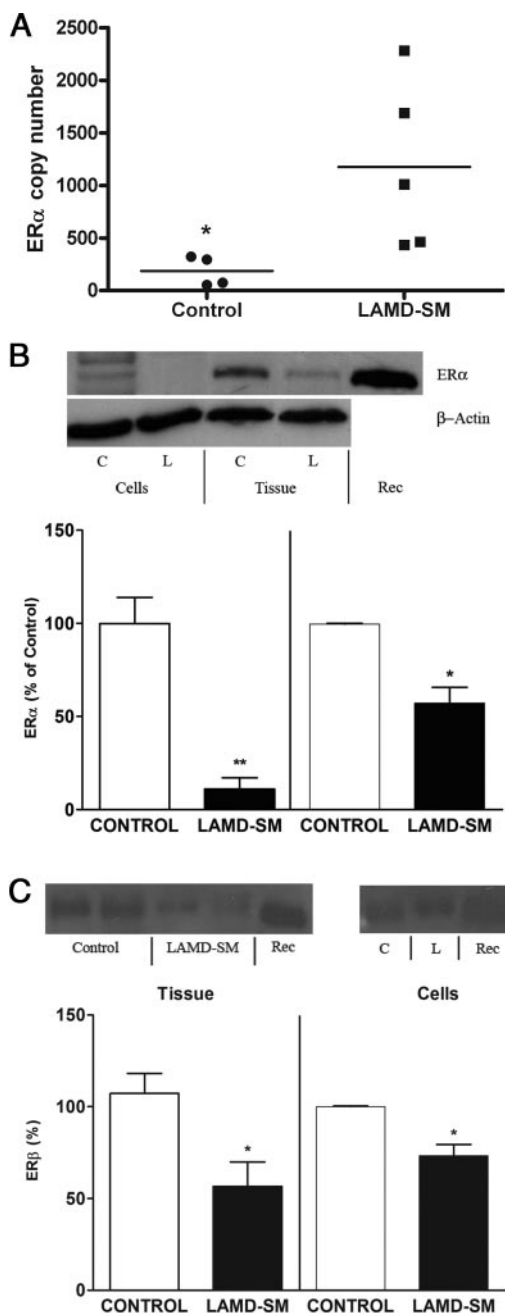


FIG. 1. A, ER α mRNA copy number in LAMD-SM and control SM cells. Real time RT-PCR was performed on four individual control cell lines and five individual LAMD-SM cell lines as described in *Subjects and Methods*. Each point represents an individual cell line. *, $P < 0.05$. B and C, Expression of ER α (B) and ER β (C) protein in LAM tissue and LAMD-SM cells (L), compared with lung tissue and SM cells from controls (C). Twenty-five micrograms of protein were loaded onto a 10% polyacrylamide gel. Western analysis was performed as described in *Subjects and Methods*. ER α protein was measured in three LAMD-SM cell lines isolated from three individual patients with LAM. ER β protein was determined in five individual LAMD-SM and control SM cell lines. Data on lung tissue and cell lines (mean \pm SEM) are expressed as a percentage of the mean of control patients. The insets show representative Western blots of ER expression in lung tissue and cell lines. *, $P < 0.05$ and **, $P < 0.005$. Membranes were stripped and reprobbed with an antibody to actin as a control for protein loading. Rec, Recombinant protein for ER α and ER β .

0.05) in control-SM cell lines (n = 5, Fig.1C). These results were somewhat unexpected because the expression of ER mRNA had

been higher in LAM tissue and LAMD-SM cells, compared with their respective controls.

ERs in LAMD-SM cells are transcriptionally active

LAMD-SM and control SM cell lines were transfected with a luciferase-based reporter gene under the control of four consecutive estrogen-responsive elements (EREs) in its promoter region. Although normal pulmonary smooth muscle cells are responsive to E₂, the transcriptional response to E₂ was higher in LAMD-SM than control-SM cells (n = 5, Fig. 2). E₂ (1 nM) induced a 3-fold increase in luciferase activity in LAMD-SM cells (Fig. 2A, $P < 0.05$) but only a 1.5-fold increase in control SM cells (Fig. 2B, $P < 0.05$). On the basis of these findings coupled with the data on ER mRNA and protein levels, we postulated that there was increased turnover/degradation of unbound ER in LAMD-SM, compared with control SM cells.

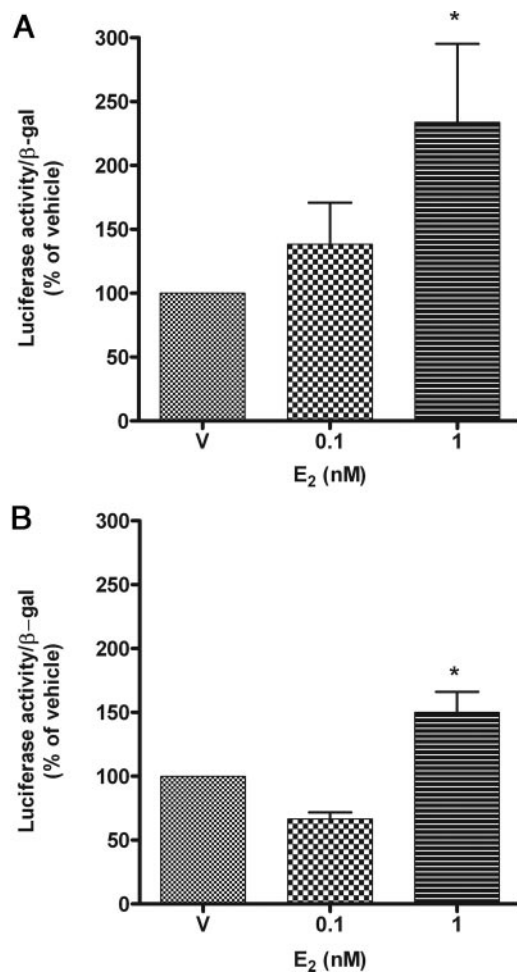


FIG. 2. E₂-mediated activation of a reporter plasmid containing four EREs differs between LAMD-SM and control SM cells. LAMD-SM (A) and control SM cells (B) were cotransfected with an 4ERE-TATA-Luc reporter plasmid and β -galactosidase (β -gal) to correct for transfection efficiency. Twenty-four hours later, the cells were stimulated with E₂ (0.1 and 1 nM). Shown are the mean \pm SEM of cell lysates collected from five individual LAMD-SM and five age-matched control SM cell lines. Data are expressed as percent of vehicle (V) control value of luciferase light units/ β -gal. Triplicate wells were used for each treatment in each experiment. There was a greater than 2.5-fold increase in LAMD-SM (A; $P < 0.05$ by one-way ANOVA), compared with control cells (B; $P < 0.01$ by one-way ANOVA; *, $P < 0.05$, compared with control vehicle).

Unbound ERs are degraded in LAMD-SM cells through a proteasome-mediated mechanism

To investigate the degradation of the ER through the proteasome pathway, we treated LAMD-SM cells and control SM cells ($n = 4$) with MG132, which specifically blocks activity of the 20S proteasome (19, 20). The levels of ER α and ER β protein increased 2-fold ($P < 0.05$) in LAMD-SM cells after treatment with MG132 and further increased with the addition of E $_2$ (2.5-fold, Fig. 3, $P < 0.05$). No changes in control SM cells were detected. These data suggest that the levels of unbound ER are lower in LAMD-SM cells because of ongoing proteasomal degradation.

MMP-2 protein and activity are increased by E $_2$ only in LAMD-SM

Immunohistochemical studies previously demonstrated abundant staining for MMP-2 and MMP-9 in LAM lesions (7, 10). However, the presence of ERs was not consistently reported in each of the cell types found in LAM lesions (5–7). To determine whether estrogens could modulate MMP-2 activity, we stimulated LAMD-SM and control SM cells with E $_2$ and measured MMP-2 protein and activity by Western analysis and zymography, respectively. To complement these data, we also used the Biotrak activity kit (Amersham Biosciences). Physiological concentrations of E $_2$ increased MMP-2 protein levels (Fig. 4A, $P < 0.05$) and MMP-2 activity nearly 2-fold (Fig. 4C, $P < 0.001$) in LAMD-SM but not control SM cells ($n = 5$, Fig. 4, B and D). The complete ER antagonist, ICI 182,780, inhibited the E $_2$ -mediated increase in MMP-2 activity, which is consistent with an ER-dependent regulation. Activity as measured by the kit showed that E $_2$ treatment increased MMP-2 activity in cells isolated from LAMD-SM patients approximately 165% ($n = 4$), compared with no change in control SM cells ($n = 3$). MMP-9 activity as measured by zymography increased only in LAMD-SM cells after E $_2$ treatment. Increasing concentrations of E $_2$ (0.1–1 nM) stimulated an increase of MMP-9 activity of between 135 and 168% over vehicle-treated cells. We focused next on the regulation of expression of MMP-2 transcriptional activation in LAMD-SM cells.

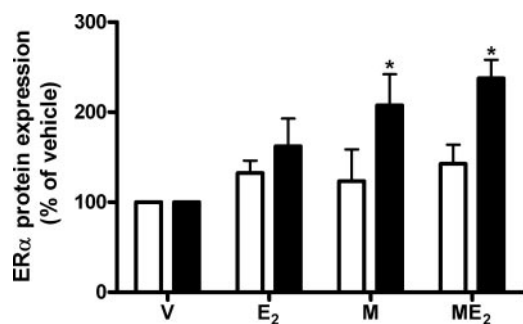


FIG. 3. MG132 prevents degradation of ER α protein in LAMD-SM cell lines. LAMD-SM (solid bars) and control SM cells (open bars) were pretreated with 1 nM of E $_2$, MG132 (M; 1 μ M), or a combination of MG132+E $_2$ (ME $_2$) as described in *Subjects and Methods*. Cell lysates were collected from four individual LAMD-SM or age-matched control SM cell lines. Western analysis was performed to assess ER α protein expression. Data are graphed as the mean \pm SEM percent of vehicle-treated cells (V). ER α expression increased in LAMD-SM cell lines in response to MG132 but not in control SM cell lines ($n = 4$, $P < 0.01$ by one-way ANOVA; *, $P < 0.05$, compared with vehicle treatment).

LAMD-SM MMP-2 transcriptional activation is not regulated by E $_2$

To determine whether the E $_2$ -mediated increase in MMP-2 activity was due to increased MMP-2 mRNA expression, we transfected LAMD-SM and SM cells with the full-length human MMP-2 promoter linked to a luciferase reporter gene (kindly provided by Dr. Hugh Watkins, University of Oxford, Oxford, UK). E $_2$ did not increase the luciferase activity in LAMD-SM ($n = 5$) or control SM cells ($n = 4$, Fig. 5). Thus, the ER-mediated increase in MMP-2 activity released from LAMD-SM cells does not appear to be due activation of the MMP-2 promoter.

LAMD-SM TIMP-2 and MT1-MMP activity are not increased by E $_2$

Treatment with E $_2$ did not increase the levels of TIMP-2 activity in both LAMD-SM cells and control SM cells, although TIMP-2 protein expression was increased from LAMD-SM cells, compared with vehicle treatment ($n = 3$). In addition, MT1-MMP activity was not increased after E $_2$ treatment in either LAMD-SM cells or control SM cells.

E $_2$ -mediated cell invasion is prevented by inhibition of MMP-2

E $_2$ -stimulated LAMD-SM cells invaded the collagen matrix of the invasion chamber in a time-dependent manner, compared with LAMD-SM cells treated with vehicle (Fig. 6A). The addition of an MMP-2 antiserum (M) reduced the invasion of estrogen-treated LAMD-SM cells by approximately 50–75% ($n = 4$ patients, Fig. 6B, *bottom panel*). Doxycycline, a nonspecific MMP inhibitor, was equally as effective in reducing the invasion of estrogen-treated LAMD-SM cells (data not shown). Treatment with IgG antisera did not change the degree of E $_2$ -stimulated invasion by LAMD-SM cells. The ER antagonist, ICI 182,789, partially inhibited the invasion of the E $_2$ -stimulated LAMD-SM cells (data not shown). This was expected because this complete ER antagonist blocked the estrogen-mediated increase in MMP-2 activity as assessed by zymography.

The number of control SM cells migrating through the matrix was much less and was not altered by E $_2$ treatment ($n = 4$ patients, Fig. 6B, *top panel*, quantification, Fig. 6C, *left panel*). This suggested that any migration of control SM cells that occurred was estrogen/ER independent. Treatment of control SM cells with MMP-2 antisera or doxycycline did not alter their little baseline ability to migrate (Fig. 6B, *top panel*, quantification, Fig. 6C, *right panel*).

Discussion

LAM is a destructive, eventually fatal lung disease, which exclusively affects women. The majority of women are at child-bearing age at the time of diagnosis (1, 21). Pregnancy and use of oral contraception are associated with a higher frequency of exacerbations and a more aggressive disease course (22, 23). LAM has also been found in postmenopausal women taking hormone replacement therapy (24). Based on these clinical findings, estrogens were hypothesized to invoke or, at least, contribute in a

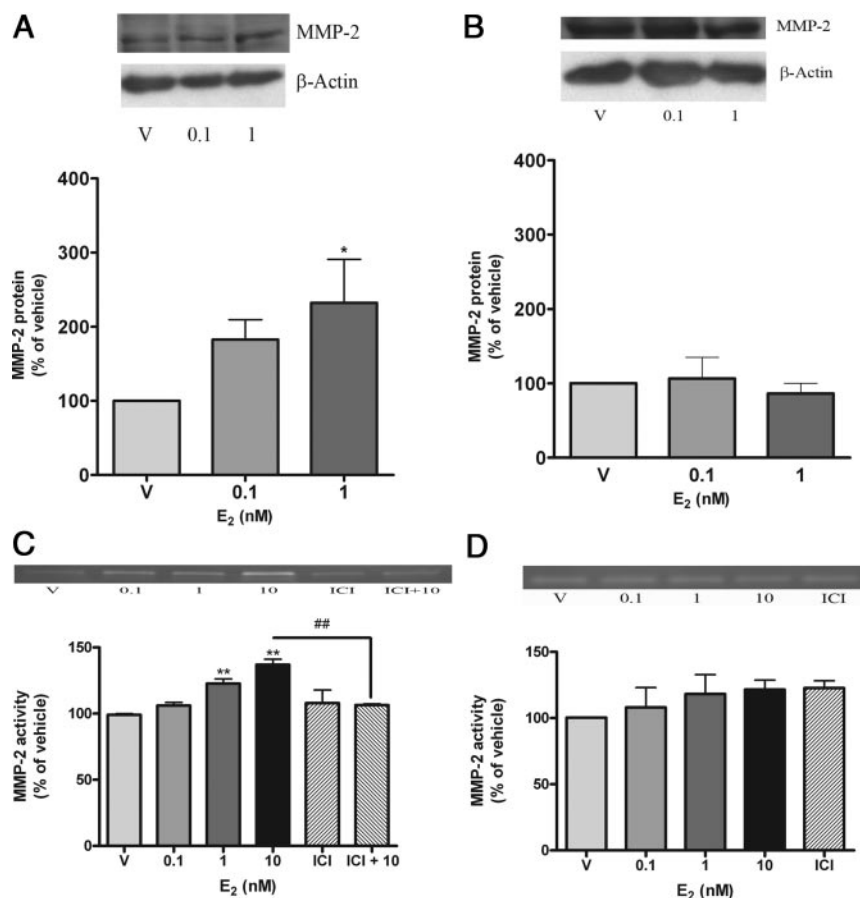


FIG. 4. E₂ increases expression of MMP-2 protein and activity in LAMD-SM cells but not control SM cells. Cell lysates or supernatants of LAMD-SM or control SM cell lines were collected for protein analysis (A and B) or zymography (C and D) as described in *Subjects and Methods*. A, Representative Western blot and graph of MMP-2 protein after vehicle or E₂ stimulation (0.1 and 1 nM) of LAMD-SM (A) and age-matched control cell lines (B). Membranes were stripped and reprobed with an antibody to actin as a control for protein loading. B, Data on graphs are expressed as percent vehicle. Shown are the mean \pm SEM. Representative zymograms and graphs depicting data are shown in C from LAMD-SM cells, which were treated with vehicle (V), E₂ (0.1–10 nM), or ICI (1 μ M) or ICI (1 μ M) + 10 nM E₂ (ICI + 10). Representative zymograms and graphs depicting MMP-2 activity after E₂ stimulation of control cells are shown in D. Data on graphs are expressed as percent vehicle control. Shown are the mean \pm SEM (*, $P < 0.05$, **, $P < 0.001$, compared with vehicle; ##, $P < 0.001$ 10 nM E₂ vs. ICI + 10 nM E₂). Cell lysates collected from five individual patients with LAMD-SM and five age-matched female controls.

principal manner to the progression of LAM (estrogen hypothesis). In contrast, therapeutic interventions aimed at diminishing the effects of estrogens showed little, if any, efficacy in improving the outcome of LAM. In addition, the immature-appearing, spindle-shaped, HMB-45 antigen-negative LAM cells, which are in the center of LAM lesions and appear to be the cell type crucial for mediating the infiltrative and destructive process by secreting MMPs, apparently lack ER expression (7).

These conflicting data gave the impetus to the current study, which we undertook to obtain further insights into the role estrogens play in the pathogenesis of LAM. Therefore, we focused our investigations on the expression and function of ERs, the expression of MMPs and their regulation by estrogens, and the potentially estrogen-driven, MMP-mediated invasiveness of LAM cells. We had to rely on *in vitro* studies because of the absence of appropriate animal models that permit reproducing the lung lesions characteristic of LAM (25, 26).

We first isolated and propagated cells from the outgrowth of

LAM lesions, which represent the most aggressively proliferating cell type (14). As expected, these cells, which we termed LAMD-SM, were spindle-shaped, exhibited positive immunoreactivity for the fibroblast antigen and antismooth muscle α -actin, and were immunonegative for HMB-45. Despite their aggressively infiltrating and destructive behavior *in vivo*, we found that LAMD-SM cells are quite distinct from cancer cells. LAMD-SM cells are diploid and show no signs of chromosome instability such as chromosomal breaks, rearrangements, or telomeric associations by cytogenetic analysis of the G-banded metaphases (Donahue, R., personal communication). We also isolated smooth muscle-like cells from the lungs of age-matched women without LAM to serve as control cells in our studies (SM cells). The ideal control cell for LAM remains a debatable issue. We chose normal female age-matched pulmonary smooth muscle cells as control cells primarily because they do not demonstrate the cancer-like aggressiveness of a LAM cell.

The action of estrogens is primarily mediated via two ERs, termed subtype ER α and ER β (27, 28). We therefore focused first on ER expression and function

in LAMD-SM cells to determine whether spindle-shaped, HMB-45-negative LAM cells are, in fact, estrogen responsive, a basic requirement for the estrogen hypothesis. We found that LAMD-SM cells (as well as control SM) expressed both ER α and ER β mRNA and protein. The function of ERs as ligand-activated transcription factors was confirmed by transfecting LAMD-SMs with an estrogen-responsive promoter linked to a luciferase reporter gene. These studies ultimately confirmed that spindle-shaped, HMB-45-negative LAMD-SMs represent an estrogen-responsive cell type.

Increased estrogen sensitivity and responsiveness, which partly depend on the level of functional ERs (15, 18, 29), could potentially account for the development of an estrogen-driven disease and could also be responsible for the accelerated rate of its progression. We therefore compared the level of ER α and ER β expression and their transcriptional activity in response to E₂ in LAMD-SM and control SM cells. Interestingly, in the absence of E₂, baseline mRNA levels for ER α and ER β were about 10-fold

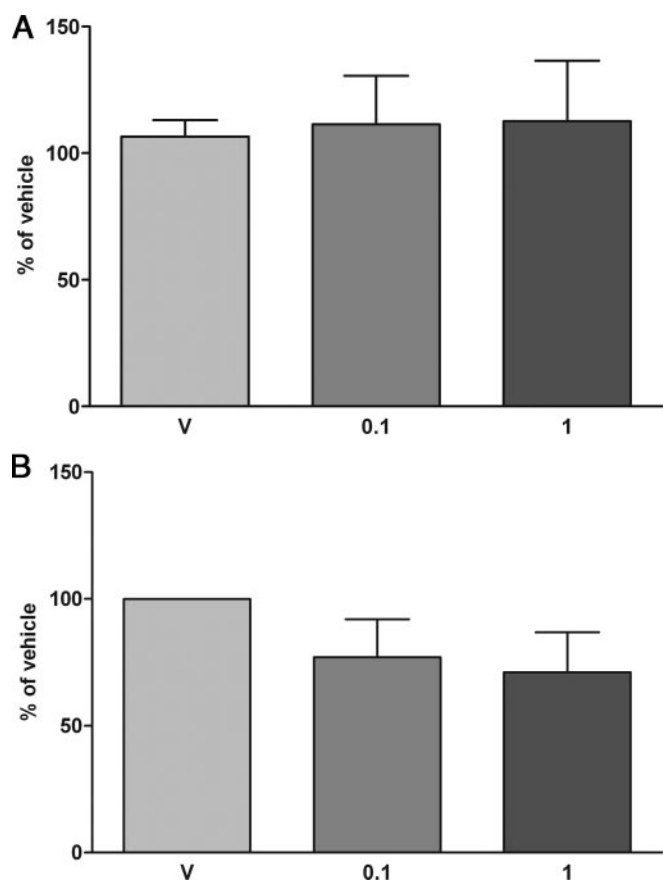


Fig. 5. E₂ does not regulate the MMP-2 promoter activity. Transfection with a full-length MMP-2 promoter construct in LAMD-SM (A) and age-matched control SM cell lines (B) demonstrates no induction by E₂ (0.1–1 nM). Data are derived from five LAMD-SM and four age-matched control SM cell lines and are expressed as percent of vehicle (V)-treated cells.

higher, whereas protein concentrations were lower in LAMD-SM than those in control SM cells. In contrast, the ability of E₂ to initiate transcription from an estrogen-responsive reporter gene via ER activation was about 2-fold higher in LAMD-SM, compared with control SM cells. This suggested that LAMD-SM cells are more responsive to estrogens than control SM cells, despite their apparently lower steady-state levels of unbound ER protein.

Steroid receptor degradation plays an obligatory role in efficient ER transcription and is integrally connected with the receptor activation process. In the same context, the intrinsic activation of ubiquitin-proteasome-mediated protein degradation appears to be necessary for the temporally dynamic exchange of cofactors binding to the ER, a process that is required for an efficient ER-mediated transcription to ensue (30, 31). Furthermore, the carboxyl terminus of constitutively expressed heat shock protein 70-interacting protein has been identified as a mediator of basal degradation of functional ERs (32). To address this issue, we treated LAMD-SM and control SM cells with the proteasome inhibitor, MG132. ER protein levels increased in LAMD-SM but not control SM cells in the presence of MG132. This suggested that ERs in their unbound state undergo rapid degradation in LAMD-SM cells by an intrinsically active ubiquitin-proteasome-mediated pathway (19, 20, 31, 33). This may

account for the apparently lower ER protein levels in LAMD-SM and might explain why ER expression could not be detected *in vivo* in the HMB-45-negative LAM cells by immunohistochemical analysis.

Sufficiently high levels of coactivators are an additional prerequisite for effective transcription to occur (28, 34). This is especially important in the context of LAMD-SM cells because coactivators are themselves targets of ubiquitin-proteasome-mediated protein degradation (35, 36). However, we have found in preliminary studies that there is no difference in steroid receptor coactivator-1 levels between LAMD-SM and control SM cells (Glassberg, M. K., S. J. Elliott, and M. Karl, unpublished data).

Based on our results, we speculated that E₂-induced activation of ER in LAMD-SM cells could have profound effects on the estrogen-mediated expression of proteases involved in the destruction of lung parenchyma in LAM. In fact, early on, Hayashi *et al.* (37) showed abundant staining for MMP-2 and MMP-9 within the spindle-shaped LAM cells located in the center of LAM lesions. The investigators postulated that these metallo-proteinases were responsible for the structural damage to elastic fibers and collagen fibrils in LAM lesions, in the absence of neutrophil or pancreatic types of elastases (4). In the present study, we confirmed that LAMD-SM cells synthesize and secrete MMP-2 (Fig. 4) and MMP-9 (data not shown). We subsequently focused on the effects of E₂ on MMP-2 synthesis and function. The degree of MMP expression and activity is regulated at multiple levels including the rate of gene transcription, posttranscriptional processing, and by the positive or negative regulation of the proteolytic MMP capabilities (38–42). We found that E₂ in physiological concentration increased MMP-2 activity released from LAMD-SM, whereas there was no estrogen-mediated modulation of MMP-2 in control SM cells. Pretreatment with the complete ER antagonist ICI 182,780 prevented the E₂-mediated increase in MMP-2 activity consistent with the notion that the activation of MMP-2 by estrogens is ER-dependent in LAMD-SM cells. Furthermore, we found that E₂ treatment increased MMP-2 protein levels but did not modulate the transcriptional activity of an MMP-2 promoter in LAMD-SM cells. E₂ did not increase the levels of TIMP-2 activity in LAMD-SM cells, suggesting that the three components of the trimolecular TIMP-2/MMP-2/MT1-MMP-2 complex do not display equivocal roles in the activation of MMP-2 (11, 39–41). Taken together, these findings showed that estrogens increase MMP-2 activity in LAMD-SM cells and confirmed that LAMD-SM cells synthesize, secrete, and activate MMP-2. Most importantly, they provided, for the first time, direct evidence for the estrogen/ER-mediated regulation of MMP expression and activity in LAM cells. Nevertheless, the biological relevance of the estrogen/ER-driven MMP synthesis and activation in respect to lung destruction and invasion remained to be established.

In collagen invasion assays, we demonstrated that the ability of LAMD-SM cells to digest collagen matrix is enhanced by the E₂-mediated activation of ERs. Most importantly, the ability of LAMD-SM cells to destroy and invade extracellular matrix is inhibited by the ER antagonist ICI 182,780, antiserum against MMP-2, or doxycycline. In contrast, control SM cells, which

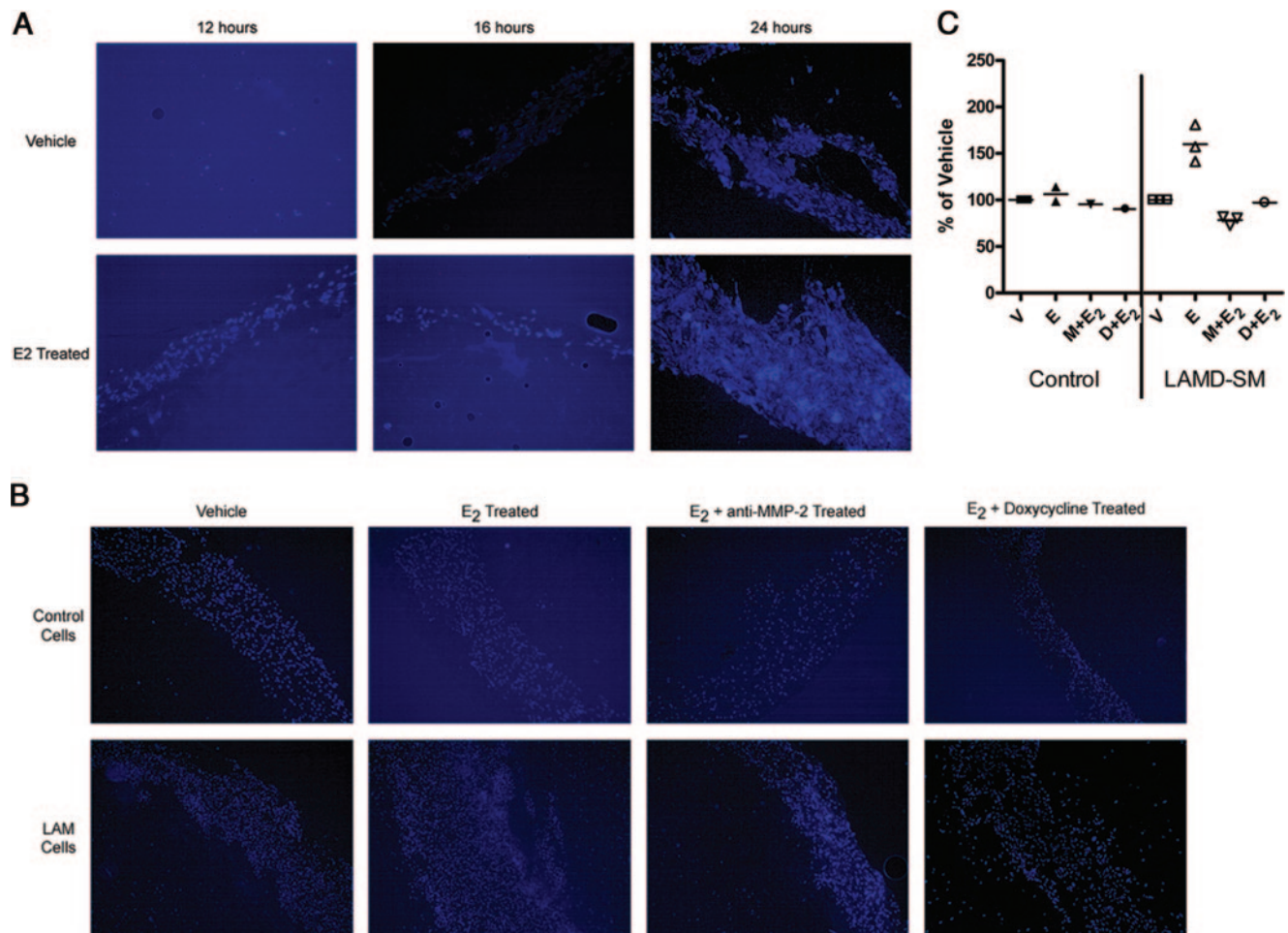


FIG. 6. MMP-2 regulation of LAMD-SM cell invasiveness. LAMD-SM and age-matched control SM cell lines were plated on matrigel-coated inserts as described in *Subjects and Methods*. **A**, Time course of cell invasion into matrigel with either vehicle (V) or E_2 treatment is shown. *n*, Two individual patients. Magnification, $\times 5$. **B**, E_2 -mediated invasion of LAMD-SM cells is prevented by an antibody to MMP-2 and doxycycline. LAMD-SM and age-matched control SM cell lines (*n* = 4) were plated on invasion chambers as described in *Subjects and Methods*. E_2 treatment induced invasion only in LAMD-SM cell lines. Invasion was partially prevented by the exposure of cells to an antibody against MMP-2 or doxycycline. Blue color represents nuclear DAPI staining. *n*, Four individual patients repeated twice. Magnification, $\times 5$. **C**, Quantification of invading cells was determined as described in *Subjects and Methods*. The data were graphed as percent of vehicle (V)-treated cells for both control SM cell lines and LAMD-SM cell lines. V, Vehicle; E_2 +M, E_2 +antibody against MMP-2; E_2 +D, E_2 +doxycycline.

exhibit only a very basic ability for cellular locomotion, remained unaffected by estrogen exposure or MMP inactivation. However, the clinically relevant consequences of our findings are that the invasive phenotype of LAMD-SM cells depends on the E_2 /ER-mediated up-regulation of MMP-2 activity. In other words, estrogens play a critical role in the destruction of lung parenchyma in LAM through stimulating MMP synthesis and activation. The therapeutic impact of treatments antagonizing this pathway could therefore have great implications for women with LAM.

In summary, we found that LAMD-SM cells express the ER subtypes, ER α and ER β . In the unbound state, ERs rapidly undergo proteasome-mediated turnover in LAMD-SM cells. However, the transcriptional activity of ERs, upon hormonal stimulation, is greater in LAMD-SM than control SM cells. Furthermore, we provide the first evidence for estrogen/ER-mediated activation of MMPs in LAMD-SM cells, and we demonstrate that inhibition of the estrogen-mediated increase in MMP activity reduces the invasiveness of LAMD-SM cells. This study

demonstrates that blocking ER action and inhibiting MMP activity would be reasonable approaches to successfully treat LAM lung disease. Future therapeutic modalities could be based on the pure ER antagonist, Fulvestrant (Faslodex or ICI 182,780), which does not have partial agonist activity like tamoxifen, and the antibiotic doxycycline, a nonspecific MMP inhibitor. In a pilot study, we have started treating women with LAM with doxycycline. We observed substantial improvements in the women's exercise capacity and oxygen desaturation. Of note, the advanced stage of LAM forbids invasive procedures such as lung biopsies or bronchoalveolar lavage to collect lung cells or tissue from doxycycline-treated women. Furthermore, noninvasive biomarkers such as measuring the excretion of urinary MMPs did not prove useful to monitor the effects of therapy. However, the encouraging results of this small trial could be interpreted as the clinical proof of the basic concept. Thus, rapid translation of this research into clinical practice may hold promise for women affected by this devastating lung disease.

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