Enhanced Endothelial Cell Senescence by Lithium-induced Matrix Metalloproteinase-1 Expression*

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Endothelial cell (EC) senescence and dysfunction occurring after chronic injury and inflammation are highly associated with the development and progression of cardiovascular diseases. However, the factors involved in the establishment of EC senescence remain poorly understood. We have previously shown that lithium, an inhibitor of glycogen synthase kinase (GSK)-3β and activator of the Wnt/β-catenin signaling pathway, induces an EC senescent-like phenotype. Herein, we show that lithium induces a rapid and pronounced up-regulation of the matrix metalloproteinase (MMP)-1, an inflammation and senescent cell marker, at the mRNA and protein levels, whereas the induction of two other senescent cell markers is either weak (interleukin-8) or delayed (plasminogen activator inhibitor-1). Lithium effect on MMP-1 expression is also specific among other MMPs and not mediated by GSK3β inhibition. Lithium affects MMP-1 expression mainly at the transcriptional level but neither the AP1/Ets regulatory sites nor the redox sensitive (~1607/2G) site in MMP-1 promoter are involved in lithium-dependent MMP-1 regulation. However, down-regulation of p53, a target of lithium in EC, dampens both basal and lithium-induced MMP-1 expression, which further links MMP-1 up-regulation with the establishment of cell senescence. Although increased MMP-1 levels have been extensively studied, less is known about their effects in the ECM microenvironment, MMP-1 up-regulation has been associated with increased tumor growth and metastasis. The human matrix metalloproteinase (MMP)-1 and the plasminogen-activator inhibitor (PAI)-1 belong to this class of proinflammatory and proangiogenic factors, which control vascular remodeling and angiogenesis by regulating extracellular matrix (ECM) degradation and release of growth factors such as transforming growth factor-β and vascular endothelial growth factor. By inducing both angiogenesis and changes in the ECM microenvironment, MMP-1 up-regulation has been associated with increased tumor growth and metastasis. PAI-1 has a dual effect on angiogenesis depending upon its level of expression and the status of the ECM proteolytic system. On the other hand, the expressions of MMP-1 and PAI-1 are increased in senescent EC in culture and in vivo within atherosclerotic lesions. MMP-1 activity is in particular associated with instability of the atherosclerotic plaque and subsequently with infarct events in humans. Increased PAI-1 levels are also associated with a poor prognostic in atherosclerosis and cardiovascular disease progression.

Interestingly, PAI-1 up-regulation has been associated with the establishment of cell senescence both in vitro and in vivo. PAI-1 is a downstream target gene of the tumor suppressor p53 (14) and is required for p53-induced cell senescence in primary fibroblasts via its ability to inhibit a proliferative pathway mediated by the urokinase-type plasminogen activator and its receptor, urokinase-type plasminogen receptor. More recently, another proinflammatory system, the chemokine receptor CXCR2 and its ligands, which include IL-8, a target gene of the Wnt/β-catenin signaling pathway (15), was shown to be crucial for the reinforcement of cell senescence in epithelial tissues (16, 17). A possible involvement of MMPs in the establishment of cell senescence has also been recently studied. Down-regulation of MMP-7, which is another target gene of the Wnt/β-catenin signaling pathway (18), has been shown to enhance senescence of primary human mammary epithelial cells (19). Similarly, the down-regulation of MMP-9 in medulloblastoma cells triggered a cell cycle arrest and a senescent-like phenotype both in vitro and in a tumorigenesis model in vivo (20). Although MMP-1 expression is highly correlated with cell

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2 The abbreviations used are: EC, endothelial cell; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor-1; ROS, reactive oxygen species; ECM, extracellular matrix; IL, interleukin; GSK3β, glycogen synthase kinase-3β; SA, senescence associated; BAEC, bovine aortic endothelial cell; CMV, cytomegalovirus; UTR, untranslated region; siRNA, small interfering RNA; DQ-FITC, dye-quenched fluorescein isothiocyanate; CM-H2DCFDA, chloromethyl-2′,7′-dichlororhydrofluorescein diacetate.
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senescence and age-related diseases (6, 8, 10, 21), the involvement of MMP-1 in the establishment of cell senescence remains to be investigated.

Lithium, a therapeutic agent in bipolar disorder and neurodegenerative diseases (22), exerts pleiotropic effects on various cellular processes via inhibition of several Mg^{2+}-dependent enzymes including inositol monophosphatase-1 (23) and glycerol-3-phosphate kinase (GSK)-3β (24, 25). We have previously shown that lithium at doses commonly used to inhibit GSK3β and activate the Wnt/β-catenin signaling pathway in transformed cell lines and tumor cells, induces instead a G_{2}/M cell cycle arrest in primary EC and the appearance of a senescent-like phenotype with cells positive for the senescence-associated (SA)-β-galactosidase marker and a flattened morphology (26).

The lithium-induced EC senescence was also associated with the activation of the tumor suppressor p53 and increase of p21 expression (26), a cascade triggering senescence in various cell types (27). Both positive and negative regulations of cell proliferation and cell senescence by GSK3β inhibition have also been documented (13, 28–30). Similarly, the stabilization of β-catenin, which is controlled by GSK3β inhibition during activation of the Wnt/β-catenin signaling pathway (28), was shown to induce either cell senescence in primary fibroblasts (31), thymocytes (32), and muscle progenitor cells (33), or proliferation of undifferentiated cells and tumorigenesis (34).

In this report, we have further characterized the lithium-induced senescent phenotype in primary EC and its relationship with GSK3β inhibition. Our data show that the up-regulation of MMP-1 expression is an early event during lithium-induced EC senescence, which is mediated neither by GSK3β inhibition nor β-catenin stabilization. Down-regulation of GSK3β and stabilization of β-catenin lead rather to an anti-inflammatory and anti-senescence program. However, our studies reveal that both basal and lithium-induced MMP-1 expressions are dependent upon the levels of the tumor suppressor and cell senescence inducer p53. Moreover, our data demonstrate that MMP-1 up-regulation participates via an amplification loop in the lithium-induced senescent phenotype in EC. Taken together our data further links MMP-1 expression and the establishment of an EC senescence program.

EXPERIMENTAL PROCEDURES

Materials—The chemicals lithium chloride (purity >99%) and actinomycin-D were purchased from Sigma. The purified native anti-GSK3β, anti-phospho-S63-Jun, anti-Jun, anti-phospho-S9-GSK3β and anti-GSK3β antibodies were from Cell Signaling. The secondary horseradish peroxidase-conjugated antibodies were from Cell Signaling.

Cell Culture and Transfection—Primary BAEC were either kindly provided by Dr. Paul Dicorleto (26) (The Cleveland Clinic Medical School-CWRU, OH) or purchased from Cambrex. BAEC were maintained in Dulbecco’s modified Eagle’s medium containing 1 g/liter d-glucose and supplemented with 5% fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). HEK293T cells (Invitrogen) were maintained in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter d-glucose and supplemented with 10% fetal bovine serum and antibiotics. All DNA transfections were performed using the Exgen500 reagent (MBI Fermentas) as recommended by the manufacturer and at least two different DNA preparations for each construct were tested.

Mammalian Expression Constructs—The S37A-β-catenin-hemagglutinin-tagged construct was a kind gift from Dr. Steve Byers (Georgetown University, Washington, D. C.) and previously described (35). The wild-type-, S9A-, and K85R-GSK3β- His-tagged constructs were previously described (36).

MMP1 Promoter Luciferase Reporter Constructs—The human MMP-1 promoter sequences from −2235 to +60 were amplified from HEK293 genomic DNA using the primers: 5′-CTAGATTCGACATTTGACATGGA-3′ and 5′-CTAGATGAGCCTTCTCTTCTCATG-3′, and then cloned into the Xhol/HindIII sites of pGL3-basic vector (Promega). The pGL3−600/+60 MMP-1 promoter luciferase reporter construct was a kind gift from Dr. Kathleen McGuire (San Diego State University, CA) (37). The genomic DNA from HEK293 cells contains the 2 guanine nucleotides (G) polymorphism at position −1607, which creates by insertion of 1 guanine nucleotide an additional Ets binding site cooperating with the AP1 binding site at −1602 to increase both basal and redox-dependent activity of the (2G)−2235/+60-MMP1 promoter luciferase construct (38, 39). To generate the (1G)−2235/+60-MMP1 promoter luciferase construct, the 2G (GGA) were mutated to 1G (GA) using the QuikChange mutation kit (Stratagene) and the following mutated primers: 5′-GTAGTTAATAATGAAGAATGACTTACTCTCATAAATC-3′ and 5′-GATTTGAGATAGCCTATCTTCTTCTTAATTTAATAC-3′.

Reporter Assays—BAEC and HEK293 cells were plated in 12-well culture clusters and transiently co-transfected 24 h later with 5 ng of pCMV-β-galactosidase for normalization purposes, 125 ng of either pGL3-basic as control or the MMP1 promoter luciferase reporters: −600/+60-MMP1P-Luc, (1G)−2235/+60-MMP1P-Luc, and (2G)−2235/+60-MMP1P-Luc. In all cases, the pCR3.1 vector was added to obtain a total of 1 μg of DNA per well. After either 24 or 36 h post-transfection the cells were lysed and the firefly luciferase and β-galactosidase activities were quantified using luminescent Luciferase assay kits (Promega) and Galacto Light Plus assays (Tropix, Applied Biosystems), respectively, and an LMax-II luminometer (Molecular Devices). The activities of firefly luciferase were normalized with those of β-galactosidase. At least three independent transfection experiments were performed in duplicate.

Analysis of Changes in MMP-1 mRNA Stability by 3′-UTR Renilla Luciferase Reporter Constructs—The 3′-UTR of MMP-1 and IL-8 mRNAs were amplified by PCR with the primer sets for: 3′-UTR-MMP-1, 5′-GACTCTTCTAGACCTAATAGCTGTGTTCTAACTGAG-3′/5′-GACTCTTCTAGACCTAATAGCTGTGTTCTAACTGAG-3′; and 3′-UTR-IL-8, 5′-GTCATATTCTAGACCTAATAGCTGTGTTCTAACTGAG-3′/5′-GACTCTTCTAGACCTAATAGCTGTGTTCTAACTGAG-3′.
and cloned into the XbaI site of the phRG-thymidine kinase promoter-vector (Promega) located downstream of the Renilla luciferase stop codon and upstream of the SV40 late poly(A) site. BAEC were co-transfected with 5 ng of pCMV-β-galactosidase vector for normalization purposes and with 250 ng of either phRG-TK (3'-UTR-Null) vector as control, 3'-UTR-MMP1, or 3'-UTR-IL8 for 24 h prior to being treated with either 10 mM NaCl or 10 mM LiCl for an additional 24-h period. The activities of Renilla luciferase (Promega) and β-galactosidase (Tropix, Applied Biosystems) were determined using specific luminescent assays. At least three independent transfection experiments were performed in duplicate.

RNA Extraction, Reverse Transcription, and Real-time PCR—RNA extractions were performed using TRIzol reagent (Invitrogen). Total RNA (1 μg) was subjected to DNase I treatment and reverse transcription as previously described (36). Standard PCR was performed using an equivalent of 20 ng of total RNA for 35 cycles. Quantitative real-time PCR was performed in duplicates, with an equivalent of 16 ng of total RNA per reaction using the SYBR Green PCR core reagent and the manufacturer recommendations. Twenty-four hours post-transfection, the cells were treated with either 10 mM NaCl or 10 mM LiCl prior to being harvested 24 h later for the analysis of mRNA and protein levels.

Short Interfering RNAs and Transfection—The custom siRNA duplexes were designed to target both the human and bovine mRNA sequences whenever possible and synthesized by Dharmacon Inc. (Lafayette, CO). The sequences are as follows: bo/huGSK3β, ACACUAUGUGGCAAAAUu; bo-p53, CGGAACACCUUUAGACACAAu; boMMP1-1, GGACCAA-GCCAUGAGAAAUu; boMMP1-2, GCUGAGAGUCUGGGAAAUu. The on-target-plus Non-targeting siRNA-1 was used as control (Dharmacon). Transfection of siRNAs (35 nm) in BAEC was achieved using Dharmafect I reagent accordingly to the manufacturer recommendations. Twenty-four hours post-transfection, the cells were treated with either 10 mM NaCl or 10 mM LiCl prior to being harvested 24 h later for the analysis of mRNA and protein levels.

Senescence-associated β-Galactosidase Assays—BAEC were plated in 24-well clusters and treated 24 h later with either 10 mM NaCl or 10 mM LiCl followed 24 h later by the addition of 5 μM activated MMP-1. The cells were processed for β-galactosidase staining 4 days after lithium treatment. For the experiments involving transfection of siRNAs, BAEC were transfected 24 h after plating with 35 nm siRNAs as described above. Eighteen hours post-transfection, the cells were treated with either 10 mM NaCl or 10 mM LiCl for 3 days. In all the cases, BAEC were fixed in 3.7% formaldehyde and stained overnight for β-β-galactosidase activity as previously described (26, 41). The number of positive SA-β-galactosidase cells and the total number of cells were determined in parallel. Color images were taken with an inverted microscope (Nikon, TE2000).

Determination of the Intracellular H2O2 Levels—BAEC were treated with either 10 mM NaCl or 10 mM LiCl from 30 min up to 36 h and in some cases 1 μM/ml lipopolysaccharide was added for positive control in the last hour of treatments. Then the cells were washed three times with Hanks’ balanced salt solution buffer supplemented with 1 mM CaCl2. The redox probe CM-H2DCFDA was added in the same Hanks’ balanced salt solution buffer for a 2 μM final concentration. The staining was performed at 37 °C for 2 h in the dark before quantification of the fluorescence intensity using λ = 485 nm excitation and λ = 535 nm emission in a Fusion™ plate reader (PerkinElmer Life Sciences). The cell numbers were determined in parallel wells using CyQUANT fluorescent staining as recommended by the manufacturer (Invitrogen). The levels of intracellular H2O2 were corrected for changes in cell numbers. At least three experiments were performed in triplicate.
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Statistical Analysis—After verification of the normal distribution of the values obtained in at least three independent experiments, either one sample t tests or one-way analysis of variance with Bonferroni post tests were performed as required using GraphPad Prism version 4.0 (GraphPad Software). Results were considered statistically significant at \( p < 0.05 \).

RESULTS

MMP-1 mRNA Up-regulation Is an Early Event Induced by Lithium in Endothelial Cells—We have previously shown that lithium induced a G2/M cell cycle arrest and the appearance of a senescent cell phenotype in primary EC that was accompanied by the activation of p53 and the up-regulation of the cell senescence in primary BAEC, we have assessed the changes in MMP-1 and PAI-1 expression, two markers associated with cell senescence in fibroblasts (42), keratinocytes (43), and EC (8, 9). As shown in Fig. 1A, lithium treatment for 24 h resulted in a significant 13-fold increase of MMP-1 and a 2.7-fold increase of PAI-1 mRNA levels. The levels of IL-8 mRNA, an inflammatory marker as well as a Wnt/β-catenin target gene (44), were only slightly increased (1.5-fold) without reaching statistical significance (Fig. 1A). The effects of lithium on MMP-1 expression were also rather specific among various MMPs, because in the same time the mRNA levels of MMP-2 increased ~1.8-fold, the mRNA levels of MMP-14 decreased by 50% (Fig. 1A). The expression of MMP-3 mRNA was undetectable in BAEC even after lithium treatment (not shown). The effects of lithium on MMP-1 mRNA expression were also dose-dependent as a significant increase of MMP-1 mRNA levels could be detected with 2.5 and 5 mM LiCl treatments (not shown). Moreover, the lithium-dependent increase in MMP-1 mRNA levels was rapid, as a time-dependent increase was observed from 12 h (5-fold) to 36 h (20-fold) (Fig. 1B). In contrast, the effects of lithium on PAI-1 expression were biphasic with first a significant decrease at 12 (80%) and 18 h (50%) before being significantly increased at 36 and 48 h (8-fold) (Fig. 1B). Although the initial decrease of PAI-1 expression is consistent with an anti-inflammatory role for GSK3β inhibition (45), the late increase of PAI-1 expression is typical of a secondary response. The latter might be triggered by lithium-induced p53 activation (26) because PAI-1 is also a p53 target gene (14). Nonetheless, our data indicate that in contrast to PAI-1 up-regulation, MMP-1 up-regulation is an initial event during lithium-induced EC senescent phenotype.

Lithium Increases Collagenase Activities and MMP-1 Protein Levels—To assess the changes in collagenase activity induced by lithium, in situ collagen degradation was imaged using BAEC grown on DQ-FITC-collagen type I-coated slides as previously described (46). As shown in Fig. 2A, in sodium-treated BAEC, a faint and intracellular vesicular staining was observed, which was mainly due to the uptake of DQ-FITC-collagen and its intracellular degradation as reported in various cell types (47). In contrast BAEC treated with lithium exhibited an
increase of bright collagen degradation spots in close contact with the collagen matrix (arrows, Fig. 2A), indicative of increased collagenase activity at focal sites. At the same time, an increase of pro-MMP-1 levels were detected in conditioned media and whole cell extracts from lithium-treated BAEC as compared with sodium-treated control cells (Fig. 2B).

**Lithium-induced MMP-1 Expression Is Mediated Neither by GSK3β Inhibition Nor β-Catenin Stabilization**—We have previously shown that lithium-dependent cell cycle arrest and the cell senescent phenotype in BAEC were not triggered by inhibition of the inositol phosphate cycle (26). Similarly, the addition of 1 mm myo-inositol to prevent lithium-induced inositol depletion had no effect on the induction of MMP-1 mRNAs by lithium (Fig. 3A), indicating that lithium-dependent inhibition of the inositol monophosphatase-1 is not sufficient for triggering MMP-1 up-regulation. To determine the involvement of GSK3β inhibition on MMP-1 expression, we tested the effects of indirubin, another direct GSK3β inhibitor (48) and valproate, an indirect GSK3β inhibitor (49). As shown in Fig. 3B, MMP-1 mRNA levels were increased 25-fold in response to lithium treatment, whereas only a 2-fold increase was observed after valproate treatment and no change was observed after indirubin treatment. Moreover, the ectopic expression of neither the constitutively active S9A-GSK3β nor the kinase-dead K85R-GSK3β had a significant effect on MMP-1 expression in BAEC (Fig. 3C), although the expression of IL-8 mRNA was increased 3.5-fold by the expression of the inactive K85R-GSK3β (Fig. 3C). On the other hand, the ectopic expression of the S37A-β-catenin mutant (35), a non-GSK3β-phosphorylatable form of β-catenin and downstream effector of the Wnt/β-catenin signaling pathway, resulted in a significant 60% decrease of the basal MMP-1 mRNA levels in BAEC (Fig. 3C).

To further rule out a possible role of GSK3β inhibition in lithium-induced MMP-1 expression, we tested the effects of GSK3β down-regulation using short interfering RNAs. In BAEC transfected for 48 h with control siRNAs, lithium increased dramatically GSK3β phosphorylation on Ser9 as previously reported in numerous cell lines (50), whereas a slight increase of GSK3β levels were also observed (Fig. 3D). After transfection with specific siRNAs against GSK3β, down-regulation of GSK3β levels greater than 95% was reproducibly observed in BAEC (Fig. 3D). Although, lithium was still able to increase GSK3β levels slightly, no Ser9 phosphorylation could be detected in GSK3β-siRNA-transfected cells (Fig. 3D). Nonetheless, despite this efficient GSK3β down-regulation, no significant changes in cell proliferation, cell number, and cell morphology were noted (not shown), which contrasts with the effects of lithium in these cells (26). Similarly, the basal levels of MMP-1 mRNAs were slightly decreased by about 10%, but not increased as expected if lithium-induced MMP-1 expression was dependent on GSK3β inhibition (Fig. 3E). Thus, the inhibition of GSK3β either by inactivation (K85R-GSK3β, Fig. 3C) or by down-regulated expression (siRNAs-GSK3β, Fig. 3E) fails to increase MMP-1 expression. Moreover, the lithium-dependent effects on MMP-1 mRNA up-regulation were significantly lower in siRNA-GSK3β-transfected cells as compared with siRNA-control cells (2.5-fold), whereas the effects of lithium on PAI-1 and IL-8 mRNA expression were not affected (Fig. 3E).

Similar lower levels of lithium-induced p21<sup>Cip</sup> mRNA expression were also observed (Fig. 3E). Thus, down-regulation of GSK3β activity in BAEC has rather the opposite effects of lithium on the expression of two senescent-cell markers, p21<sup>Cip</sup> and MMP-1, suggesting that inhibition of GSK3β is rather associated with the inhibition of EC senescence. Our data in EC are thus consistent with the reported positive role for active GSK3β on human fibroblast senescence and SAHF formation (13, 29) and demonstrate that lithium affects another pathway controlling proliferation/senescence.

**Lithium Had No Specific Effect on MMP-1 mRNA Stability**—Many stress and growth-regulated mRNAs are rapidly regulated post-transcriptionally at the levels of mRNA stability via in particular AU-rich sequences (51). Although there are 4 putative AU-rich sequences within the MMP-1 3′-UTR, the levels of MMP-1 mRNA were not significantly decreased during 3 h of actinomycin D treatment in BAEC and longer treatment led to confounding results because of cell apoptosis (not shown). Thus to assess the effects of lithium on MMP-1 mRNA stability, phRG-TK-Renilla luciferase reporters containing downstream of the Renilla luciferase stop codon, either the 3′-UTR of MMP-1 mRNA (3′-UTR-MMP1) or the 3′-UTR of IL-8 mRNA (3′-UTR-IL8), were generated and transfected into BAEC. As shown in Fig. 4, lithium treatment induced a similar increase of Renilla luciferase activity for all the 3′-UTR constructs tested, indicating general effects of lithium on either translation efficiency or thymidine kinase promoter dependent transcription rather than specific effects on mRNA stability and AU-rich sequences. Together, these data indicate that the main effects of lithium on MMP-1 mRNA expression occur at the transcriptional level.

**The Lithium-induced MMP-1 Expression Is Independent of the Common AP1/Ets and Redox-sensitive Regulatory Sites**—The transcriptional regulation of MMP-1 expression during cell senescence is poorly understood, although a ROS-dependent mechanism is suspected in fibroblasts and EC (52–55), via redox-sensitive transcription factors such as AP-1 and Ets family members (39, 56). To determine whether the effects of lithium on MMP-1 expression were dependent on redox-sensitive sites, we used the −600/+60-MMP1P-Luc reporter construct that contains the proximal AP-1 (−73) and Ets (−88) regulatory sites conserved among many MMP promoters (56), as well as the (1G) and (2G)−2285/+60-MMP1P-Luc reporters that contain additional distal AP-1/Ets binding sites, whereas differing for the −1607/2G polymorphism creating an additional Ets1 and redox-dependent site (38, 39). In BAEC, lithium treatment did not affect significantly the transcriptional activity of the tested MMP-1-promoter reporters including (2G)−2285/+60-MMP1P-Luc, whereas a 4-fold increase was observed following treatment with phosphor myristate acetate, a known activator of AP-1 transcriptional activity (Fig. 5A). The absence of lithium-induced AP-1 activity is in agreement with the absence of changes in the levels of phosphorylated S63-Jun and total c-Jun in BAEC (Fig. 5B). This is in contrast with the 1.5-fold increase of c-Jun levels in lithium-treated HEK293 cells (Fig. 5B), which is associated with reproducible 1.6–2.3-fold increases of the MMP-1-promoter reporters (Fig. 5A). The latter corresponds also to a 7-fold increase of the levels of MMP-1...
mRNAs in HEK293 cells (not shown). Thus whereas lithium affects MMP-1 expression both in the immortalized HEK293 cells and primary BAEC, some of the mechanisms involved differ. Moreover, in agreement with the absence of activation of the redox-sensitive AP-1/Ets regulatory sites in the MMP-1 promoter (39), lithium treatment in BAEC from 30 min to 36 h failed to increase the levels of H₂O₂ as detected using the redox probe CM-H₂DCFDA (Fig. 5C). Instead, a slight decrease of H₂O₂ basal levels was noted and the increase of H₂O₂ after lipopolysacchride-induced oxidative stress in BAEC was significantly dampened, in agreement with the previously reported antioxidant role of lithium in neuronal cells (57). Thus taken together our data demonstrate that the effects of lithium on MMP-1 expression are independent of the AP-1/Ets transcrip-
tion factors activated during inflammation and redox changes and thus suggest a novel mechanism for MMP-1 regulation during lithium-induced senescence.

**Basal and Lithium-induced MMP-1 Expressions Are Dependent on p53 Levels in BAEC**—p53 is a master regulator of the balance between apoptosis and cell senescence in response to stress (27). Because we have previously shown that lithium increases p53 stability and activity in BAEC (26), the expression of MMP-1 was assessed in BAEC transfected with specific p53-siRNAs (Fig. 6, A and B), which was followed by the down-regulation of known p53 targets, such as Bax (Fig. 6A) and p21CIP (Fig. 6B). Surprisingly the basal expression of MMP-1 was also dramatically decreased by 90% in p53-siRNA-transfected BAEC (Fig. 6B). Lithium treatment in these transfected cells failed to induce a decrease in cell number, a change in cell morphology, and the appearance of SA-β-galactosidase positive cells (not shown) as well as failed to increase p21CIP expression (Fig. 6B), in agreement with the dependence of lithium-induced cell cycle arrest and senescence on the activation of the p53/p21CIP cascade (26). Surprisingly, lithium failed also to increase significantly MMP-1 expression in these conditions (Fig. 6B). The basal activities of the MMP-1-promoter luciferase reporters, −600-MMP1-Luc and (1G)−2235-MMP1-Luc, were also significantly decreased by 50% in BAEC transfected with p53-siRNAs (Fig. 6C). This decrease was not affected by lithium treatment. Taken together these data reveal that MMP-1 is a novel p53-target gene in EC and that p53 is required for lithium-induced MMP-1 expression, and thus reinforce the link between MMP-1 expression and cell cycle arrest and/or senescence in EC.

**Modulation of Lithium-induced Senescent-like Phenotype by MMP-1 Levels in EC**—Because MMP-1 expression is highly associated with EC senescence both in vitro and in vivo, next we tested the role of MMP-1 in the lithium-induced senescent phenotype in BAEC. First, the appearance of SA-β-galactosidase positive cells was monitored in lithium-treated BAEC in the absence or presence of exogenously activated MMP-1. As shown in Fig. 7A, lithium treatment for 5 days increased ~4.5-fold the number of SA-β-galactosidase positive cells as compared with sodium-treated control cells. The number of SA-β-galactosidase positive cells was further increased about 1.6-fold by the presence of 5 nm activated MMP-1 during the last 4 days of lithium treatment (Fig. 7A). In sodium-treated cells, the presence of activated MMP-1 led to a slight (1.3-fold) although not significant increase of the SA-β-galactosidase positive cells. Of note, a similar effect was detected using purified MMP-1 from

### Figure 4

**Lithium does not affect specifically MMP-1 mRNA stability.** BAECs were transfected with the indicated 3'-UTR Renilla luciferase reporters in the presence of 5 ng of CMV-β-galactosidase vector for normalization purposes. Twenty-four hours after transfection, BAECs were treated with either 10 mM NaCl or 10 mM LiCl for an additional 24-h period prior to quantification of the Renilla luciferase and β-galactosidase activities. The graph represents the relative ratio of the normalized Renilla luciferase activity in lithium-treated cells versus sodium-treated cells (mean ± S.E. (error bars), n = 4–5 independent experiments).

### Figure 5

**Lithium-dependent transcriptional activation of MMP1 expression in EC does not involve proximal AP1 and distal AP1/Ets1 redox sensitive binding sites.** A, BAECs and HEK293 cells were transfected with 5 ng of CMV-β-galactosidase vector for normalization purposes and with 125 ng of pGL3-basic as control. −600/+60-MMP1-Luc, (1G)−2235/+60-MMP1-Luc, or (2G)−2235/+60-MMP1-Luc reporter constructs for 24 h prior to being treated with 10 mM NaCl, 10 mM LiCl, or 100 nM phorbol myristate acetate (PMA) as indicated for an additional 24-h period. The firefly luciferase and β-galactosidase activities were determined and the results are expressed as the fold-induction of the normalized luciferase activity in treated cells as compared with NaCl-treated control cells (mean ± S.E. (error bars), n = 4–5 independent experiments). B, BAECs and HEK293 cells were treated with either 10 mM NaCl or 10 mM LiCl for 36 h prior to being lysed for the analysis of Jun activation (phospho-S63-Jun) and Jun levels (c-Jun) by Western blotting. A representative experiment is shown using c-Jun as normalization control. C, BAEC were treated with either NaCl or LiCl (10 mM) for the indicated times prior to staining with 2 μM CM-H2DCFDA for 30 min. BAEC were also challenged for the indicated times with 1 μg/ml lipopolysaccharide as positive control. The fluorescence intensity was determined at λexc = 485 nm/λem = 535 nm (Fusion, PerkinElmer Life Sciences) and normalized for variations in cell number. The results are expressed as the relative levels of the normalized fluorescence intensity in treated versus control cells (mean ± S.E. (error bars), n = 6). * results were considered statistically different from NaCl-treated control cells at p < 0.05 (t test).
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Figure 6. Down-regulation of p53 abrogates basal and lithium-induced MMP-1 expression. A and B, BAEC were transfected with 35 nm of either siRNA-CT-1 or siRNA-p53 for 24 h prior to being treated with either 10 mM NaCl (−) or 10 mM LiCl (+) for a 24-h period. A, after cell lysis, the levels of p53 and Bax, a p53 target, were analyzed by Western blotting using β-actin as control (CT). B, after RNA extraction and reverse transcription, the levels of the target mRNAs were determined by real-time PCR using rpl30 mRNAs for normalization. The results are expressed as fold-change in target mRNA levels with NaCl-treated cells being equal to 1 (mean ± S.E., n = 3–4 independent experiments). C, BAEC were co-transfected with either control (CT) or p53 siRNA duplexes (35 nm) and the indicated MMP-1-promoter luciferase reporters for 48 h prior to cell lysis and determination of the firefly luciferase and β-galactosidase activities. Treatments with either 10 mM NaCl or 10 mM LiCl were performed in the last 24 h of transfection. The graph represents the relative levels of normalized luciferase activity with si-Control equal to 1 for each of the reporters (mean ± S.E., n = 3 independent experiments performed in duplicates), * results were considered statistically different from NaCl-treated siRNA control-transfected cells at p < 0.05 (t test).

Two different sources: recombinant MMP-1 (Chemicon) and synovial fibroblast-conditioned media (EMD-Calbiochem). Associated with the increase of SA-β-galactosidase positive cells, there was also a slight 1.35-fold increase of p21Cip mRNA levels in BAEC co-treated with lithium and activated MMP-1, whereas the mRNA levels of other senescent cell markers, MMP-1, IL-8, and PAI-1, were not significantly different from lithium-treated cells (Fig. 7B). In BAEC treated with activated MMP-1, a 2-fold increase of p21Cip mRNA levels was also observed (Fig. 7B), suggesting that activated MMP-1 affects EC cell senescence in part by increasing the levels of the cell cycle inhibitor p21Cip.

The effects of FN-439, a MMP inhibitor, which displays a higher specificity for collagenases including MMP-1, were also observed in BAEC treated with 10 to 20 μM FN-439 for 24 h, as characterized by an increase of PAI-1 (9-fold), IL-8 (2-fold), and MMP-1 (6-fold) mRNA levels (not shown). So to further demonstrate the possibility of a MMP-1-dependent amplification loop in EC senescence, the effects of MMP-1 down-regulation by siRNAs were determined. Two sets of siRNA duplexes against the bovine MMP-1 mRNA sequences were designed (see “Experimental Procedures”) and transfected into BAEC. Both MMP-1 siRNAs were specific and efficient as none of them affected significantly the levels of the MMP-2 and MMP-14 and their regulation by lithium, whereas a significant 80 and 60% decrease of basal MMP-1 mRNA levels were observed with siRNA-MMP1-1 and siRNA-MMP1-2, respectively (Fig. 8A). Although a slight increase of MMP-1 mRNA levels occurred after lithium treatment in siRNA-MMP1-transfected cells, those levels still corresponded to a 95 and 80% decrease as compared with the cells transfected with the siRNA-control and treated with lithium (Fig. 8A). This decrease of MMP-1 mRNA expression is followed by a significant decrease of MMP-1 proteins in conditioned media even after treatment with lithium (Fig. 8B). At this point, it is worth noting that in the siRNA experiments, there is a 24-h delay for subsequent cell treatments. Because the cells are less proliferative at the time of lithium treatment, lower levels of MMP-1 and p21Cip up-regulations were reproducibly observed in siRNA-control (Figs. 4B and 8C) as compared with untransfected cells (Fig. 1A), whereas no significant changes were noted for the effects of lithium on PAI-1 and IL-8 (Figs. 4B and 8C). Similarly, a lower increase of the number of SA-β-galactosidase positive cells (2.2- instead of 5-fold) was also noted although the difference between lithium-treated and sodium-treated control cells remained significant. Nonetheless, down-regulation of MMP-1 levels in BAEC was accompanied by a dampened number of SA-β-galactosidase positive cells after lithium treatment (Fig. 8D). Taken together our data with both exogenously added MMP-1 and prevention of MMP-1 up-regulation are consistent with a role for MMP-1 in the reinforcement of EC senescence.

Discussion

In the present study, we have shown that the expression of MMP-1, a crucial regulator of collagen turnover and a senescent cell marker (1, 8, 43), is significantly increased during lithium-induced cell senescence in EC. This lithium effect is neither mediated by lithium-induced depletion of the inositol phosphate cycle nor lithium-induced GSK3β inhibition, but linked to EC cell cycle arrest and senescence. Indeed, we further demonstrate that MMP-1 expression is controlled by p53 levels in EC. However, in contrast to the delayed up-regulation of PAI-1, another target of p53 and marker of senescent cells (9, 13), MMP-1 up-regulation is an early event in the lithium-induced EC senescent phenotype. In turn, MMP-1 participates in the reinforcement of EC senescence in part through up-regulation of the cell cycle inhibitor p21Cip.

In contrast to MMP-2, MMP-14, MMP-7, and MMP-26, which are downstream targets of the Wnt/β-catenin signaling pathway in various cell types (58–61), our data show that this is not the case for MMP-1 in EC. The inhibition of GSK3β by indirubin and by ectopic expression of the kinase-dead GSK3β had no significant effect on MMP-1 expression (Fig. 3). Down-
regulation of GSK3β by specific siRNAs decreased both basal and lithium-induced MMP-1 expression in EC (Fig. 3) and β-catenin stabilization, the downstream GSK3β target in the Wnt/β-catenin signaling pathway (28), had the same inhibitory effect on basal MMP-1 expression (Fig. 3). Although consensus DNA binding sites for the downstream effectors of the Wnt/β-catenin signaling pathway, the transcription factors belonging to the TCF/LEF1 family (62), are present and functional within the MMP-2, MMP-14, MMP-7, and MMP-26 promoters (58–61), such consensus sites are absent within the 4300-bp sequences that characterize the full-length human MMP-1 promoter (63). Accordingly, the stable S37A-β-catenin forms failed to increase the transcriptional activity of the MMP1P-Luc constructs in BAEC (not shown). Moreover, we have previously shown that lithium does not activate β-catenin/TCF activity in BAEC (26), which agrees also with the absence of MMP-14 up-regulation (Fig. 1). The opposite effects of lithium (Fig. 1) and GSK3β down-regulation (Fig. 3) on MMP-1 expression seem to reflect rather opposite effects on cell cycle progression with the activation of cell cycle arrest and senescent program by lithium, whereas GSK3β down-regulation favors an anti-inflammatory program and cell quiescence at confluence. In this respect, our data on GSK3β down-regulation in primary EC are thus in agreement with the proposed positive role of active GSK3β in the control of pro-inflammatory programs (64) and establishment of cell senescence (13) in primary cells.

Up-regulation of MMP-1 expression during cell senescence has been so far linked to increased levels of IL-1β, another inflammatory cytokine marker of senescent cells (63), and increased production of reactive oxygen species (ROS) by both mitochondrial dysfunction and increased proinflammatory signaling (2), which converge to AP-1 activation and MMP-1 transcriptional regulation (39, 56, 63). Changes in MMP-1 mRNA stability by IL-1β have been also reported (63). However, a posttranscriptional mechanism is unlikely the main trigger for the increased levels of MMP-1 mRNAs after lithium treatment because experiments with the 3′-UTR-MMP-1 reporter construct failed to show a specific effect of lithium (Fig. 4) and IL-1β up-regulation is a delayed event like PAI-1 up-regulation (not shown). In agreement with a specific effect of lithium on MMP-1 among other MMPs, our data have to rule out the involvement of the conserved AP-1/Ets regulatory sequences within MMP promoters (56). Because these AP-1/Ets regulatory sites have been shown to be essential for redox regulation of MMP-1 promoter activity (39), their unresponsiveness to lithium treatment is also consistent with the absence of change in ROS in BAEC after normalization for lithium-induced mitochondrial biogenesis (36).

Nonetheless, our studies further pinpoint a tight association of lithium-induced MMP-1 expression with either lithium-induced cell cycle arrest or lithium-induced EC senescence. Indeed, the induction of MMP-1 expression paralleled the expression of the cell cycle inhibitor p21Cip both in kinetics and levels. We have previously shown that activation of p53 is the main trigger of p21Cip up-regulation in lithium-treated EC and fibroblasts (26) and herein we demonstrate that down-regulation of p53 expression by siRNAs affect concomitantly the basal expression of both p21Cip and MMP-1 mRNAs in EC (Fig. 6B). Thus, in addition to confirming the p53-dependent effects of lithium on p21Cip expression, our data indicate that MMP-1 is also a target of p53 in EC. A significant 2-fold decrease of the MMP-1 promoter luciferase reporters was also observed in BAEC after down-regulation of p53 (Fig. 6C), although there are no obvious p53 binding sites within the 4300-bp MMP-1 promoter. Previously, p53 overexpression was shown instead to blunt MMP-1 induction by IL-1β in sarcoma HT1080 cells by inhibiting the formation of functional complexes between AP-1 and the coactivator p300 (65). Thus it is possible that p53 con-

**FIGURE 7. Activated MMP-1 increases lithium-induced SA-β-galactosidase marker and p21Cip expression.** A, 24 h after plating, BAEC were treated with either 10 mM NaCl or 10 mM LiCl in the absence or presence of 5 mM activated MMP-1. Five days later, the cells were fixed and stained for SA-β-galactosidase activity. Representative phase-contrast color images are shown. The numbers of SA-β-galactosidase positive cells were counted as well as the total number of cells for each treatment and the results are presented in the graph (mean ± S. E. (error bars), n = 4–5 independent experiments performed in duplicates). *, results were considered statistically different from NaCl-treated control cells at p < 0.05 (one-way analysis of variance-Bonferroni post test). B, BAEC were treated as described in A for 36 h prior to RNA extraction and gene expression analysis by real-time PCR. *, results were considered statistically different from NaCl-treated control cells at p < 0.05 (t test).
trols MMP-1 expression indirectly by regulating co-activators and/or co-repressors. This possibility is further supported by the very recent finding that p53 was present in p300 complexes bound to the MMP-1 promoter in primary dermal fibroblasts after UV irradiation (66). Alternatively, the p53-dependent changes in cell cycle progression might also affect basal MMP-1 expression.

Among MMPs, MMP-1 is so far the most up-regulated during cell senescence in vitro and implicated in vivo in human age-related diseases such as arthritis and atherosclerosis (8, 10, 21). Our data pinpoint a novel role for MMP-1 during cell senescence by its ability to enhance cell dysfunction and reinforce the lithium-induced EC senescent phenotype. This MMP-1 effect on pre-senescent cells is thus quite opposite to the previously reported proangiogenic activities on proliferative EC (6). For determining the SA-β-galactosidase activity, BAECs were transfected with the indicated siRNAs and treated with either 10 mM NaCl or 10 mM LiCl for 4 days. The graph represents the number of SA-β-galactosidase positive cells per 10⁴ cells (mean ± S. E. (error bars), n = 4–5 independent experiments performed in duplicates). *, results were considered statistically different at p < 0.05 (t test).

Very recently, a positive feedback loop between MMP-1 up-regulation, its consequential collagen fragmentation, and cell senescence has been reported in photo-aging skin and old dermal fibroblasts (21). MMP-1-cleaved collagen fragments have been shown to induce ROS production and subsequently to further up-regulate MMP-1 expression (21). Our data in EC also further link the up-regulation of MMP-1 levels with an amplification of the appearance of senescent cell markers and features, including MMP-1 expression (Figs. 7 and 8). Whether the effects of MMP-1 on EC senescence are also mediated by MMP1-induced cleaved collagen fragments and secondary

**FIGURE 8. Down-regulation of MMP-1 expression affects lithium-induced senescent cell markers.** A–C, BAECs were transfected with 35 nM of the indicated siRNA duplexes for 24 h prior to being treated with either 10 mM NaCl or 10 mM LiCl for an additional 24-h period. Then, the conditioned media were collected for Western blot analysis of MMP-1 levels (A) and the cells were harvested for RNA extraction and real-time PCR analysis of the indicated target genes (A and C). D, for determining the SA-β-galactosidase activity, BAECs were transfected with the indicated siRNAs and treated with either 10 mM NaCl or 10 mM LiCl for 4 days. The number of SA-β-galactosidase positive cells per 10⁴ cells (mean ± S. E. (error bars), n = 4–5 independent experiments performed in duplicates). *, results were considered statistically different at p < 0.05 (t test).
ROS production remain to be investigated. Nonetheless, these senescent-enhancing effects of MMP-1 during skin aging and in lithium cell cycle-arrested EC further stress the dual roles of an increasing number of factors, which have only been considered so far as regenerative and/or angiogenic factors based on their effects on enabled proliferative cells.

Our study also highlights the pleiotropic effects of lithium on different cellular processes including induction of cell senescence in primary cells and changes in cell adhesion via regulation of MMPs. The inhibition of the considered two main targets of lithium, GSK3β and IMP-1 (28), is insufficient to recapitulate these specific effects of lithium (Figs. 3 and 4). Because lithium is a broad competitive inhibitor of Mg2+-dependent factors (69), these data are not surprising but further stress the fact that other lithium targets await to being discovered. Recently, a novel target of lithium, the Golgi-resident-3-phosphoaddenosine, 5′-phosphate 3′-phosphatase, has linked lithium toxicity with the decreased sulfation of secreted factors and ECM components in chondrocytes (70). Interestingly, this novel lithium target also pinpoints changes in ECM structure and ECM components in chondrocytes (70). Thus, MMP-1 up-regulation might be associated with both the beneficial effects of lithium and some side effects of lithium therapy because the latter are more prominent in the elderly (72).

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