Int J Clin Exp Pathol 2016;9(6):6023-6030
www.ijcep.com /ISSN:1936-2625/IJCEP0022919

Original Article

Matrix metalloproteinase-1 promotes keratinocytic proliferation and migration

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Received December 29, 2015; Accepted March 17, 2016; Epub June 1, 2016; Published June 15, 2016

Abstract: Matrix metalloproteinases (MMPs) has been reported to be involved in epidermal proliferation. However, little information is available regarding the effect of MMP-1 on keratinocytes. Therefore, we aimed to investigate the effect of MMP-1 on keratinocytes proliferation and migration. Human keratinocyte cell line HaCaT was transfected with target sequence for MMP-1-specific small interfering RNA (siRNA). After transfection, the MMP-1 mRNA levels were determined using quantitative real-time RCR (qRT-PCR). Cell proliferation, apoptosis, and migration ability were analyzed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay, TUNEL method, and microchemotaxis chambercell, respectively. In addition, protein levels of E-cadherin were assessed by Western blotting. The mRNA levels of MMP-1 were statistically decreased by transfection with siMMP-1 compared to control group and scrambled group ($P < 0.05$). After knockdown of MMP-1, the cell viability and migration ability were both significantly decreased, while the apoptotic rate was significantly increased in the siMMP-1 group ($P < 0.05$). Moreover, the levels of E-cadherin were significantly up-regulated by transfection with siMMP-1. MMP-1 plays significant roles in keratinocytic proliferation and migration. These effects may be through regulating E-cadherin.

Keywords: Matrix metalloproteinase-1, keratinocytes, proliferation, migration, E-cadherin

Introduction

Cutaneous wound healing and/or scar formation is one of the most complex biological and critical processes in clinical practice which is accomplished by multi-step process [1, 2]. Re-epithelialization is an essential feature of the development of wound healing [3, 4]. The proliferation and migration of keratinocyte in or around the wound is believed to play significant roles in re-epithelialization [5]. However, the mechanism initiating the proliferation and migration of keratinocytes has not been fully elaborated.

In addition to the keratinocytic proliferation and migration, several molecules, such as extracellular matrix (ECM) components, play significant roles in epidermal wound healing [6]. Excessive deposition and alterations in ECM molecules, especially collagen, is also a fundamental element of wound healing [7, 8]. The proteolytic degradation of ECM molecules and cleavage of collagen are the functions of matrix metalloproteinases (MMPs). MMPs are one of zinc-dependent neutral endopeptidases. A great deal of studies has been performed to analyze the differential expression and functional role of MMPs and their tissue inhibitors (TIMPs) in the epidermis or in diseased skin [9-11]. For example, Hattori et al. have demonstrated that MMP-13 is involved in migration of keratinocyte, angiogenesis, and contraction in wound healing, while MMP-9 plays a significant role in keratinocyte migration [12]. Sadowski et al. suggested that the dysregulation of MMP-19 expression in epidermis was implicated in the cutaneous infections and proliferative skin diseases [11]. However, the exact mechanism by which MMP-1 on keratinocytes proliferation and migration is yet to be understood. Additionally, E-cadherin has been reported to mediate cell-cell interactions and is responsible for the establishment and maintenance of tissue architecture [13]. It contributes to the regulation of cell attachment, survival, motility, and

growth. Loss of E-cadherin is consistently found in many malignancies [14].

In our study, we assumed that the effect of MMP-1 on keratinocytes proliferation and migration might be involved with the expression of E-cadherin. First, the expression of MMP-1 was knockdown by small interfering RNA (siRNA) method. Then we investigated the effect of knockdown of MMP-1 on keratinocytes proliferation and migration. Thereafter, the effect of knockdown of MMP-1 on the expression of E-cadherin was explored. Our research might provide new insights into skin disease or wound healing.

Material and methods

Cell culture of HaCaT cells

The human keratinocyte cell line HaCaT obtained from the American Type Culture Collection (ATCC, USA) was used in our study. The cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 4.5 g/L D-glucose, L-glutamine, and 110 mg/L sodium pyruvate (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen). The cells were maintained at 37°C with 5% CO_2. The cells were passaged by using 0.25% trypsin (Sigma-Aldrich, St. Louis, MO) and harvested when the cells reached 70-80% confluent for further analysis.

Transfection

For transfection studies, HaCaT cells (1×10^6 per dish) were plated on 60-mm dish and subjected to transfection. Using siRNA approach, the expression of MMP-1 was knockdown with Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. The target sequence for siMMP-1 and a scrambled RNA were synthesized and constructed by GenePharma Corporation (Shanghai, China). After 48 h of transfection, the cells were harvested for further experiments.

Quantitative real-time RCR (qRT-PCR)

Total RNA was extracted from HaCaT cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed into complementary DNA (cDNA) using Taq-Man Reverse Transcription reagents (Perkin Elmer, Applied Biosystems Division, Darmstadt, Germany). To assess the relative gene expression levels of MMP-1, cDNA was amplified by qRT-PCR using the SYBR Green-based detection system (iQ SYBR Green Supermix, Bio-Rad Laboratories). Reactions were carried out in triplicate. A housekeeping gene GAPDH was used as a reference. The expression levels of target gene were determined using the comparative CT ($2^{-\Delta\Delta CT}$) method. The primers utilized were listed as below: GAPDH: F5'-GGTCTCCTCTGACTTCAACA-3', R5'-AGCCAAATTCGTTGTCATAC-3'; MMP-1: F5'-GAGCAAACACACTGACCTACAGGA-3', R5'-TTGTCCCGATGATCTCCCCTGACA-3'.

Cell proliferation assay

HaCaT cell proliferation was determined after 72 h and 7 days of transfection by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. In brief, HaCaT cells were placed in 96-well plate and incubated at 37°C in a 5% CO_2 incubator. At different time points (0 h, 72 h, and 7 days), a volume of 10 μL of 12 mM MTT was added to each well, and then maintained at 37°C for another 4 h in the darkness. After wash with phosphate-buffered saline solution (PBS), the absorbance at 570 nm was monitored by a Synergy 4 Multi-Mode Microplate Reader (BioTek Instruments, USA).

Cell migration assay

HaCaT cell migration ability was determined by using a 48-well microchemotaxis chambercell (Neuroprobe). Briefly, DMEM medium supplemented with 10% FBS was placed in the lower chamber and serum-starved HaCaT cells (1.0×10^5 cells/mL) were added to the upper chamber. A polycarbonate membrane (10 μM) (Sigma-Aldrich, St. Louis, MO, USA) treated with 0.01% collagen type I (Coll-I) was placed between the upper and lower chamber for 4 h. Thereafter, the membrane was removed and the upper chamber was wiped. The cells were then fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The number of migrated cells in each well was calculated in five random fields under an inverted phase contrast microscope (Nikon, Tokyo, Japan). Each experiment was conducted out in triplicate.

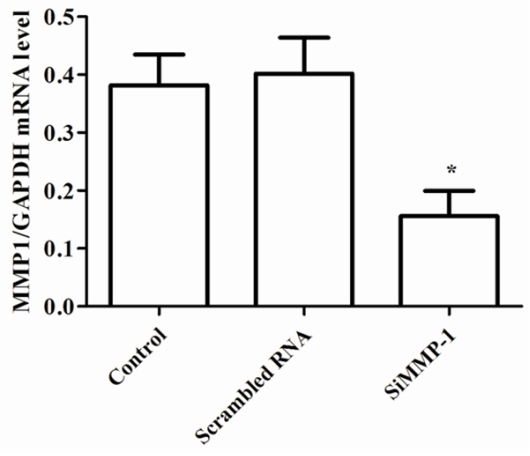

Figure 1. Effect of transfection on expression of MMP-1. Matrix metalloproteinase-1; siMMP-1, small interfering MMP-1 *P < 0.05 compared to the control group and the scrambled group.

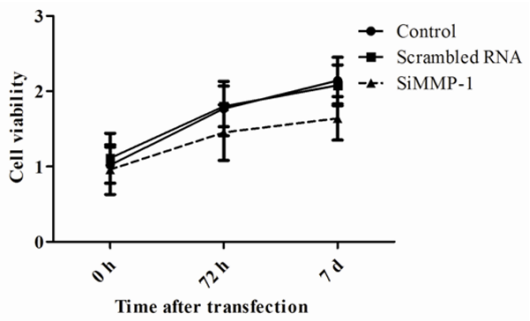

Figure 2. Effect of transfection on cell proliferation. Matrix metalloproteinase-1; siMMP-1, small interfering MMP-1.

Cell apoptosis assay

Cell apoptosis was determined using a TUNEL kit (APO-DIRFCTTM Kit, BD Biosciences) according to the manufacturer's instructions. Briefly, HaCaT cells were harvested, washed, centrifuged and re-suspended in paraformaldehyde in PBS. Thereafter, the cells were washed twice with PBS, centrifuged, and fixed in 70% ethanol at -20°C for 12 hours. The cells were then stained with terminal deoxynucleotidyl transferase (TdT) and FITC-labeled deoxyuridine triphosphates (FITC-dUTP). Apoptosis of HaCaT cells was determined using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Western blotting

HaCaT cells were harvested, washed with PBS, and then lysed in RIPA buffer for protein extraction. Protein concentration was determined using a BCA assay kit (Sigma-Aldrich). Thereafter, the protein extracts was subjected to 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride (PVDF) (Bedford, MA, USA) membrane, and then blocked with 5% skim milk in Tris-buffered saline containing 0.5% Tween 20 for 2 h at room temperature. Then the membranes were incubated overnight at 4°C with anti-E-cadherin antibody (Sigma-Aldrich Corporation) or anti-GAPDH antibody (Sigma-Aldrich Corporation), subsequently horseradish peroxidase-conjugated secondary antibody was performed. Immunoreactive bands were detected by a SuperSignal West Pico chemiluminescence substrate (Thermo Scientific, Waltham, MA, USA).

Statistical analysis

One-sample K-S test was first performed to identify normal distribution. Chi-square test or rank-sum test was used to analyze enumeration data. The collected data were tested by student t-test (for two groups) or analysis of variance (ANOVA, for more than three groups). The pairwise comparisons were then performed by post-hoc Tukey test. The statistical analysis was subjected to statistic package for social science (SPSS, version 18.0, Chicago, IL). A statistical significance was defined when $P < 0.05$.

Results

Effect of transfection on expression of MMP-1

To determine the silencing effect of MMP-1 on HaCaT cells, siRNA technology was performed to down-regulate the expression of MMP-1. After 48 h of transfection, mRNA levels of MMP-1 were measured by qRT-PCR. The results showed that the mRNA levels of MMP-1 were significantly reduced by transfection with siMMP-1 compared to the scrambled RNA group and control group ($P < 0.05$) (**Figure 1**). However, no significant differences were found between the control group and scrambled RNA.

Effect of transfection on cell proliferation

To investigate the silencing effect of MMP-1 on HaCaT cells proliferation, cell viability was evaluated by MTT assay. We found that silencing of MMP-1 could significantly decrease the cell viability (**Figure 2**) at different points (72 h and

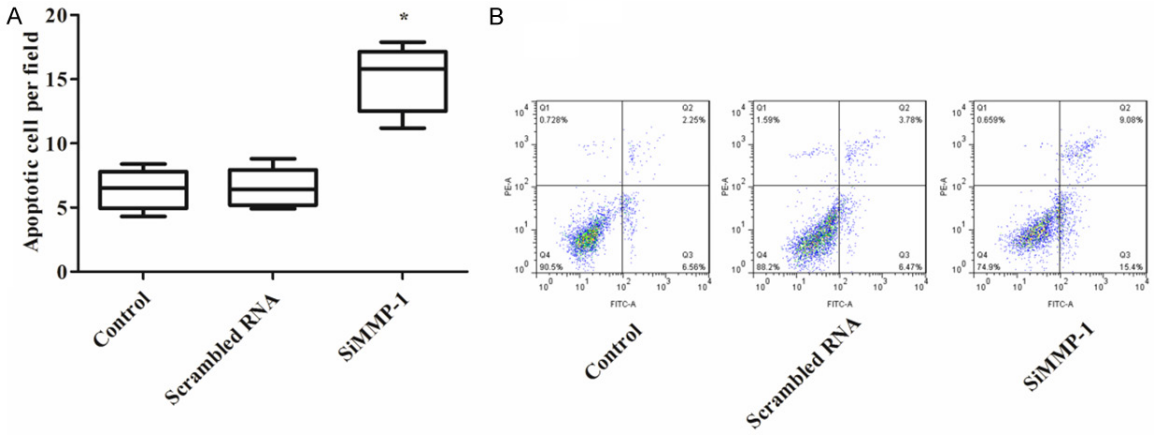

Figure 3. Effect of transfection on cell apoptosis. A: Quantitative analyses of cell apoptosis; B: The picture of cell apoptosis. Matrix metalloproteinase-1; siMMP-1, small interfering MMP-1. *P < 0.05 compared to the control group and the scrambled group.

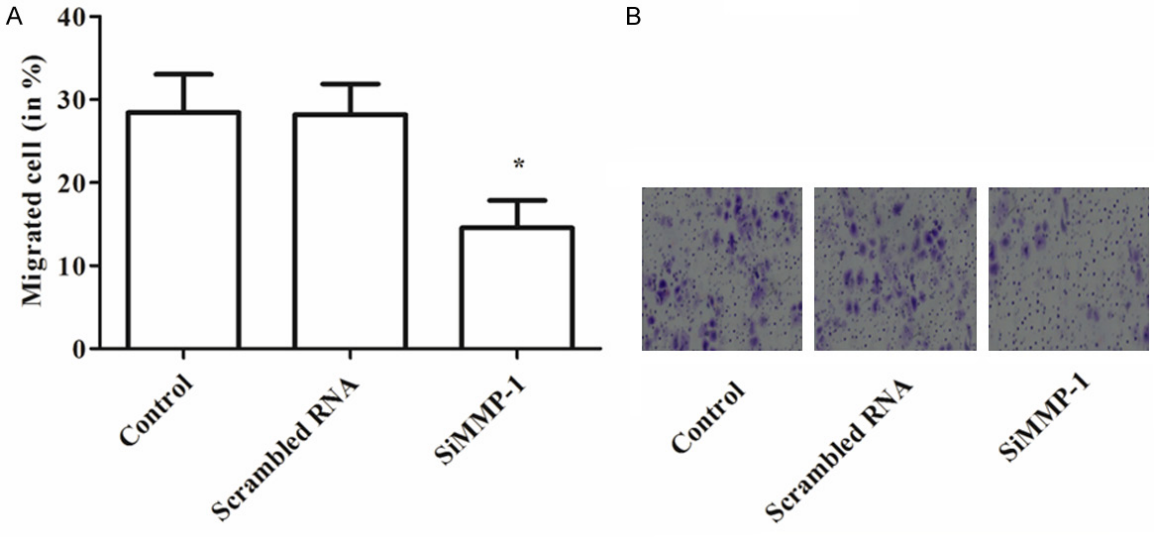

Figure 4. Effect of transfection on cell migration. A: Migrated cell after transfection in each group; B: The picture of cell migration. Matrix metalloproteinase-1; siMMP-1, small interfering MMP-1. *P < 0.05 compared to the control group and the scrambled group.

7 days) compared with the scrambled RNA group and control group (P < 0.05), indicating that MMP-1 promotes the keratinocytes proliferation.

Effect of transfection on cell apoptosis

To validate the silencing effect of MMP-1 on HaCaT cells apoptosis, cell apoptosis was examined by TUNEL method. The apoptotic cell per field was calculated. Although there were no significant differences between the control group and scrambled RNA group, the apoptotic cell per field was statistically higher in the siMPP-1 group than that in the control group or scrambled RNA group (P < 0.05) (**Figure 3A** and **3B**). This result suggested that MMP-1 inhibits the keratinocytes apoptosis.

Effect of transfection on cell migration

To search the effect of silencing of MMP-1 on cell migration ability, we performed the cell migration assay. As shown in **Figure 4A** and **4B**, the percentage of migrated cell was significantly lower in the siMMP-1 group than that in the

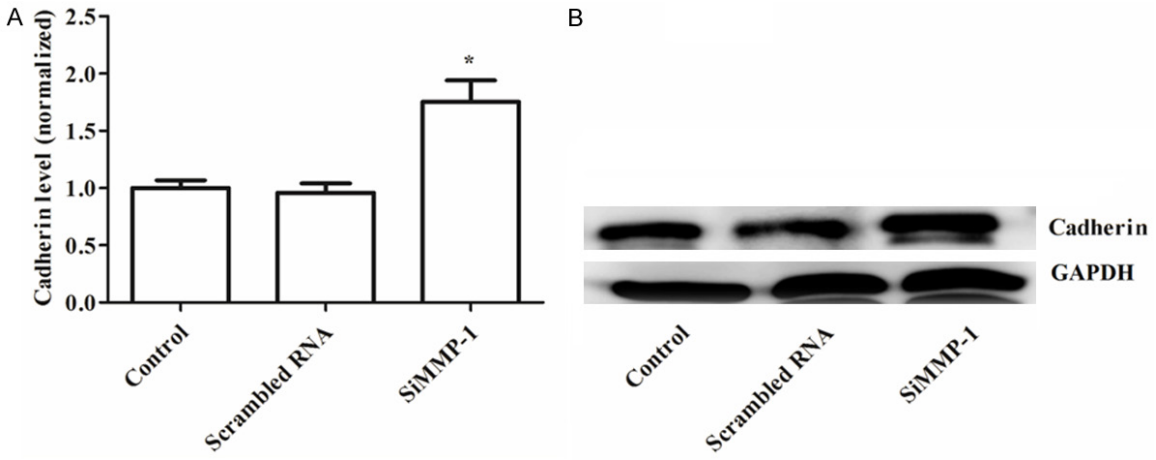

Figure 5. Effect of transfection on expression of E-cadherin. A: Relative expression of E-cadherin; B: The pictures of Western blotting. Matrix metalloproteinase-1; siMMP-1, small interfering MMP-1. *P < 0.05 compared to the control group and the scrambled group.

control group and the scrambled group (P < 0.05). But there were no significant differences between the control group and the scrambled group. Our results demonstrated that silencing of MMP-1 significantly reduced keratinocytes migration ability.

Effect of transfection on expression of E-cadherin

We further explored how MMP-1 regulates keratinocytes migration. The protein expression levels of E-cadherin were determined. As shown in **Figure 5A** and **5B**, the protein expression levels of E-cadherin were significantly increased by transfection with siMMP-1 compared to the control group and scrambled group (P < 0.05), indicating that MMP-1 contributes to keratinocytes migration ability by through modulating the expression of E-cadherin.

Discussion

Keratinocyte proliferation and migration are crucial events of re-epithelization, which is essential for wound healing, and numerous factors have been reported to affect these processes [15, 16]. In the present study, we found that knockdown of MMP-1 significantly decreased the keratinocytes viability and migration ability, while significantly increased the keratinocytes apoptotic rate. Moreover, the levels of E-cadherin were significantly up-regulated by knockdown of MMP-1. These results confirm that MMP-1 is responsible for the proliferation and migration of keratinocytes by regulating the expression of E-cadherin.

After injury, the skin repairs itself, namely wound-healing. Wound healing is a dynamic, complex, well-coordinated, and progressive process that involves in a sequence of cellular and biochemical materials [17, 18]. A series of overlapping stages have been demonstrated in the process of wound healing: haemostasis, inflammation, migration and proliferation, and remodeling [19]. During the proliferation, migration, and differentiation stage, it has been reported that the basal keratinocytes resolve their hemidesmosomes, interrupt the interaction with the basement membrane, and let migration through the wound matrix during the wound healing process [6]. MMPs, a type of ECM-degrading proteinases, contribute a lot in this process by regulating the wound matrix during keratinocyte migration. Then, keratinocytes can interact with the underlying dermis, exhibiting their special functions on wound repair.

The MMP family is a large family of extracellularly acting proteases, including 25 distinct extracellular endopeptidases and of which 24 are found in mammals [20, 21]. They can be grouped into seven different types: collagenases, gelatinases, stromelysins, matrilysins, metalloelastases, membrane-type MMPs (MT-MMPs) and other MMPs [22]. After stimulation by cell contact with ECM or other materials (e.g. cytokines and oncogenes), the MMPs can be transcriptionally activated and expressed in

many cell types, such as keratinocytes, fibroblasts, endothelial cells, and some inflammatory cells [23]. In addition to degrading or remodeling ECM, MMPs also exhibit other numerous and varied functions, such as regulation of cell-cell and cell-matrix which is involved in modulating matrix biology and cellular behavior signaling [24]. Among all the members of MMPs, MMP-1 could be expressed rapidly in response to wounding and/or injury. Moreover, it is only produced when the cells are in contact with native type I collagen [25, 26]. Furthermore, it has been demonstrated that keratinocyte migration on type I collagen depends on the communications among MMP-1, $\alpha_2\beta_1$ integrin (type I collagen integrin receptor) and type I collagen [6, 27]. In a human study, they found that the expression of MMP-1 represented with the highest level in migrating basal keratinocytes at 1 day after the skin was injured [28]. MMP-1 has been reported to be declined either in mRNA levels or activity in hypertrophic scars [29-31]. In contrast to hypertrophic scars, Dong et al. found that the expressions of MMP-1 and MMP-9 were up-regulated in scarless fetal rat wounds [32].

In our study, we explored the effect of knockdown of MMP-1 on keratinocytes proliferation and migration, as well as the possible mechanism. The expression of MMP-1 was down-regulated by using siRNA approach. The mRNA level of MMP-1 was identified by qRT-PCR. The results confirmed the level of MMP-1 was successfully down-regulated. Thereafter, we further analyzed the effect of knockdown of MMP-1 on keratinocytes proliferation, apoptosis, and migration. Our results showed that the keratinocytes proliferation and migration was significantly decreased by knockdown of MMP-1, while the apoptosis was markedly up-regulated, suggesting that MMP-1 promotes the keratinocytes proliferation and migration. We further investigated the underling possible mechanism. E-cadherin has been reported to be involved in maintenance of tissue integrity and wound healing [33, 34]. It is responsible for calcium-dependent cell-cell adhesion and acts as junctional proteins. The conserved cytoplasmic domain of E-cadherin is essential for the stabilization of intercellular adhesion. E-cadherin could be shed by MMPs [35], and then the cell-cell adhesions are loosened. Subsequently, E-cadherin shifts to a repair phenotype which is characterized by dedifferentiation, proliferation, and migration [36]. McGuire et al. has found that MMP-7 (matrilysin) modulates wound re-epithelialization by cleavage of E-cadherin within the adherens junction, which helps the epithelial cells migrate away from the wound edge. In addition, loss of MMP-7 leads to impaired E-cadherin cleavage in vivo [24]. Similarly, our results found that MMP-1 could regulate the re-epithelialization by cleavage of E-cadherin, suggesting that MMP-1 plays an important role in cleaving cellular adhesion molecules and thereby promotes the keratinocytes proliferation and migration.

In conclusion, the results of our experiment indicate that the stimulatory effects of MMP-1 on the cell proliferation, apoptosis and migration of HaCaT cells may be mediated by the expression of E-cadherin.

Disclosure of conflict of interest

None.

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