Differential effects of tumor necrosis factor-α on matrix metalloproteinase-2 expression in human myometrial and uterine leiomyoma smooth muscle cells

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STUDY QUESTION: Does tumor necrosis factor-α (TNF-α) differentially regulate matrix metalloproteinase-2 (MMP-2) expression in leiomyomas compared with normal myometrium?

SUMMARY ANSWER: TNF-α up-regulates MMP-2 expression and stimulates cell migration through the activation of extracellular signal-regulated kinase (ERK) signaling pathway in leiomyoma smooth muscle cells (SMCs), but not in normal myometrial SMCs.

WHAT IS KNOWN ALREADY: Uterine leiomyoma, the benign smooth muscle cell tumor, is the single most common indication for hysterectomy. High expression of MMPs or TNF-α has been reported in uterine leiomyomas; however, the molecular mechanism underlying these observations remains unknown.

STUDY DESIGN, SIZE, DURATION: Samples were obtained between 2009 and 2013 from 12 women of reproductive age at the proliferative phase of the menstrual cycle by hysterectomy. Leiomyomas and matched normal myometrium from each woman were analyzed in vitro.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Western blot, RT-qPCR and a wound-healing assay were used to investigate the effects of TNF-α on MMP-2 expression and intracellular signal transduction in cultured SMCs from leiomyomas and matched myometrium.

MAIN RESULTS AND THE ROLE OF CHANCE: Western blot and RT-qPCR analyses using tissues from clinical patients showed that the levels of MMP-2 protein (P=0.008) and mRNA (P=0.009) were significantly higher in uterine leiomyomas compared with their matched myometrium. Treatment with TNF-α significantly up-regulated the protein (P=0.039) and mRNA (P=0.037) levels of MMP-2 in cultured leiomyoma SMCs but not in matched myometrial SMCs. The extracellular signal-regulated kinase (ERK) and nuclear factor-kappa B (NF-κB) pathways were activated by TNF-α in leiomyoma SMCs. Specific inhibitors of the ERK or NF-κB pathway (PD98059 or Bay11-7082) suppressed TNF-α-induced MMP-2 expression in leiomyoma SMCs. The wound-healing assay revealed that TNF-α promoted the migration of cultured leiomyoma SMCs (P=0.036); however, PD98059 compromised the cell migration triggered by TNF-α.

LIMITATIONS, REASONS FOR CAUTION: This study is descriptive and although we observed clear differential regulation of MMP-2 by TNF-α at mRNA and protein levels in leiomyoma, future studies are needed to identify why the difference in TNF-α response exists between human leiomyoma tissue and normal myometrium. Including some of the experiments such as transfection studies for TNF-α and MMP-2 promoter mapping could have added more insight as to why this difference exists. In addition, further studies in vivo are needed to verify the results obtained from primary cultured SMCs.

WIDER IMPLICATIONS OF THE FINDINGS: Considering the positive effect of TNF-α on leiomyoma SMC migration, strategies targeting TNF-α, in parallel with the production of more specific inhibitors of MMPs, may provide alternative therapeutic approaches for the treatment of leiomyoma.
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**Key words:** tumor necrosis factor-α (TNF-α) / matrix metalloproteinase (MMP) / uterine leiomyoma / extracellular signal-regulated kinase (ERK)

**Introduction**

Uterine leiomyoma, the most common gynecologic neoplasm, occurs in > 70% of reproductive-age women (Lumsden and Wallace, 1998; Rein, 2000; Flake et al., 2003). Steroid hormones are believed to play critical roles in the pathogenesis of the tumor (Burroughs et al., 2000; Di et al., 2008); however, despite much investigation, including genetic and molecular approaches, the underlying mechanism of uterine leiomyoma remains unclear.

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine involved in inflammation, immunity, migration, cellular homeostasis and tumor progression (Balkwill, 2009). Several investigators have shown that the abundance of TNF-α is regulated by steroid hormone levels. Indeed, TNF-α levels dropped in ovariectomized mice, whereas the administration of estradiol and progesterone resulted in the reappearance of this cytokine (Roby and Hunt, 1994). The expression of TNF-α in uterine leiomyoma was higher than that in the adjacent myometrium and was significantly down-regulated by addition of progesterone (Kurachi et al., 2001). Although, some evidence has suggested a role for TNF-α in the pathogenesis of uterine leiomyoma, details on the TNF-α-mediated signaling pathways in leiomyoma smooth muscle cells (SMCs), such as downstream adaptor proteins or phosphorylation steps, are still largely unknown.

Tissue remodeling involving ‘extracellular matrix (ECM) turnover’ plays a key role in leiomyoma growth and is regulated by the combined actions of matrix metalloproteinases (MMPs), a family of > 20 zinc-dependent endopeptidases (Islam et al., 2013). Previous studies have shown that several forms of MMPs are expressed in myometrium and leiomyomas (Dou et al., 1997; Bodner-Adler et al., 2004; Wolarska et al., 2004; Bogusiewicz et al., 2007). Moreover, the activity of MMP-2 is higher in human leiomyoma tissues compared with normal myometrium (Inagaki et al., 2003). In vitro studies have shown that cytokines such as TNF-α and interleukin-1β increase the expression and activity of MMP-2 in vascular SMCs (Galis et al., 1994; Fabunmi et al., 1996), which serves as a stimulus for us to examine whether TNF-α has different effects on MMP-2 expression in myometrial and leiomyoma SMCs.

The present study was designed to characterize the effects of TNF-α on MMP-2 expression and intracellular signaling pathways in primary cultured SMCs obtained from human leiomyomas and matched myometrium. Our study was expected to establish a potential role for TNF-α/MMP-2 in the pathogenesis of uterine leiomyoma.

**Materials and Methods**

**Antibodies**

Antibodies against phospho-p44/42 (ERK) MAPK (Thr202/Tyr204), total p44/42 (ERK), MAPK, phospho-ELK-1, phospho-IkBa (Ser32/36) and total IkBa (L35A5) were obtained from Cell Signaling (Beverly, MA, USA). The MMP-2 and MMP-9 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Primary cell culture**

Human leiomyoma (HL) and matched myometrium (HM) SMCs were isolated from uterine leiomyoma and normal myometrial tissues, respectively. Uterine leiomyoma and matched myometrial tissues from the same patient were cut into small pieces (~1 mm³) and placed into dissociation solution (Dulbecco’s modified Eagle’s medium (DMEM), 20% v/v fetal bovine serum (FBS), 0.2% v/v collagenase II (Invitrogen, Carlsbad, CA, USA) and 50 mg/ml trypsin inhibitor (Sigma-Aldrich)), followed by incubation for 2–6 h at 37°C, with the dissociation solution changed every 2 h. The dissociated tissues were filtered through a sterile 100-μm mesh filter, and the isolated cells were washed three times with phosphate-buffered saline (PBS) using 400 g centrifugation for 5 min. Cells were resuspended in phenol red-free DMEM (Hyclone, Logan, UT, USA) with 10% v/v heat-inactivated FBS (Hyclone) and 1% v/v antibiotic solution (Sigma-Aldrich) for further culture. The cultures were maintained in a standard 95% air, 5% CO₂ incubator at 37°C. When 95–100% cell confluence was reached, cells were passaged by the standard method of trypsinization, plated in culture dishes and grown in phenol red-free DMEM supplemented with 10% FBS. Cells from passages three to five were used for all the experiments. Monolayer HM- and HL-SMCs were rendered quiescent by incubation in phenol red-free DMEM containing no FBS for 24 h. The cells were then used to test the effect of human recombinant TNF-α (Sigma-Aldrich) on the expression of MMP-2.
and 9 protein and mRNA. The following inhibitors were added to the cells 30 min before treatment with 10 ng/ml TNF-α for 24 h at 37°C: 10 μM PD98059 (Sigma-Aldrich), 25 μM Bay11-7082 (Santa Cruz, CA, USA) or 5 μM U0126 (Sigma-Aldrich). All treatments were performed in phenol red-free media containing no FBS. Each experiment was performed in triplicate.

Western blot
Tissues and cultured SMCs were lysed with ice-cold lysis buffer [150 mM NaCl, 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1% v/v NP-40, 0.1% v/v sodium dodecyl sulfate, 0.25% v/v sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM Na2VO3, and protease inhibitor cocktail tablets (Roche Molecular BioChemicals, Indianapolis, IN, USA)], followed by centrifugation at 4°C for 10 min (1000 g). Protein concentrations were determined using a bichoninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. Aliquots containing 25-40 μg of protein were mixed with loading buffer, followed by denaturation at 95°C for 5 min. Proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% v/v nonfat milk in Tris-buffered saline/Tween-20 (TBST) buffer (20 mM Tris–HCl, pH 7.6, 136 mM NaCl and 0.1% v/v Tween-20) and then probed for 1.5 h or overnight with primary antibodies: MMP-2, 1:500; MMP-9, 1:500; β-actin, 1:5000. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (dilution: 1:5000) for 1.5 h at room temperature. Protein signals were visualized using the West Pico Chemiluminescent Substrate Kit (Pierce). Images were acquired by a Molecular Image Chemidoc XRS System and analyzed using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Wound-healing assay
The cultured human leiomyoma SMCs (passages three to five) were seeded in six-well plates. When 70% cell confluence was reached, the leiomyoma SMCs were rendered quiescent by incubation for 24 h in phenol red-free media containing no fetal bovine serum. Confluent monolayer cells were scratched in a straight line with a 200 μl pipette tip; the cell debris and suspended cells were removed by washing the cells with serum-free DMEM. The cells were then incubated in serum-free DMEM, and pretreated with or without PD98059 (10 μM) for 30 min in the absence and presence of TNF-α (10 ng/ml). After 24 h of incubation at 37°C, additional images were then captured by aligning the dishes with the reference point made at 0 h. The distance between one side of the scratch and the other was measured using Image Pro-Plus software (Media Cybernetics, Inc., Warrendale, PA, USA). Cell migration was indicated as wound exposure and expressed as percentage of unhealed distance of treated cells versus that of control cells.

Statistical analysis
The data were presented as the means with the 95% confidence intervals (CIs) (n = replicate number of experiments). The normality of the data distributions was first assessed using the Kolmogorov–Smirnov test. Differences were analyzed with a two-tailed paired t-test or one-way ANOVA followed by the Newman–Keuls post hoc test as appropriate. The statistical analysis was performed using Instat 3.0 (GraphPad Software, San Diego, CA, USA), and P < 0.05 was considered statistically significant.

Results
MMP-2 expression is up-regulated in human uterine leiomyoma
Uterine leiomyomas and their matched myometrial tissues were sampled for protein and mRNA for western blot (n = 12) and RT-qPCR (n = 12) analyses, respectively. Western blot analysis showed that the levels of MMP-2 protein in leiomyoma tissues were significantly higher compared with the matched myometrium tissues (P = 0.008, Fig. 1A and B). Consistently, as shown in Fig. 1C, the levels of mmp-2 mRNA from these patients were significantly higher in leiomyoma tissues than their matched myometrium tissues (P = 0.009), suggesting up-regulation of mmp-2 gene expression in uterine leiomyomas. Immunohistochemistry was performed to confirm this finding. As shown in Fig. 1D, MMP-2 signals appeared outside the nuclei and were much stronger in uterine leiomyomas than that in matched normal myometrium or adjacent myometrium.

ERK activity is down-regulated in human uterine leiomyoma
We proceeded to examine the activity of the ERK pathway in uterine leiomyomas and their matched myometrial tissues. As described...
in our previous study (Cui et al., 2011), leiomyoma tissues (n = 12) have significantly lower levels of phospho-ERK1/ERK2 than myometrium tissues, but the total ERK1/ERK2 levels were similar in both tissues (Fig. 1E), suggesting suppression of the ERK pathway. This result was further confirmed with kinase activity assay (Fig. 1F).
TNF-α increases the expression of MMP-2 in HL-SMCs but not in HM-SMCs

To investigate whether the high expression of MMP-2 in uterine leiomyoma tissues is related to the presence of TNF-α, we established primary culture of SMCs from uterine leiomyoma and matched myometrial tissues. Both the myometrium and leiomyoma SMCs stained positive for the smooth muscle marker proteins (Supplementary Fig. S1A), as described in our previous study (Ren et al., 2011). Western blot results showed less calponin and SM α-actin present in leiomyoma SMCs (n = 3) (Supplementary Fig. S1B). The basal expression levels of MMP-2 were then evaluated in cultured HM- and HL-SMCs using western blot and RT–qPCR analyses. Our results showed that MMP-2 protein (n = 5; P = 0.006) and mRNA expression (n = 5; P = 0.002) in HL-SMCs was much higher than that in HM-SMCs (Fig. 2A and B).

Subsequently, we examined the effects of various concentrations of TNF-α on MMP-2 expression in both SMCs types. As shown in Fig. 2C, treatment with TNF-α for 24 h concentration-dependently increases the expression of MMP-2 in HL-SMCs but not in HM-SMCs. Primary cell culture of SMCs from myometrium and matched uterine leiomyoma was performed using the enzyme digestion approach. Protein and total RNA were extracted from cells in passages three to five for western blot and RT–qPCR analyses, respectively. (A) Cumulative western blot results showing the expression of MMP-2 in cultured HL-SMCs relative to HM-SMCs (*P < 0.05, n = 5). Inserts: Representative western blots of MMP-2 and β-actin. (B) RT–qPCR data showing the mRNA levels of mmp-2 in HL- relative to HM-SMCs (*P < 0.05, n = 5). (C) RT–qPCR data showing the mRNA levels of mmp-2 relative to the no TNF-α controls in HM- and HL-SMCs in response to TNF-α at different concentrations, as indicated on the x-axis, for 24 h. (*P < 0.05, n = 3). (D) RT–qPCR data showing the mRNA levels of mmp-2 relative to zero time (t0) in HM- and HL-SMCs in response to 10 ng/ml TNF-α treatment for different times (*P < 0.05 versus cells at t0, n = 3). (E) Representative western blots of MMP-2 and β-actin. (F) Western blot results showing MMP-2 expression relative to t0 in response to treatment with 10 ng/ml TNF-α for different times (*P < 0.05 versus cells at t0, n = 3). The data are presented as means with 95% confidence intervals (CIs).
stimulated the expression of MMP-2 mRNA ($P = 0.012$) in cultured leiomyoma SMCs ($n = 3$), but not in the myometrium SMCs ($n = 3$). Treatment with 10 ng/ml TNF-α for 48 h in HL-SMCs ($n = 3$) maintained the significantly up-regulated expression of mmp-2 mRNA ($P = 0.037$) and protein ($P = 0.039$; Fig. 2D–F).

Because matrix metalloproteinase-9 (MMP-9) and MMP-2 have similar specificities towards the substrates, we also detected the effects of TNF-α on the expression of MMP-9 using RT–qPCR and western blot analyses. Conversely, treatment with 10 ng/ml TNF-α did not affect the MMP-9 protein expression in leiomyoma SMCs, but time-dependently increased the MMP-9 mRNA expression (data not shown).

**TNF-α activates the ERK pathway in HL-SMCs**

Because leiomyoma tissues have significantly lower levels of phospho-ERK1/ERK2 than myometrium tissues (Fig. 1E and F), we examined the effects of TNF-α on ERK1/ERK2 phosphorylation in primary cultured HL- and HM-SMCs. As shown in Fig. 3A, 10 ng/ml TNF-α treatment reduced the level of phospho-ERK1/ERK2 in HM-SMCs ($n = 4$) but increased the ratio of phospho-ERK1/ERK2 and total ERK1/ERK2 in HL-SMCs ($n = 4$) as early as 5 min ($P = 0.032$), reaching a peak at 15 min ($P = 0.006$). Pretreatment with PD98059 (10 μM, 30 min), a specific MEK inhibitor, suppressed the ERK1/ERK2 phosphorylation induced by TNF-α in HL-SMCs (Fig. 3B).

Having shown that the ERK signaling pathway is activated by TNF-α in HL-SMCs, we addressed whether ERK1/ERK2 phosphorylation is required for TNF-α-induced MMP-2 expression. Pretreatment of HL-SMCs ($n = 4$) with PD98059 (10 μM) abolished TNF-α-induced MMP-2 expression at both protein (Fig. 3C and D) and mRNA levels (Fig. 3E), as detected by western blot and RT–qPCR, respectively. Consistently, U0126 (5 μM), a mechanistically different inhibitor of ERK activation, also inhibited the levels of MMP-2 mRNA and protein up-regulated by TNF-α in HL-SMCs (data not shown).

**TNF-α activates the nuclear factor-kappa B (NF-κB) pathway in HL-SMCs**

To investigate whether the transcription factor NF-κB is also stimulated by TNF-α in leiomyoma SMCs through the canonical pathway, we

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**Figure 3** Tumor necrosis factor-α (TNF-α) activates the extracellular signal receptor kinase (ERK) pathway in human leiomyoma smooth muscle cells (HL-SMCs). (A) Cumulative western blots showing the phosphorylation of ERK1/ERK2 in HL- and matched human myometrium (HM) -SMCs treated with or without 10 ng/ml TNF-α for different times. Inserts: Representative western blot results showing the phosphorylation of ERK1/ERK2 in HM- and HL-SMCs. The y-axis represents the proportion of total ERK1/ERK2 that is phosphorylated relative to control ($*P < 0.05$ versus control cells, $n = 4$). (B) Cumulative western blots showing the phosphorylation of ERK1/ERK2 in HL-SMCs treated with or without TNF-α (10 ng/ml) and PD98059 (10 μM). Inserts: Representative western blot results showing the phosphorylation of ERK1/ERK2 in HL-SMCs. The y-axis represents the proportion of total ERK1/ERK2 that is phosphorylated relative to control ($*P < 0.05$ versus control cells, $n = 4$). (C) Representative western blots of MMP-2 and β-actin. (D) Cumulative western blot results showing the expression of MMP-2 in HL-SMCs treated with or without TNF-α (10 ng/ml) and PD98059 (10 μM). The y-axis represents MMP-2 protein expression relative to control ($*P < 0.05$ versus control cells, $n = 4$). (E) RT–qPCR data showing the mRNA levels of mmp-2 in HL-SMCs treated with or without TNF-α (10 ng/ml) and PD98059 (10 μM). The y-axis represents MMP-2 mRNA expression relative to control ($*P < 0.05$ versus control cells, $n = 4$). The data are presented as means with 95% confidence intervals (CIs).
evaluated the phosphorylation of cytoplasmic inhibitor IκBα using western blot analysis. Our results revealed that IκBα was transiently phosphorylated as early as 5 min (P = 0.04) after treatment with 10 ng/ml TNF-α, reaching a peak at 60 min (P = 0.008) in HL-SMCs (n = 3); however, TNF-α did not induce IκBα phosphorylation in HM-SMCs (n = 3) (Fig. 4A). To explore whether IκBα is phosphorylated through the ERK pathway, we examined IκBα phosphorylation by pre-treatment with PD98059 (10 μM, 30 min). As shown in Fig. 4B, PD98059 was able to inhibit TNF-α-activated IκBα phosphorylation, demonstrating the ERK dependence of IκBα phosphorylation in HL-SMCs. Moreover, pretreatment of HL-SMCs with Bay 11-7082 (Pierce et al., 1997), an IκBα phosphorylation inhibitor (25 μM, 30 min), suppressed the IκBα phosphorylation induced by TNF-α, as determined by western blot.

We also examined whether IκBα phosphorylation is required for TNF-α-induced MMP-2 expression. As shown in Fig. 4C and D, pretreatment of HL-SMCs (n = 3) with Bay11-7082 (25 μM) inhibited TNF-α-induced MMP-2 expression at both protein and mRNA levels, as detected by western blot and RT–qPCR, respectively. Therefore, it is concluded that the NF-κB signaling pathway is also involved in the up-regulation of MMP-2 induced by TNF-α in cultured leiomyoma SMCs.

**TNF-α promotes the migration of HL-SMCs**

It is generally accepted that MMPs are important for vascular SMC migration into the intima (Suh et al., 2012). Because TNF-α induced MMP-2 expression in cultured leiomyoma SMCs, we investigated whether TNF-α promotes cell migration of HL-SMCs with an in vitro wound-healing study. As shown in Fig. 5A and B, TNF-α treatment of

![Figure 4 Tumor necrosis factor-α (TNF-α) activates the nuclear factor-kappa B (NF-κB) pathway in human leiomyoma smooth muscle cells (HL-SMCs). (A) Cumulative western blots showing the phosphorylation of iκBα in HL- and matched HM-SMCs treated with or without TNF-α for different times. Inserts: Representative western blot results showing the phosphorylation of iκBα in HL- and HM-SMCs. The y-axis represents the proportion of total iκBα phosphorylated relative to t0 (*P < 0.05 versus cells at t0, n = 3). (B) Cumulative western blots showing the phosphorylation of iκBα in HL-SMCs treated with or without TNF-α (10 ng/ml), PD98059 (10 μM) and Bay11-7082 (25 μM). The y-axis represents the proportion of total iκBα that is phosphorylated relative to control (*P < 0.05 versus control cells, n = 3). (C) Representative western blots of MMP-2 and β-actin. (D) Cumulative western blot results showing the expression of MMP-2 in HL-SMCs treated with or without TNF-α (10 ng/ml) and Bay11-7082 (25 μM). The y-axis represents MMP-2 protein expression relative to control (*P < 0.05 versus control cells, n = 3). (E) RT–qPCR data showing the mRNA levels of mmp-2 in HL-SMCs treated with or without TNF-α (10 ng/ml) and Bay11-7082 (25 μM). The y-axis represents MMP-2 mRNA expression relative to control (*P < 0.05 versus control cells, n = 3). The data are presented as means with 95% confidence intervals (CIs).
HL-SMCs promoted cell migration compared with the control cells \((n = 5; P = 0.038)\), which might be due to the increased expression of MMP-2. Moreover, pretreatment with PD98059, the ERK specific inhibitor, greatly compromised this cell migration effect, possibly via the inhibition of MMP-2 expression.

**Discussion**

To the best of our knowledge, this is the first report showing that TNF-\(\alpha\) regulates the ERK and NF-\(\kappa\)B pathways differently in SMCs from leiomyoma and matched myometrium, in particular promoting the migration of cultured leiomyoma SMCs (Fig. 5C). Stimulation of MMP-2 expression by TNF-\(\alpha\) in leiomyoma SMCs suggests a role for TNF-\(\alpha\) in the overexpression of MMP-2 in uterine leiomyoma tissues.

MMPs have long been associated with tumor progression by virtue of their ability to degrade all structural components of the ECM (Visse and Nagase, 2003). In several cases, the stage of tumor progression has been positively correlated with the expression of MMP family members (Setzler-Stevenson, 1996). Consistently, tumor growth and angiogenesis are reduced in MMP-2-knockout mice (Itoh et al., 1998). Our results show high levels of MMP-2 in human uterine leiomyoma, further supporting a role for MMP-2 in tumor progression. Under normal physiological conditions, MMPs are tightly regulated at the transcriptional and post-transcriptional levels and are also controlled at the protein level via their activators, inhibitors and cell surface localization (Sternlicht and Werb, 2001). Our in vitro studies also revealed that TNF-\(\alpha\) treatment significantly up-regulates MMP-2 expression, suggesting that high MMP-2 expression in uterine leiomyoma tissues may result from TNF-\(\alpha\) effects in vivo. Therefore, our data strongly indicate that the up-regulation of MMP-2 expression plays a potential role in the pathogenesis of human uterine leiomyoma. Since mice without MMP-2 are viable (Itoh et al., 1998), it will be interesting to examine whether uterine leiomyoma develops in these animals.

The increased biosynthesis of TNF-\(\alpha\) was reported in uterine leiomyoma tissues (Kurachi et al., 2001), strongly suggesting a role for TNF-\(\alpha\) in its pathogenesis. Therefore, our observation that TNF-\(\alpha\) treatment increased mmp-2 gene expression in leiomyoma SMCs further supports a role for the up-regulation of mmp-2 gene expression in the development of uterine leiomyoma. Moreover, TNF-\(\alpha\)-related cancers, such as breast cancer (Hagemann et al., 2004) and osteosarcoma (Nyormoi et al., 2003), exhibited MMP-2 up-regulation, further supporting a role for up-regulated MMP-2 expression in tumor growth and the potential role of TNF-\(\alpha\) in this up-regulation. The difference in TNF-\(\alpha\) response existing between human leiomyoma tissue and their
matched myometrium may be regulated by hormone levels. Hormones such as estrogen (E2) and progesterone (P4) are considered to be promoters of leiomyoma growth (Park et al., 2011). We did not assess the hormonal status of the patients included in our study; however, the underlying mechanism should be explored in the future investigations.

Notably, our previous study has shown that leiomyoma tissues have significantly lower levels of phosho-ERK1/ERK2 than myometrium tissues (Cui et al., 2011). Therefore, in cultured SMCs, we predicted that TNF-α may decrease the phosphorylation of ERK1/ERK2. Unexpectedly, our results showed that TNF-α increased the ERK1/ERK2 phosphorylation in leiomyoma SMCs. To date, there has been no explanation for these contradictory observations. However, there is no doubt that cells in tissue culture conditions may not behave in precisely the same way as in vivo due to the microenvironment of the cell.

NF-κB is one of the major transcription factors stimulated by TNF-α and plays a pivotal role in controlling the initiation and progression of cancer (Basseres and Baldwin, 2006). Our results revealed that the NF-κB pathway is activated by TNF-α in leiomyoma SMCs, which contributes to the up-regulation of MMP-2 expression. Therefore, our results further support a role for MMP-2 up-regulation in tumor progression. A previous report has demonstrated that the Akt pathway is involved in regulating TNF-α-activated NF-κB in cardiac myocytes (Condorelli et al., 2002). We observed that activation of the NF-κB pathway was effectively inhibited by PD98059, a specific inhibitor of ERK, suggesting a role for ERK in regulating NF-κB function in leiomyoma SMCs. However, as the precise function of NF-κB transcription factors on MMP-2 regulation is not clearly demonstrated in this study, further experiments are required using cell transfection to over-express constitutively active and/or mutated NF-κB constructs and/or deletion of the NF-κB binding site in the MMP-2 promoter.

Previous studies demonstrate that TNF-α is a potential migration factor for different cell types, such as fibroblasts and inflammatory cells (Fure and McHugh, 1989; Postlethwaite and Seyer, 1990). However, several investigators have revealed that TNF-α specifically inhibits trophoblast migration and invasion (Bauer et al., 2004). Although it is well known that the migration of vascular SMCs (VSMCs) is dependent on the ERK pathway activated by TNF-α, little is known about the effects of TNF-α on uterine leiomyoma SMC migration and the chemoattractant signaling pathways mediating this action. Our results reveal that TNF-α stimulates the migration of HL-SCMs through the ERK signaling pathway. Using the ERK specific inhibitor PD98059, we are able to reveal that activation of the ERK pathway is a critical signaling step for TNF-α-induced leiomyoma SMC migration. On the other hand, we previously described that treatment with G-1, a G-protein-coupled receptor 30 (GPR30) agonist and epidermal growth factor (EGF) stimulated the phosphorylation of ERK in both SMC types (Ren et al., 2011; Tian et al., 2013); however, these studies did not investigate whether the ERK pathway is involved in the migration of cultured leiomyoma SMCs. The migration of uterine leiomyoma SMCs has also been linked to other molecules, such as CCN5 and heparin, which inhibit the motility of both human myometrial and leiomyoma SMCs (Mason et al., 2003, 2004).

MMPs directly modulate cell—matrix adhesion either by removal of sites of adhesion or by exposing a binding site, which can be translated into an effect on cell migration (McCawley and Matrisian, 2001). It is generally accepted that MMP-2 and -9 are possibly involved in the migration potential of VSMCs (Suh et al., 2012). The precise effect of MMP-2 on leiomyoma SMC migration is not demonstrated in this study, and further experiments may require the use of endogenous MMP inhibitors known as tissue inhibitor of metalloproteinases (TIMPs).

Taken together, our results reveal that MMP-2 is overexpressed in human uterine leiomyomas compared with the matched myometrium. TNF-α up-regulates the MMP-2 expression and promotes the migration of human leiomyoma SMCs via the ERK signaling pathway. Given the stimulatory effect of TNF-α on MMP-2 expression, we speculate that TNF-α may result in the up-regulation of MMP-2 in vivo, contributing to tumor growth. Considering the positive effect of TNF-α on leiomyoma SMC migration, strategies targeting TNF-α, in parallel with the production of more specific inhibitors of MMPs, may provide alternative therapeutic approaches for the treatment of leiomyoma.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors’ roles

Y.W., G.F., J.W., Y.Z., Y.L., Y.S., Y.Z., W.L., Y.X. and Z.L. participated in study design, execution, analysis, manuscript drafting, critical discussion and made final approval of the version to be published.

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Conflict of interest

None declared.

References


