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Original Article

High glucose environment induces M1 macrophage polarization that impairs keratinocyte migration via TNF-α: An important mechanism to delay the diabetic wound healing

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ABSTRACT

Background: Macrophages play important roles during wound healing, and delayed healing in diabetics is associated with sustained inflammation. M1 type macrophage is recognized to secrete excessive amount of tumor necrosis factor-alpha (TNF-α) as compared to its M2 counterpart.
Objectives: We hypothesized that macrophage polarization is different between diabetic and normal rats during skin wounding and has direct impact on keratinocyte function in the context of re-epithelialization.
Methods: Skin wounds were created in diabetic and control rats. The phenotypes of infiltrating macrophages, the levels of TNF-α, and the rate of wound closure were determined. Using cell model, the effects of M1 type macrophage on keratinocyte migration were evaluated, and the potential regulatory pathways were determined.
Results: The percentage of M1 macrophages and the levels of TNF-α expression were significantly higher in the perilesional area of diabetic rats as compared to control. The condition media (CM) from M1 type macrophage upregulated tissue inhibitor metalloproteinases (TIMP)-1 expression in keratinocytes and significantly reduced keratinocyte migratory capacity. Addition of neutralizing TNF-α antibody to the CM or gene-silencing of TIMP1 in keratinocytes restored the keratinocyte migratory capacity. Treating wounds of diabetic rats with TNF-α antagonist improved the wound healing process.
Conclusions: In summary, high glucose wound environment harbored more M1 macrophages infiltration, an event that created excess TNF-α micro-environment. TNF-α upregulated TIMP1 expression in keratinocytes and resulted in impaired keratinocyte migration. Taken together, these events contributed to impaired wound healing during diabetic condition, and targeting TNF-α is a potential therapeutic option to improve diabetic wound healing.

1. Introduction

Dysregulation of glucose homeostasis and elevated glucose levels are central etiologies of diabetes, a medical condition commonly encountered [1]. WHO has projected that the incidence of diabetes will continue to rise around the globe [2]. An important complication associated with diabetes is poor wound healing that may lead to ulceration, infection, and ultimately amputation [3]. Clearly, understanding the mechanisms responsible for poor diabetic wound healing is an important public health issue that needs to be addressed.

Abbreviations: TNF-α, tumor necrosis factor-alpha; CCR7, C-C chemokine receptor type 7; TIMP1, tissue inhibitor of metalloproteinases 1; MMP1, matrix metalloproteinases 1; IL, interleukin; STZ, streptozotocin; CXCR4, C-X-C chemokine receptor type 4; PCR, polymerase chain reaction; DM, diabetes mellitus; IHC, immunohistochemistry; DAB, 3,3'-diaminobenzidine; PMA, phorbol 12-myristate 13-acetate; IFN-γ, interferon gamma; LPS, lipopolysaccharides; RNA, ribonucleic acid; FBS, fetal bovine serum; Ab, antibody; siRNA, small interfering RNA.

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Wound healing is a complex multicellular process involving keratinocytes, fibroblasts, endothelial cells, and inflammatory cells. During the early phase of healing process, platelet aggregates and disrupted keratinocytes near the wound site release various growth factors that enhance recruitment of inflammatory cells to the disrupted skin. Following the inflammatory cell infiltration, re-epithelialization process also begins [4]. Macrophages are recognized to play an important role during wound healing as depletion of macrophages results in significant delay of wound repair [5]. It has been recognized that significant amount of mononuclear cells infiltration were seen at edge of diabetic ulcers [6]. Therefore, it is likely that under high glucose conditions, the mononuclear cells were able to infiltrate the wound area but were dysfunctional in regards to their reparative capacity for stimulating proper re-epithelialization [7].

Most previous studies on diabetic wound healing focused on various cellular aspects of healing including inflammatory cells [6,8,9], fibroblasts [10–12], and endothelial cells [13–15]. The impacts of diabetes on keratinocytes, the main cell type responsible for re-epithelialization, have not been carefully examined. Recent studies suggest that macrophages exist in several different phenotypic states within the healing wound and may impart important impacts on proper wound healing [16]. More specifically, the “classically” activated (M1) and the “alternatively” activated (M2) subclasses have been defined [17]. The M1 phenotype is derived from resting macrophages, responding to pathogen associated molecular patterns (PAMPs) or to Th1 cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). M1 macrophages represent a pro-inflammatory phenotype, demonstrating increased phagocytic capacity, antigen presenting activity, pro-inflammatory cytokine secretion, and oxidative metabolites to promote host defense and elimination of damaged tissues [18,19]. During early inflammatory phase, M1 macrophages were important in clearance of dead cells and debris within the wound [20]. On the other hand, M2 macrophages represent a phenotype important in resolution of inflammation [21]. M2 macrophages are derived from resting macrophages after exposure to Th2 cytokines, such as IL-4 or IL-13 [22]. It has been reported that delayed wound healing in diabetic mice were associated with sustained M1 macrophage infiltration [23].

During the wound healing process when the intact basement membrane zone is disrupted, type I collagen is the provisional matrix used by migrating keratinocytes in the wound bed [24]. Previous studies demonstrated that proper functioning of matrix metalloproteinase (MMP) is required for migration of keratinocytes on type I collagen [25–27]. The function of MMPs is regulated by their inhibitors, tissue inhibitor metalloproteinases (TIMPs) [28]. In keloid, a condition characterized by excessive deposition of collagen on the dermis, gene-silencing of TIMP-1 leads to enhanced collagen degradation [29].

Recently, we demonstrated that high glucose conditions lead to keratinocyte dysfunction which contributes to impaired diabetic wound healing [27,30,31]. Although macrophages play a critical role during wound healing, the effects and impacts of high glucose environment on macrophage polarization in the context of re-epithelialization have not been carefully examined. The current study was launched to examine this clinically important issue and propose potential treatment strategy to promote wound healing in high glucose conditions.

2. Materials and methods

2.1. Streptozotocin (STZ)-induced diabetes mellitus animal model and TNF-α antagonist treatment

Male Wistar rats (BioLASCO Taiwan) with initial weight of 200−300 g were acclimated for 1 week before experimentation. Diabetic condition was induced by intravenous injection of STZ (55 mg/kg, Sigma-Aldrich). After 24 h, the rats with blood glucose levels over 250 mg/dL were considered as achieving successful induction. Four weeks after diabetes induction, the control, diabetic, saline-treated diabetic and anti-TNF-α treated diabetic rats were used for wound healing studies. One day before wounding and two and four days after wounding, Enbrel® (25 mg etanercept, Wyeth Pharmaceuticals) were injected to the peri-wound sites to neutralize TNF-α, and saline injection was used as control. Samples of rat skin were obtained under the protocols approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University (IACUC103053). Prior to injury, the animals were anesthetized by injection of a Zoletil 50 solution (50 mg/kg body wt i.p.). After shaving of the dorsal hair and cleansing of the skin with 70% ethanol, full-thickness excisional wounds were created using 6-mm biopsy stamps (Stiefel). Three days after wounding, the perilesional rat skin was harvested with 8-mm punch for IHC staining.

2.2. Immunohistochemical staining

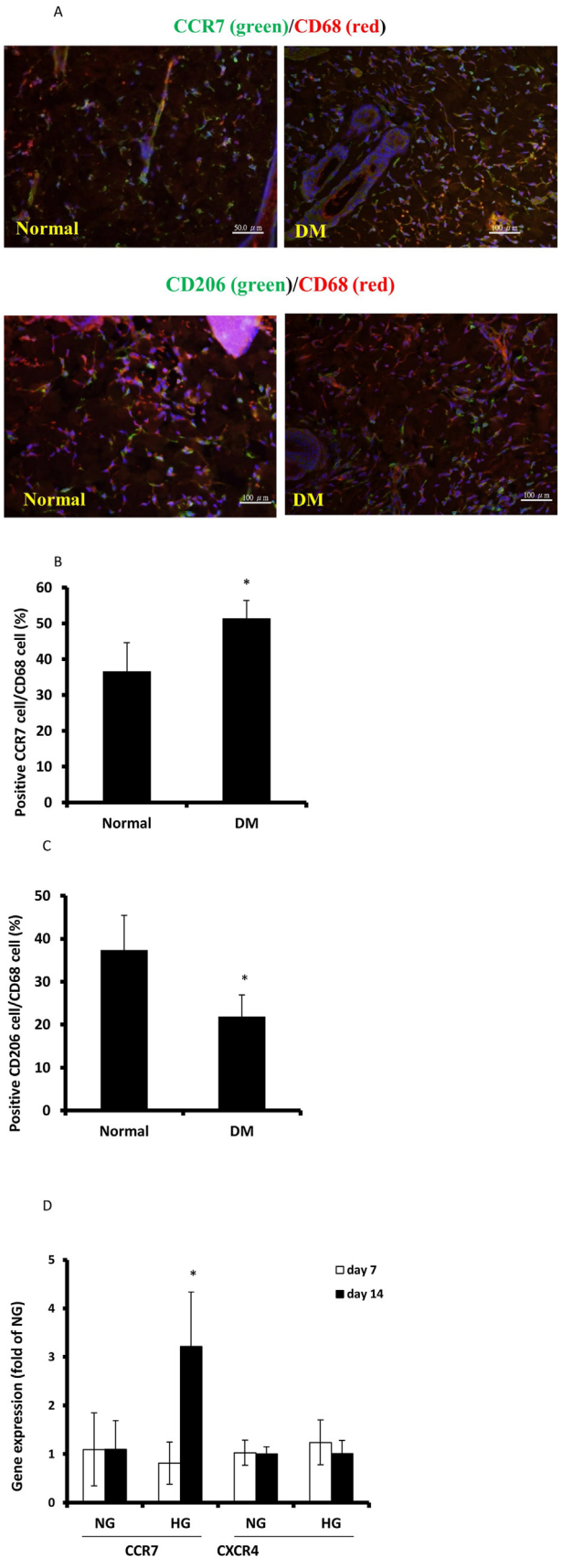
Rat skin specimens were embedded in paraffin wax, and tissue sections of 5-μm thickness were prepared as described previously [1]. The skin sections were incubated with anti-CD68, −CCR7, -CD206 antibodies that were pre-conjugated with Zenon® Alexa Fluor (ABcam) overnight at 4°C. Indicated skin samples were incubated with anti-TNF-α and −CCR7 antibodies (ABcam) overnight at 4°C, and subsequently incubated with EnVision/HRP reagent (DakoCytomation) for 30 min at room temperature. The staining was developed with DAB substrate chromogen solution (DakoCytomation). A DP70 light microscope (Olympus Corporation) was used to examine the skin sections.

2.3. Cell culture and treatment

THP-1 was purchased from BCRC (BCRC No. 60430, Hsinchu, Taiwan). The suspended THP-1 cells (2×10^6) were grown in RPMI

Table 1
Primer sequence of genes investigated in the study primer sequence.

gene	forward sequence	reverse sequence
CCR7	5'-GGTGGTGGCTCTCCTTGTCATT-3'	5'-GCTTTAAAGTTCCGCACGTCCTT-3'
CXCR4	5'-TCTGTGACCGCTTCTACC-3'	5'-AGGATGAGGATGACTGTGG-3'
MMP1	5'-GGGAGATCATCGGGACAACTC-3'	5'-GGGCCTGGTTGAAAAGCAT-3'
MMP2	5'-TTGACGGTAAGGACGGACTC-3'	5'-ACTTGCAGTACTCCCCATCG-3'
MMP3	5'-TGATCCTGCTTTGTCCTTTG-3'	5'-TTCAAGCTTCCTGAGGGATT-3'
MMP9	5'-TTGACAGCGACAAGAAGTGG-3'	5'-CCCTCAGTGAAGCGGTACAT-3'
TIMP1	5'-AAGGCTCTGAAAAGGGCTTC-3'	5'-GAAAGATGGGAGTGGGAACA-3'
TIMP2	5'-CCAAGCAGGAGTTTCTCGAC-3'	5'-GACCCATGGGATGAGTGTTT-3'



Medium (Gibco), treated with PMA (200 nM, Sigma-Aldrich) for 8 h to differentiate into attached macrophages (M0). To obtain M1 type macrophage, IFN-r (50 ng/ml, R&D Systems) and LPS (10 ng/ml, Sigma-Aldrich) were added for 24 h. The conditioned media (CM) were then collected and stored at −20 °C for the subsequent experiments. The cell pellets were used for mRNA extraction. For high glucose cultivation studies, 26 mM glucose (Sigma-Aldrich) was used for high glucose THP-1 cultivation.

The HaCaT keratinocyte cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Cultured cells were maintained in a humidified incubator with 5% CO_2 and atmosphere at 37 °C. The HaCaT cells were treated with 10% CM obtained from macrophages. In neutralization studies, the TNF-α neutralizing Ab (ABcam) was pre-incubated with indicated CM for 1 h.

2.4. RNA isolation, reverse transcription, and real-time quantitative PCR

Total RNA was extracted from THP-1 and HaCaT using the TRIzol reagent (Invitrogen). Three microgram of total RNA was reverse transcribed using the GoScript Transcription System (Promega) according to the manufacturer's specification. Quantitative real-time PCR was performed using QuantiFast SYBR Green PCR Kit (Qiagen), and the results were normalized according to the expression levels of GAPDH RNA. The primer sequences used are listed in Table 1. Amplification and detection of target mRNA were performed with a StepOne sequence detection system (Applied Biosystems). The expression of messenger RNA (mRNA) were calculated as 2-△△CT.

2.5. Migration assay

For keratinocyte migration evaluation, the *in vitro* scratch wound assay was performed. Cultured HaCaT cells were plated in tissue culture dishes precoated with type I collagen (10 μg/ml, Sigma-Aldrich). The cells were preincubated with 10 μg/ml of mitomycin-C (Sigma-Aldrich) for 2 h to inhibit cell proliferation. Monolayer of cells was scratched in a standardized manner with a sterile 200-μL pipette tip to create a cell-free zone in each dish. The medium was aspired, followed by extensive washing to remove cellular debris. The denuded surfaces were recoated with 10 μg/ml type I collagen in HaCaT culture medium for 1 h at 37 °C. The culture dishes were replaced with fresh HaCaT medium and 10% of indicated CM was added. Photographs were taken 0 and 24 h after wounding by Nikon ECLIPSE Ti inverted microscope with NIS-Elements F3.2 software (Nikon). Migration was quantified by ImageJ 1.48 s software (National Institutes of Health). The data were expressed as recovered area of the scratch filled by keratinocytes.

2.6. Small interfering RNA (siRNA) experiment

The HaCaT were transfected with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Fig. 1. High glucose environment enhanced M1 type macrophages induction *in vivo* and *in vitro*.(A). The representative figure of M1 type macrophages (CCR7, green; CD68, red) and M2 type macrophages (CD206, green; CD68, red) found at the perilesional skin of the normal and the diabetic (DM) rats 3 days after wounding (N = 5 for each group). (B). The percentage of CCR7 positive cells/CD68 cell and (C). the percentage of CD206 positive cells/CD68 cell in (A). (D). The expressions of CCR7 (M1 macrophages marker) and CXCR4 (M2 macrophages marker) in THP-1 cells treated with normal glucose (NG) or high glucose (HG) for 14 days were evaluated by real-time quantitative PCR. * Indicates $P < 0.05$ compared with normal control.

Briefly, 3 μl of TIMP1 siRNA or control siRNA and 3 μl of Lipofectamine RNAiMAX were each diluted with 50 μl of Opti-MEM® I Reduced Serum Medium (Invitrogen) and incubated at room temperature for 5 min. The sequences of double-strand siRNA directed against TIMP1 were 5′-UGCAGAAGGCCGUCU-GUGG-3′; and 5′−CCACAGACGGCCUUCUGCA-3′ (Sigma-Aldrich). The Stealth RNAi duplexes (Invitrogen) were used as a negative control. Subsequently, they were mixed and incubated at room temperature for 20 min. One hundred μl of the mixture (oligomer-Lipofectamine RNAiMAX complex) was added to each well which had 1 mL Opti-MEM and incubated at 37°C for 6 h. The reaction medium was changed to culture medium and incubated at 37°C for 24 h. Subsequently, scratch wound assay was performed.

2.7. Statistical analysis

The results were expressed as mean ± standard deviation. Student's-t test was used for statistical evaluation between the control and experimental groups. For all analyses, a P value < 0.05 was considered to be statistically significant.

3. Results

3.1. Streptozotocin-induced diabetic rats contained increased infiltration of proinflammatory M1 macrophages near the wounding site as compared to control

Normal and STZ-induced diabetic rats were used for *in vivo* studies. Previously, we had shown that wound healing capacity of diabetic rats was impaired [31]. At three days after wound induction, immunofluorescent (IF) staining showed that at the perilesional dermis (Fig. 1A), the ratio of CCR7 (marker for M1 macrophages) /CD68 (marker for macrophages) was significantly higher in the dermis of diabetic rats as compared to control rats (Fig. 1B). In contrast, the ratio of CD206 (M2 type macrophages marker) / CD68 was significantly higher in the perilesional dermis of control rats as compared to diabetic rats (Fig. 1C). These results corroborated with previous finding indicating that high glucose environment favors M1 type macrophage polarization [23]. Subsequently, *in vitro* model was employed to validate this finding. Human monocyte cells (THP-1) were cultivated at high glucose or normal glucose environment. Fourteen days after cultivation, THP-1 cells cultivated under high glucose condition expressed significantly higher CCR7, but not CXCR4 (M2 type macrophages marker) as compared to their normal glucose cultivated counterpart (Fig. 1D). This result corroborated with the notion that high glucose environment favors M1 macrophage polarization

3.2. The condition media (CM) of M1 type macrophage reduced the migratory capacity of cultured keratinocytes

Since high glucose environment resulted in M1 type macrophage predominance near the wound area, we next aimed to clarify the effect of M1 type macrophage on keratinocyte migration, a crucial step during re-epithelialization process. The monocytes were polarized to M0 and M1 macrophages as described in the method section. The CM from M0 and M1 macrophage cultures were collected. As demonstrated in Fig. 2, the migratory capacity was significantly reduced in keratinocytes treated with CM derived from M1 macrophages (wound closure: 56.2 ± 1.99%) as compared to their M0 CM treated counterpart (wound closure: 69.2 ± 2.98%). As aforementioned, MMPs play an important role regulating the migratory capacities of keratinocytes during the wound healing process. Therefore, the expressions of different MMPs and TIMPs were evaluated. As demonstrated in Fig. 2C, addition of M1 CM significantly reduced MMP1 expression

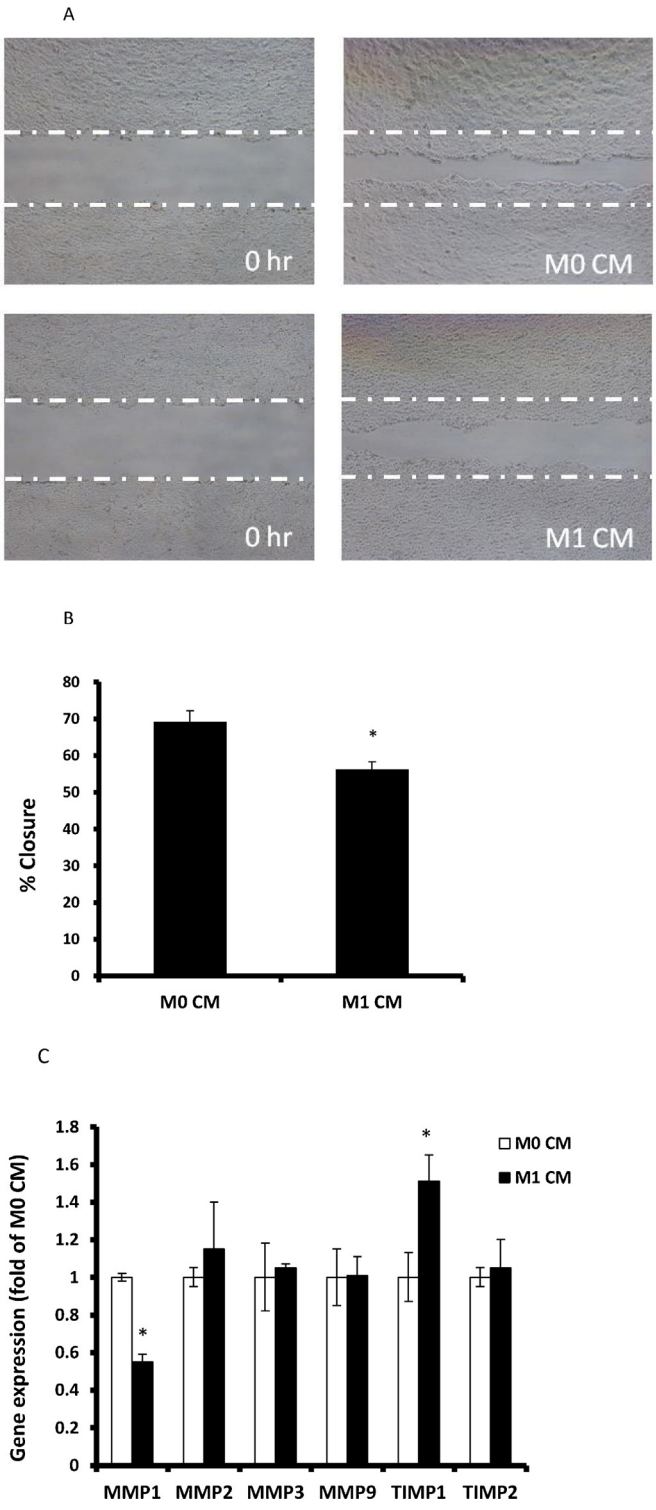


Fig. 2. Conditioned media (CM) from M1 type macrophages inhibited the cell migration of cultured keratinocytes. (A). Migration capacity of cultured human keratinocytes after treatment with either M0 conditioned media (CM) or M1 CM as evaluated by *in vitro scratch wound assay*. Left panel indicated starting point (0 h) of assay; right panel represented keratinocyte migration 24 h after indicated treatments. The representative photograph of three independent experiments is shown in (A). (B). The percentage of wound closure in (A) measured with Image J software from three independent experiments. (C). The expressions of MMPs and TIMPs mRNA in keratinocytes after treatment with either M0 CM or M1 CM measured with real-time quantitative PCR. *Indicates P < 0.05 as compared to M0 CM group.

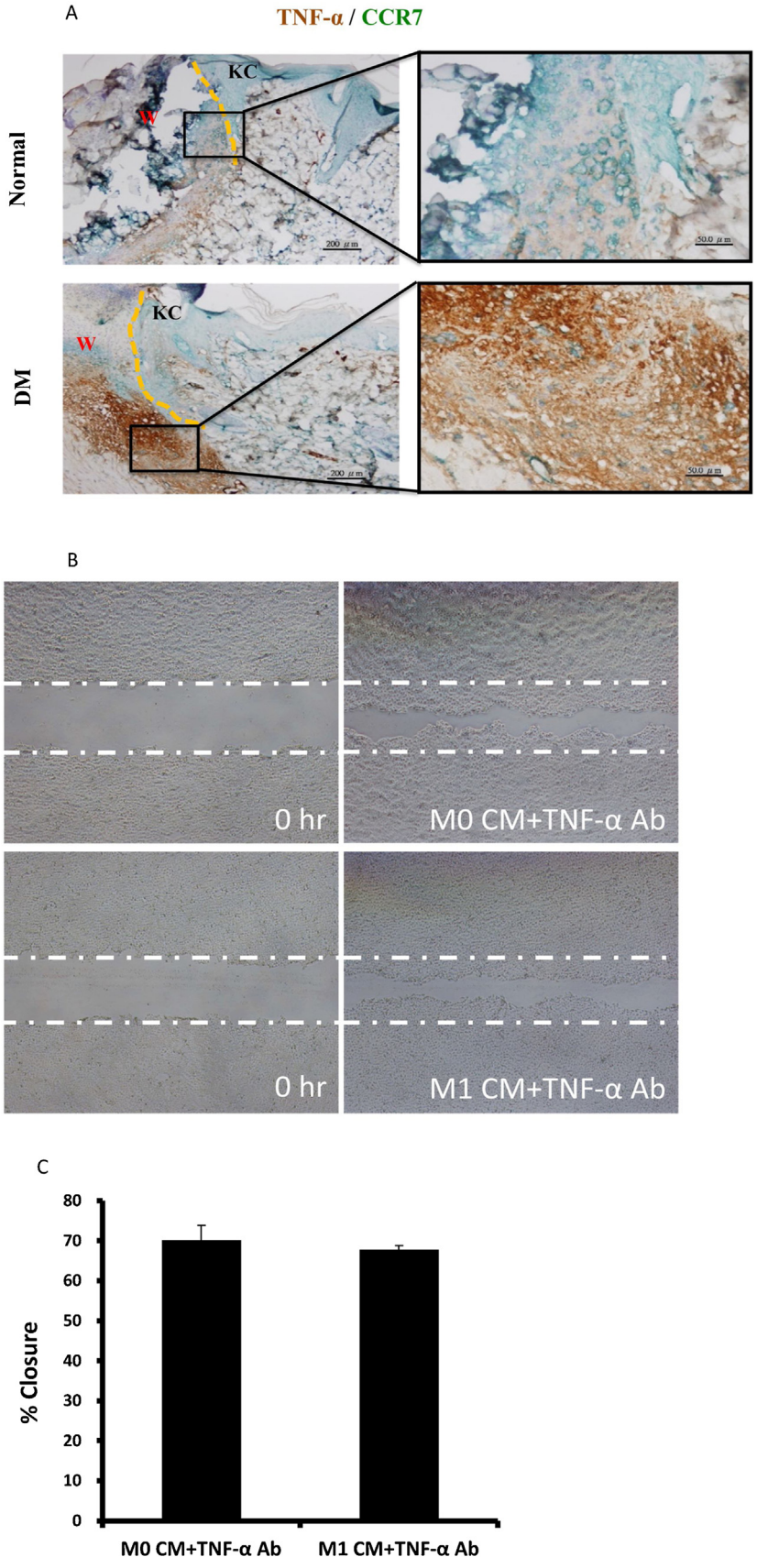


Fig. 3. M1 derived CM impaired keratinocyte migration *via* **elevated TNF-α and enhanced TIMP1 expression in keratinocytes.** (A). Immunohistochemical staining of TNF-α (brown) from the perilesional skin of the normal and the diabetic rats 3 days after wounding. W: wound area; KC: epidermal keratinocyte. (B). Migration capacity of cultured human keratinocytes after treatment with either M0 CM or M1 CM with TNF-α neutralizing antibody pretreatment evaluated by *in vitro scratch wound assay*. (C). The percentage of wound closure in (B) calculated with Image J software from three independent experiments. (D). The expressions of TIMP1 and MMP1 mRNA in cultured human

and increased TIMP1 expression in cultured keratinocytes as compared their M0 CM treated counterpart. These results suggest that CM from M1 macrophages contained certain molecule(s) that may impair keratinocyte migration. It has been recognized that tumor necrosis factor-α (TNF-α) is the most important factor secreted by M1 macrophages and that TNF-α expression is abundant in diabetic wound environment. In this study, using immunohistochemical staining analyses, higher TNF-α expression was noted in diabetic wound as compared to control (Fig. 3A). Therefore, we subsequently evaluated the cytokine profiles of CM from cultured macrophages. As demonstrated in Additional file 1 (Figure S1), the TNF-α level was significantly elevated in CM derived from M1 macrophages as compared to their M0 counterpart. Since elevation of TNF-α level was predominate in CM derived from M1 macrophage, the mechanistic link between elevated TNF-α in M1 CM and impaired keratinocyte migration were explored.

3.3. *TNF-α from M1 type macrophage reduced migratory capacity of cultured keratinocytes* via *induction TIMP1 expression*

To evaluate the functional role of elevated TNF-α in CM from cultured M1 macrophages, a TNF-α neutralizing antibody was introduced. As demonstrated in Fig. 3B and 3C, this treatment restored the migratory capacity of cultured keratinocytes, indicating that elevated TNF-α level in the M1 CM contributed to impaired migration of keratinocytes. More specifically, after neutralizing TNF-α in the environment, the wound closure of cultured keratinocytes after treatment with M0 and M1 CM were $70.12 \pm 3.62\%$ and $67.75 \pm 1.02\%$, respectively, and no significant difference was found between these groups. Subsequently, exogenous TNF-α was added to cultured keratinocytes, and real time PCR analyses revealed upregulation of both MMP1 and TIMP1 expressions (Fig. 3D). Since MMP1 was downregulated while TIMP1 was upregulated in keratinocytes after treatment with CM from M1 macrophage, we subsequently focused on the significance of altered TIMP1 expression on keratinocyte migration. Gene silencing of TIMP1 enhanced the migratory capacity of keratinocytes treated with CM from cultured M1 macrophage. More specifically, the wound closure increased from $39.35 \pm 4.99\%$ to $57.65 \pm 5.77\%$ (Fig. 3E, 3 F). This result indicated that TIMP upregulation induced by TNF-α released from M1 macrophage significantly contributed to impaired migration of keratinocytes. It is noted that MMP1 reduction resulting from M1 CM treatment may also contributed to the impaired migratory capacity of keratinocytes. However, in our experimental condition, neutralization of TNF-α abrogated the hampering effect of M1 CM on keratinocyte migration, and exogenous TNF-α treatment increased MMP1 expression in keratinocytes. Taken together, reduction of MMP1 in keratinocytes induced by M1 CM was induced by factors other than TNF-α.

3.4. *Perilesional TNF-α neutralization improved wound healing in diabetic rat*

Since increased TNF-α release from M1 macrophage impaired keratinocyte migration *in vitro*, we hypothesized that wound healing of diabetic rats may be improved by reduction of TNF-α levels in the perilesional areas. Etanercept, a TNF-α neutralizing peptide, was injected perilesionally before and after skin wounding, and subsequently, the healing process was carefully documented. As demonstrated in Fig. 4A, TNF-α near the wound area was neutralized after etanercept treatment. As demonstrated in Fig. 4B and 4C, the healing process of diabetic wound significantly improved after perilesional injection of TNF-α neutralizing peptide. More specifically, the wound closure improved from $61.0 \pm 8.02\%$ to $85.0 \pm 3.01\%$.

4. Discussion

Previously, it was reported that significant amount of inflammatory cells (including neutrophil, monocytes and macrophages) infiltration were seen at edge of diabetic ulcers [6]. Moreover, we had demonstrated that skin wound of diabetic rats required longer time to heal compared with normal control [31]. Although diabetic wounds were characterized by excessive and prolonged mononuclear cell infiltration, how these cells affect diabetic wound healing re-epithelialization remains unclear. Since monocytes and macrophages have been recognized to play important roles during the wound healing [32], we hypothesized that under high glucose conditions, the mononuclear cells are able to infiltrate the wound area but are dysfunctional in regards to their reparative capacity for stimulating proper re-epithelialization. In this study, we demonstrated that high glucose environment renders monocytes prone to M1 type macrophages polarization using *in vivo* and *in vitro* models. Moreover, skin wounding resulted in significant increased TNF-α expression and M1 type macrophage infiltration to the perilesional skin in diabetic rats as compared to control. Additionally, administration of TNF inhibitor to the perilesional area significantly enhanced the recovery rate of wound compared with saline control in diabetic rats. Our results indicated that excessive TNF-α derived from M1 macrophages in the diabetic condition contributed to poor healing in high glucose environment. Conflicting results have been published regarding the role of TNF-α on wound healing. It has been reported that local application of TNF-α increased wound disruption strength and eventually accelerated wound healing in rats [33]. In contrast, other studies demonstrated that local application of TNF-α impaired wound healing in rats [34,35]. Additionally, Buck et al. showed that inoculating TNF-α producing cells results in impaired skin wound healing [36]. In this study, we found that TNF-α secretion by M1 macrophage inhibited keratinocyte migration *via* upregulation of TIMP1. Different experimental designs and model systems likely contributed to these conflicting results. It is noted that in a study using type 2 diabetic (db/db) mice, etanercept was also shown to enhance healing of skin wound [37]. Therefore, high TNF-α levels likely impair skin healing in high glucose environment. As impaired wound healing in diabetic patients is still an important clinical condition that frequently imposes therapeutic challenges to physicians and may lead to serious complications for patients, modulation of aberrant macrophage polarization near the wound area and reduction of excessive inflammatory factors including TNF-α present novel therapeutic strategy to promote wound healing in diabetic patients.

keratinocytes treated with recombinant human TNF-α. * Indicates $P < 0.05$ as compared to control group. (E). Migration capacity of cultured human keratinocytes treated with either negative control siRNA (upper panel) or TIPM1 siRNA (lower panel) before M1 CM treatment. (F). The percentage of wound closure in (E) calculated with Image J software from three independent experiments. * Indicates $P < 0.05$ as compared to negative control siRNA group. For *in vitro scratch wound assay*, left panel indicated starting point (0 h) of assay; right panel represented keratinocyte migration 24 h after indicated treatments.

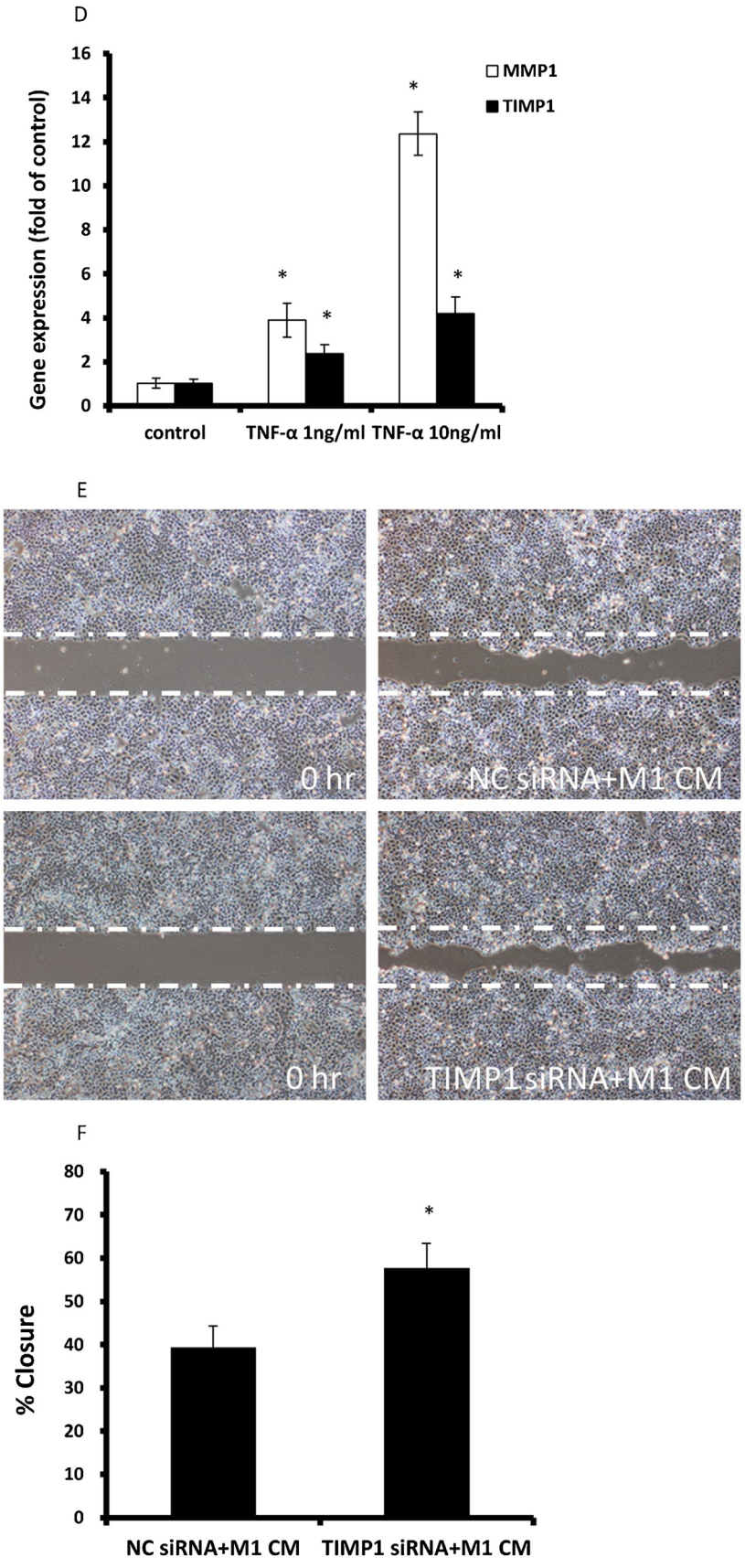


Fig. 3. (Continued)

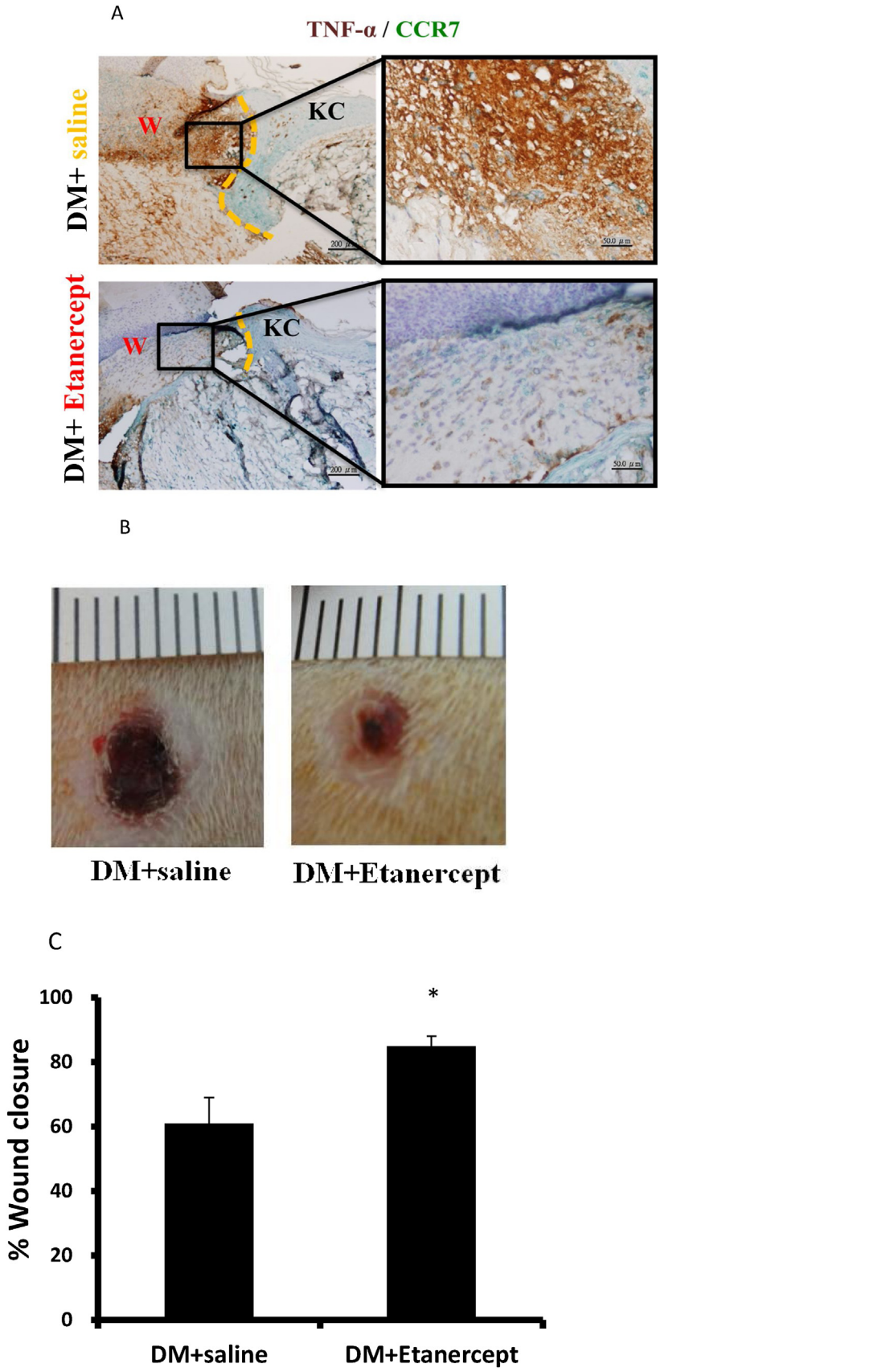


Fig. 4. Etanercept pretreatment reduced TNF-α expression and enhanced wound closure in STZ-induced diabetic rats. (A). Immunohistochemical staining of TNF-α (brown) from the perilesional skin of the diabetic rats treated with saline (upper panel) and diabetic rats treated with early administration of Etanercept (TNF-α inhibitor) (lower panel) 3 days after wounding. W: wound area; KC: epidermal keratinocyte. (B). Seven days after wounding, the comparison of wound healing between diabetic rats treated with early administration of Etanercept (right) and diabetic rats treated with saline (left). (C). The percentage of wound closure in (B) calculated with Image J software from three independent experiments. * Indicates $P < 0.05$ as compared to saline control group.

Authors' contributions

C.C.E. Lan conceived the project, designed the experiments and edited the manuscript. S.M. Huang, M.H. Chiu and C.H. Wu performed and analyzed experiments. S.M. Huang, M.H. Chiu and C.S. Wu wrote the paper. Y.T. Chang provided experimental materials and contributed to data analyses. G.S. Chen supervised the design of experiments and data analyses. All authors read and approved the final manuscript.

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Declaration of Competing Interest

The authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.11.004.

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