Ubiquitin Carboxyl Terminal Hydrolase L1 Attenuates TNF-α-Mediated Vascular Smooth Muscle Cell Migration Through Suppression of NF-κB Activation

Xiujie Gao, PhD, Lei Wu, MSc, Kun Wang, MSc, Xuesi Zhou, MSc, Meng Duan, MSc, Xinxing Wang, PhD, Zhiqing Zhang, PhD and Xiaohua Liu, PhD

Summary

Ubiquitin carboxyl terminal hydrolase L1 (UCH-L1) is one of the deubiquitinating enzymes in the ubiquitin-proteasome system. It has been shown that UCH-L1 could markedly decrease neointima formation through suppressing vascular smooth muscle cell (VSMC) proliferation in the balloon-injured rat carotid. However, whether UCH-L1 plays roles in VSMC migration remains to be determined. In this study, the primary VSMCs were isolated from aortic media of rats and TNF-α to was used to induce VSMC migration. Using a modified Boyden chamber and wound healing assay, it was found that TNF-α can dose and time-dependently induce VSMC migration with a maximal effect at 10 ng/mL. Moreover, UCH-L1 expression increased gradually with the prolonged induction time at 10 ng/mL of TNF-α. UCH-L1 content in VSMC was then modulated by recombinant adenoviruses expressing UCH-L1 or RNA interference to evaluate its roles in cell migration. The results showed that over-expression of UCH-L1 attenuated VSMC migration, while knockdown of it enhanced cell migration significantly no matter whether TNF-α treatment or not. Finally, the effect of UCH-L1 on NF-κB activation was demonstrated by NF-κB nuclear translocation and DNA binding activity, and the levels of IL-6 and IL-8 in cell culture media were examined by ELISA. It was showed that UCH-L1 over-expression inhibited NF-κB activation and decrease IL-6 and IL-8 levels, while knockdown of it enhanced NF-κB activation and increase IL-6 and IL-8 levels during TNF-α treatment. These data suggest that UCH-L1 can inhibit TNF-α-induced VSMCs migration, and this kind of effect may partially due to its suppression role in NF-κB activation. (Int Heart J Advance Publication)

Key words: Deubiquitylating enzyme, Signal pathway, Vascular remodeling

Atherosclerosis is a pathological process affecting numerous cardiovascular diseases including acute coronary syndrome. Migration of VSMC from the media into the intima has been known as one of the crucial steps in the development of atherosclerosis. Clarification of the mechanisms of VSMC migration can not only provide therapeutic target for atherosclerosis, but also reduce the morbidity and mortality of atherosclerosis-related cardiovascular diseases.

The ubiquitin proteasome system regulates various biological processes such as cell proliferation, the cell cycle, apoptosis, and signal transduction. Ubiquitination disorders have been found in contributing to the development of several cardiovascular diseases. Ubiquitin carboxyl terminal hydrolase L1 (UCH-L1), also known as protein gene product 9.5 (PGP 9.5), is an important member of deubiquitinating enzymes in the ubiquitin-proteasome system. In addition to acting as a deubiquitinating enzyme, UCH-L1 also expressed the activity of ubiquitin ligase, and owned the function in stabilizing intracellular ubiquitin monomer. It has been found that UCH-L1 was closely related with a variety of human diseases such as neural degenerative diseases, tumor, and infertility. Takami’s work demonstrated that UCH-L1 inhibited vascular lesion formation via suppression of an inflammatory response in the vasculature. UCH-L1 negatively regulated TNF-α-mediated VSMC proliferation via suppression of ERK activation. However, whether UCH-L1 also played roles in VSMC migration and its signaling mechanisms in VSMC migration remain to be examined. In the present study, we investigate the effect of UCH-L1 on migration of VSMCs stimulated by TNF-α in order to elucidate the mechanism of UCH-L1 in vascular remodeling.
Methods

Reagents and antibodies: The recombinant rat TNF-\(\alpha\) was purchased from R&D systems, Inc., Minneapolis, MN, USA. The Millicell was purchased from EMD Millipore Corporation, Billerica, MA, USA. The enhanced chemiluminescence detection kit and antibodies (anti-\(\alpha\)-smooth muscle actin, anti-UCH-L1, anti-p65, anti-lamin A/c, anti-GAPDH) were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. The vectors of pDONR221 and pAd/CMV/V5-DEST, recombination system, BLOCK-it\(^{TM}\) Pol II miR RNAi Expression Vector Kit with EmGFP, and Lipofectamine 2000 were purchased from Invitrogen, Carlsbad, CA, USA. The ELISA kits for IL-6 and IL-8 were purchased from Thermo Scientific, Inc, Waltham, MA, USA. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone, Cramlington, UK. Nuclear extract kit was purchased from Active Motif (Carlsbad, CA, USA).

Cell culture: The primary rat VSMCs were isolated from thoracic aortic media of male Wistar rats (180-200 g) according to Sachinidis’s method. The cells were cultured in DMEM supplemented with 10% FBS, and maintained in a humidified atmosphere of 5% CO\(_2\) at 37°C. Following four passages, cells were immunostained with an anti-\(\alpha\)-actin antibody to characterize the cells as VSMCs by using the method of fluorescence immunoassay.

Construction of adenovirus for up-regulating or interfering UCH-L1 expression: The recombinant adenovirus expression vector of UCH-L1 was constructed based on the methods described previously. In brief, UCH-L1 full length coding sequence was amplified using specific primers (5'-GAAGATCTG ATGCA GCTGA AACCAG ATGGAGATTA AC-3' and 5'-CGGGATCC CGGGCTGCTT TGAGACGGAGC CACTG CTCA-3') according to the sequence of rat UCH-L1 (NM_017237), and subcloned into the shuttle pEGFP-N1 (with GFP gene). Then the UCH-L sequence was amplified using specific primers of cell migration. Three fields per well were evaluated, and experiments were performed at least in duplicate.

Cell migration assay: The transwell migration assay (Boyden Chamber) was modified from a previous publication. In brief, 5 × 10\(^4\) cells were added to the upper well of 24-well plates and placed over a polycarbonate membrane. TNF-\(\alpha\) was used as a chemotactic stimulus in the lower well. Following the prescribed time at 37°C, non-migrating cells were removed using a cotton swab and the migrating cells on the bottom of the membrane were counted with a light microscope (Olympus, ix70). Each condition was tested at least in duplicate.

Cell migration was evaluated using wound healing assay. VSMCs (5 × 10\(^5\) cells/well) were grown at the bottom of 6-well plates with low serum culture medium (0.1%-0.5% FBS) overnight. A 0.5 mm-wide and cells free scratch space was achieved with a sterile tip in the center of the bottom of 6-well plates according to Taboubi’s method. Cells then lived in normal serum with or without the addition of TNF-\(\alpha\). Migration was assessed with a light microscopy at 100 × magnification.

Cell migration was expressed as a relative percentage of the scratch spacing of each time point to the initial scratch spacing, and the smaller of the relative percentage the larger of cell migration. Three fields per well were evaluated, and experiments were performed at least in duplicate.

Western blot analyses: The cultured rat VSMCs were harvested and lysed for extracting proteins. RIPA with both protease and phosphatase cocktail was applied to extract total proteins, and nuclear extract kit was performed to extract nuclear and cytoplasmic proteins. Equal amounts of protein from tissue lysates were resolved on SDS-PAGE. Membranes with the target proteins were incubated at 4°C with primary antibodies overnight. Following incubation with the appropriate peroxidase-conjugated secondary antibodies, proteins were visualized using an enhanced chemiluminescence detection kit.

Electrophoretic mobility shift assay (EMSA): EMSA was performed with a Promega gel shift system as previously described, except we used nuclear extracts rather than whole cell lysates. Nuclear extracts were prepared as described. EMSA probes with 50 biotin labels were purchased from Sigma-Aldrich. Nucleotide sequences were 5'-AGTTG AGGGG ACTTT CCCAG GC-3'. Nucleotide sequences were 5'-AGTTG AGGGG ACTTT CCCAG GC-3'.

Enzyme-linked immunosorbent assay (ELISA): IL-6 and IL-8 levels in cell culture media were detected using IL-6 and IL-8 ELISA kits, respectively. In brief, cells were seeded into 24-well plates 24 hours prior to assay performance. Supernatants from cells were collected and analyzed for IL-6 and IL-8 levels according to the manu-
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Figure 1. UCH-L1 expression increased in VSMC during TNF-α-induced VSMC migration. A: VSMCs were induced with different concentration of TNF-α for 12 hours and cell migration was detected by using a modified Boyden chamber. Results represent at least four independent experiments. B: 10 ng/mL of TNF-α was used to induce VSMC for different times and cell migration was determined using a wound healing assay. The length between two arrows represents the scratch distance. C: The scratch space analysis. A relative percentage of the scratch spacing of each time point to the initial scratch spacing (control) was calculated to show cell migration. Results represent at least three independent experiments. D: UCH-L1 expression was detected using western blot methods. Cultured VSMCs were treated with 10 ng/mL TNF-α for various times. Protein samples were then extracted and detected. GAPDH levels were measured as an internal control. *P < 0.05 versus 0 hours, #P < 0.01 versus 0 hours (n = 3).

Results

UCH-L1 expression increased during TNF-α-mediated VSMC migration: When increasing concentrations of TNF-α were introduced to VSMC for 12 hours, the number of migrating cells on the bottom of the membrane rose at first, reached maximal (75.0 ± 9.2/ field) at the concentrations of 10 ng/mL, and then reduced with higher concentration of TNF-α (Figure 1A). Therefore, VSMCs treated with 10 ng/mL of TNF-α for 12 hours were selected as the cell model for the transwell migration assay.

In wound healing assay, it was found that the distance between the scratch line was getting shorter with the prolongation of treatment time induced by 10 ng/mL of TNF-α (Figure 1B). The wound scratch distance was significantly shorter at 12 hours of TNF-α induction (P < 0.05) and approached confluence at 48 hours (Figure 1C). These results showed that TNF-α could time-dependently induce VSMC migration.

Finally, UCH-L1 protein expression was detected and results showed that UCH-L1 expression increased gradually along with the augment of cell migration induced by TNF-α (Figure 1D).

Over-expression of UCH-L1 inhibited TNF-α-mediated VSMC migration: We have found that UCH-L1 was up-regulated during TNF-α-mediated VSMC migration. In order to elucidate the relationship between UCH-L1 expression and VSMC migration, we firstly investigated the effect of UCH-L1 over-expression (Figure 2A) on TNF-α-mediated VSMC migration. In the wound healing assay, the scratch distances in control group (control) and adenovirus transfection control group (Ad-GFP) were reduced

Statistical analyses: Data are expressed as mean ± standard error of the mean (SEM). Differences in means between multiple groups were assessed using analysis of variance (ANOVA). Statistical significance was established between two groups using a Student’s t test and a post hoc test. All tests were performed in triplicate. P < 0.05 was considered statistically significant.
Knockdown of UCH-L1 expression promoted TNF-α-mediated VSMC migration: Although it had been found that UCH-L1 over-expression could suppress TNF-α-mediated VSMC migration, we then knocked down UCH-L1 expression in VSMCs by using adenovirus containing interference sequence of UCH-L1 (Figure 3A). Results showed that compared with control group (control) and adenovirus transfection control group (Ad-RNAi-C), the scratch distance became apparent narrower when knockdown of UCH-L1 expression (Ad-UCHL1-RNAi) in VSMCs after 10 ng/mL of TNF-α inducing for 36 hours by the wound healing assay (Figure 3B, C). Similarly, in the transwell migration assay, the number of migrated VSMCs also increased when knockdown of UCH-L1 expression no matter treated with TNF-α or not (Figure 3D). These results further confirmed that UCH-L1 played roles in regulating VSMC migration not only in the normal physiological condition, but also in TNF-α increased pathological status.

UCH-L1 negatively regulated TNF-α-mediated VSMC migration via suppression of NF-κB activation: Previous studies have shown that UCH-L1 could suppress TNF-α-mediated VSMC proliferation via ERK activation. As we have known, TNF-α-mediated VSMC migration requires NF-κB activation, so the effect of UCH-L1 on the NF-κB activation during VSMC migration was explored. Firstly, the downstream regulated gene of NF-κB pathway, such as IL-6 and IL-8, were analyzed. It was found that UCH-L1 over-expression inhibited IL-6 and IL-8 expression (Figure 4A), while knockdown of UCH-L1 enhanced their expression after TNF-α treatment (Figure 4B). NF-κB pathway promotes inflammation response by inducing pro-inflammatory molecules in an NF-κB nuclear translocation and DNA-binding dependent fashion. We then detected the NF-κB nuclear translocation by western blot.
**Figure 3.** Effect of UCH-L1 gene knockdown on TNF-α-induced VSMC migration. A: The knockdown of UCH-L1 expression in Ad-UCH-L1-RNAi-infected VSMCs by the use of western blot. B, C: VSMC migration from different groups (control, Ad-RNAi-C, Ad-UCHL1-RNAi) was detected using wound healing assay with or without 10 ng/mL of TNF-α induced for 0 hours and 36 hours. Data are shown as scratch distance. Results represent at least three independent experiments. D: VSMC migration from different groups (control, Ad-RNAi-C, Ad-UCHL1-RNAi) was determined using transwell migration assay with or without 10 ng/mL of TNF-α induced for 12 hours. Data are shown as cell count. Results represent at least four independent experiments. *P < 0.05 versus Ad-RNAi-C (n = 3).

**Figure 4.** Effect of UCH-L1 on the secretion of pro-inflammatory molecules of IL-6 and IL-8 in the supernatants during TNF-α-induced VSMC migration. A: Concentrations of IL-6 and IL-8 in the supernatants were measured using ELISA when UCH-L1 expression was up-regulated. *P < 0.05 versus Ad-GFP, #P < 0.05 versus Ad-GFP + TNF-α (n = 6). B: Concentrations of IL-6 and IL-8 in the supernatants were measured using ELISA when UCH-L1 expression was knocked down. *P < 0.05 versus Ad-RNAi-C, #P < 0.05 versus Ad-RNAi-C + TNF-α (n = 6).
activity at 10 ng/mL. According to a previous report\(^{34}\), 10 ng/mL of TNF-α dependence from 0 to 10 ng/mL and with maximal concentration-mediated VSMC migration in a concentration-dependent manner from 0 to 10 ng/mL and with maximal activity at 10 ng/mL. According to a previous report\(^{34}\), 10 ng/mL of TNF-α showed no effect on VSMC proliferation when treated for 18 hours. Therefore, in our study, the influence of cell proliferation might be excluded when 10 ng/mL TNF-α was used in the subsequent experiments.

UCH-L1 is firstly screened as one gene that can inhibit both vascular cells proliferation and NF-κB activity in the vasculature by Takami, et al.\(^{35}\) Then, functional researches showed that UCH-L1 inhibited vascular lesion formation via suppression of inflammatory responses\(^{36}\) and negatively regulates TNF-α-mediated VSMC proliferation via suppression of ERK activation.\(^{37}\) These data suggested that UCH-L1 might play roles in vascular remodeling. However, the exact signaling mechanisms of UCH-L1 in VSMC proliferation and its effects on VSMC migration are unknown. In the present research, we explored the role of UCH-L1 in the regulation of TNF-α-mediated VSMC migration in vitro. Firstly, we established the cell model of VSMC migration induced by TNF-α, and found that UCH-L1 protein expression changed dramatically from extremely low in untreated VSMCs to increase significantly start from the time point of 12 hours, reach a peak level at 36 hours, and remain elevated until 48 hours during TNF-α-mediated VSMC migration. These results indicated a potential link between VSMC migration and UCH-L1. Then, adenovirus was used to regulate UCH-L1 content in VSMCs. It was found that UCH-L1 over-expression could inhibit VSMC migration and knockdown of UCH-L1 enhanced cell migration no matter TNF-α treatment or not. There were other mechanisms in UCHL1 inhibiting VSMC migration without TNF-α treatment, such as ERK pathway, the mechanisms need to be further studied. However, the present results proved that UCH-L1 functions as a regulator of VSMC migration mediated by TNF-α. Finally, the mechanisms of UCH-L1 in regulating VSMC migration were explored. Because NF-κB signaling is important for TNF-α-mediated VSMC migration and related to ubiquitin mediated protein degradation, we detected the influence of

analyses. We found that overexpression of UCH-L1 significantly inhibited TNF-α-induced nuclear translocation of p65 (Figure 5A). Conversely, the knockdown of UCH-L1 enhanced it (Figure 5B). To further address the inhibition of NF-κB activation by UCH-L1, DNA-binding activity of NF-κB was detected by the EMSA assay. Results showed that UCH-L1 over-expression could inhibit the NF-κB DNA-binding activity during TNF-α treatment, while knockdown of UCH-L1 expression would enhanced the NF-κB DNA-binding activity (Figure 5C). These data suggest that UCH-L1 controls VSMC migration induced by TNF-α might through modulation of NF-κB activation.

![Figure 5](https://example.com/figure5)

**Figure 5.** UCH-L1 inhibits NF-κB nuclear translocation and DNA binding activity in VSMCs. A: The effect of UCH-L1 overexpression on the NF-κB p65 nuclear translocation. VSMCs were transfected with Ad-UCH-L1, and then treated with (+) or without (-) TNF-α (10 ng/mL) for 30 minutes. B: The effect of UCH-L1 knockdown on the NF-κB p65 nuclear translocation. VSMCs were transfected with Ad-UCH-L1-RNAi, and then treated with (+) or without (-) TNF-α (10 ng/mL) for 30 minutes. C: NF-κB DNA-binding activity in VSMC was detected using EMSA when UCH-L1 expression was regulated after treating with TNF-α for 30 minutes.

**Discussion**

The pathogenesis of atherosclerosis has not been fully elucidated, although several hypotheses have been described in an attempt to explain the genesis and progression of atherosclerotic lesions.\(^{37,38}\) VSMC migration from the vascular media to intima is considered as an important pathogenic event during lesion formation in atherosclerosis.\(^{39}\)

It has been found that many factors can promote VSMC proliferation and migration, such as PDGF,\(^{40}\) angiotensin II,\(^{41}\) IL-1 and IL-6,\(^{42}\) TNF-α,\(^{43}\) VEGF,\(^{44}\) FGF,\(^{45}\) EGF,\(^{46}\) IGF,\(^{47}\) bFGF,\(^{48}\) and neuropeptide Y.\(^{49}\) TNF-α is one of the important factors released by inflammatory cells at sites of vascular injury and expressed in the arterial wall under pathological conditions, where it is associated with lesion formation.\(^{29,30}\) Previous studies have demonstrated that TNF-α is a potent promoting migration factor for different cell types, including fibroblasts, inflammatory, and VSMCs.\(^{31,33}\) In the present study, TNF-α could induce VSMC migration in a concentration-dependent manner from 0 to 10 ng/mL and with maximal activity at 10 ng/mL. According to a previous report,\(^{34}\) 10 ng/mL of TNF-α showed no effect on VSMC proliferation when treated for 18 hours. Therefore, in our study, the influence of cell proliferation might be excluded when 10 ng/mL TNF-α was used in the subsequent experiments.

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UCH-L1 on NF-κB activity. All of these results demonstrate that both NF-κB nuclear translocation, DNA-binding activity and its downstream gene expression (IL-6 and IL-8) could be regulated negatively by UCH-L1.

In conclusion, we found that UCH-L1 can inhibit TNF-α-induced VSMCs migration, and this kind of effect of UCH-L1 may partially due to its suppression role on NF-κB activation. These results provide novel role of UCH-L1 in vascular remodeling.

Disclosures
Conflicts of interest: The authors declare that they have no competing interests.

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