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Matrix metalloproteinase 9 induces keratinocyte apoptosis through FasL/Fas pathway in diabetic wound

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Abstract

Apoptosis is a mechanism to remove unwanted cells in the tissue. In diabetic wound, which is characterized by delayed healing process, excessive apoptosis is documented and plays a crucial role. Matrix metalloproteinase 9 (MMP9), which is elevated in non-healed diabetic wound, is necessary for healing process but its abnormality resulted in a delayed healing. The classical function of MMP9 is the degradation of extracellular matrix (ECM). However, there is some literature evidence that MMP9 triggers cell apoptosis. Whether the excessive MMP9 contributes to epidermis cell apoptosis in delayed healing diabetic wound and the underlying mechanisms is not clear. In this study, we aimed to explore whether MMP9 induced keratinocyte apoptosis and investigate the plausible mechanisms. Our in vitro study showed that advanced glycation end products (AGEs) induced keratinocyte apoptosis and enhanced MMP9 level. Besides, MMP9, both intra-cellular expressions and extra-cellular supplement, promoted cell apoptosis. Further, MMP9 resulted in an increased expression of FasL, other than Fas and p53. These findings identified a novel effect that MMP9 exerted in delayed diabetic wound healing, owing to a pro-apoptotic effect on keratinocyte, which was mediated by an increase of FasL expression. This study increases understanding of elevated MMP9 which is involved in diabetic wound repair and offers some insights into novel future therapies.

Keywords Advanced glycation end products · Matrix metalloproteinase 9 · Apoptosis · Signaling pathway

Introduction

Impaired wound healing is one of the most common and serious complications of diabetes. Diabetic patients are more susceptible to chronic wounds, such as diabetic leg and foot ulcers. The overall lifetime risk is reported to be 12–25% [1]. Chronic wound in diabetic patients is a major cause of non-traumatic lower limb amputations (roughly 65,700 amputations per year in the U.S.) and results in a high financial burden [2, 3].

Wound repair process in healthy individuals depends on several interrelated processes, including the migration of inflammatory cells into the wound area to colonize the provisional matrix, proliferation of fibroblasts and vascular cells, apoptosis, and synthesis of extracellular matrix proteins to reconstitute dermal architecture [4]. In this procedure, cells decreasing and tissue repair is regulated and dynamically balanced. There is convincing evidence showing that apoptosis is the main mechanism of cell elimination [5]. Aberrant apoptosis can influence the healing process, outcomes and prognosis of a particular wound, such as impaired healing (excess apoptosis) or keloid formation (less apoptosis) [6, 7]. Diabetic

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mellitus is related to increased apoptosis in wound [8–11]. Hyperglycemia deregulates the sequential apoptotic events through multiple mechanisms, leading to delayed wound healing [4]. Keratinocytes play a crucial role in healing. Upon injury, keratinocytes not only proliferate and migrate to cover the wound but also express a wide range of molecules, which greatly regulating fibroblasts function and promoting granulation tissue formation, contribute to a successful healed wound [12–14]. Loss or decreased function of keratinocytes resulted in impaired wound healing has been documented [15, 16].

Matrix metalloproteinases (MMPs), are zinc endopeptidases capable of degrading all components of ECM. MMPs consists of 23 distinct proteases in humans and can be grouped into collagenases, gelatinases, stromelysins, membrane type metalloproteinases, matrilysins, and others. MMPs are involved in all wound healing events and chronic wounds contain a significantly higher level of proteases, especially collagenase (MMP1 and 8) and gelatinase (MMP2 and 9) [17]. Among these, MMP9 plays the most important role in wound healing. In diabetic foot ulcer (DFU) biopsies, MMP9 was significantly increased but MMP1 level remained constant when compared with the healthy skin biopsies [18]. For MMP2, there was no significant difference between good healers and poor healers, which might indicate that the expression of MMP2 in skin is more constitutive and is regulated independently and to a lesser extent than MMP9 [19]. Although both MMP8 and MMP9 levels increased and remained constant throughout the follow-up period in poor healed diabetes patients, level of MMP9 was much higher than MMP8 [20]. It is a kind of gelatinases with a primary function of degrading type IV collagen, gelatin, proteoglycans, elastin, etc. MMP9 activation is found in DFUs and regarded as a predictor of poor wound healing [21]. Importantly and interestingly, it has been established that MMP9 has many unexpected substrates besides components of ECM, which profoundly influence cell behavior, survival, and death. Among these, particularly, associations between MMP9 and cell apoptosis has been of great interest and MMP9 may serve as a pro-apoptotic factor [22–25].

Considering that chronic wounds in diabetes is characterized by increased apoptosis as well as a high level of MMP9, the purpose of this study was to investigate the hypothesis that MMP9 may have an pro-apoptotic effect on keratinocyte and to explore the underlying mechanism improving the scant knowledge of MMP9's role in diabetic wound healing.

Materials and methods

Skin biopsies and immunohistochemical analysis

Diabetic patients with chronic foot wounds (15 patients) and those with traumatic foot wounds but normal glucose tolerance (15 patients) were enrolled. A written informed consent was obtained, which was approved by the Human Studies Committee of the Sun Yat-sen Memorial Hospital of Sun Yat-sen University. A 10 mm biopsy was taken from the center of each DFU or traumatic wound (after the infection has been controlled), cut into strips, which was immediately placed into a 10% formaldehyde solution. The tissues were embedded in paraffin blocks within 24 h. The sections were evaluated by immunohistochemistry using the streptavidin–biotin complex (SABC) technique. Briefly, the sections were submerged in xylene, ethanol (absolute, 95%, 90%, 80%, and 70%, diluted in double-distilled water), and 0.1 M citrate buffer, pH 6.0, for microwave irradiation. Endogenous peroxidase was blocked by incubation with 3% H_2O_2. After antigen retrieval and the blocking of non-specific protein binding, the sections were incubated with specific antibodies (polyclonal rabbit cleaved caspase-3, diluted 1:150, CST, Beverly, MA, USA; and monoclonal rabbit MMP9, diluted 1:200, Epitomics, Burlingame, CA, USA) at 4 °C overnight, followed by incubation with a biotin-conjugated secondary antibody (goat anti-rabbit IgG, diluted 1:200, Boster, Wuhan, China). The SABC technique was used to visualize the staining. Diaminobenzidine was used as a chromogenic substrate. Staining was evaluated by two independent investigators. The percent of positive-staining cells was graded as follows: 0, none; 1, ≤ 25%; 2, ≤ 50%; 3, ≤ 75%; and 4, ≤ 100%. Intensity was classified as follows: 0, none; 1, weak; 2, moderate; and 3, intense. The Immune-Reactive-Score (IRS) was calculated as follows: IRS = percent of positive cells × staining intensity.

Cell culture

Normal *human* keratinocytes were obtained from the foreskins of children aged 4–10 years through routine circumcision as previously described [26]. Keratinocytes at passages 3–5 were used in this study. Cells were growth arrested at 90% confluence following incubation for 24 h in supplement-free K-SFM, and then subsequently subjected to different agents: bovine serum albumin (BSA; Calbiochem, La Jolla, CA, USA); AGE modified BSA (AGE-BSA; Calbiochem, La Jolla, CA, USA); rh-MMP9 (R&D, Minneapolis, MN, USA) (activated by APMA, Genmed, Shanghai, China); GM6001 (Sigma, St. Louis, MO, USA), a broad-spectrum matrix metalloprotease inhibitor.

Virus packaging and transfection

To induce over-expression of the MMP9 gene in keratinocytes, recombinant adenovirus particles containing the MMP9 gene (pDC316-mCMV-EGFP-hMMP9) were constructed by Biowit Technologies (Shenzhen, China). Keratinocytes at the logarithmic phase were inoculated

in 6-well plates and were cultured in K-SFM containing eosinophil chemotactic factor (ECF) and BPE when cell confluence reached 70–90%, the keratinocytes were transfected with Ad-hMMP9-EGFP and control adenovirus particles (Ad-EGFP) as the experimental and negative control groups, respectively. Cells in control group were conventionally cultured but received no treatment. When the cells reached confluence, they were dissociated and collected for use in analyses.

Flow cytometry analysis

Keratinocytes (2×10^5 cells/well) were seeded onto 6-well plates. Then, cells were harvested by trypsin–EDTA digestion and washed with PBS. The procedure was designed according to manufacturer's instructions on the EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA, USA).

TUNEL assay

Keratinocytes (1×10^3/well) were incubated in 96-well microplates. They were then washed with PBS and fixed in a 4% paraformaldehyde solution (100 µl/well) for 1 h at 15–25 ℃.Slides were rinsed with PBS 2 times before incubation (60 min, in dark place, 37 °C) in a permeabilization solution (fresh 0.1% Triton) for 2 min on ice (2–8 °C). Cells were washed twice with PBS and resuspended in a TUNEL reaction mixture (30 µl/ well) (Roche Applied Science, Mannheim, Germany). Then, the microplates were covered with lids and incubated for 60 min at 37 °C in a humidified atmosphere in the dark. Subsequently, the cells were washed three times for 5 min each and stained with Hoechst (50 µl/well) (Invitrogen, Carlsbad, CA, USA) in the dark at room temperature. Next, the cells were washed three times with PBS for 15 min in total before fluorescence microscopic analysis (Eclipse Ti Inverted Microscope, Nikon, Japan) at an excitation wavelength in the range of 450–500 nm and detection in the range of 520–570 nm. Apoptotic cells were characterized by red color in the nucleus. A total of 100 cells in eight random fields were counted, and the apoptotic index (AI), defined as the number of positive apoptotic cells/100 × 100%, was calculated.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and total RNA was used as a template for cDNA synthesis. Real-time RT-PCR was performed using a Light Cycler 480 SYBR Green Master (Roche Diagnostics, Mannheim, Germany) with a Light Cycler System. The following primers were used to amplify the coding regions:

5′-CGCCCATTTCGACGATGAC-3′ (forward) and 5′-CGCCATCTGCGTTTCCAA-3′ (reverse) for MMP9; and 5′-CAATGACCCCTTCATTGACC-3′ (forward) and 5′-TTGATTTTGGAGGGATCTCG-3′ (reverse) for GAPDH. Each value represents the average of at least three independent experiments.

Gelatin zymography

Proteolytic activity was assessed in conditioned cell medium using a gelatin substrate gel for gelatinases as described previously [27]. The densitometry levels of zymograms were measured with digital image analysis (NIH Image software).

Western blot analysis

Cells were washed twice with PBS and collected in radioimmunoprecipitation assay lysis buffer containing PMSF and protease and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany) to yield whole-cell extracts. Cell lysates were resolved by SDS–PAGE and transferred to nitrocellulose membranes according to standard procedures. The membranes were blocked with 5% skim milk and probed with primary antibodies (*human*) for cleaved caspase-3 (diluted 1:500; CST, Beverly, MA, USA), MMP9 (diluted 1:1000; Epitomics, Burlingame, CA, USA), FasL (diluted 1:1000; CST, Beverly, MA, USA), Fas (diluted 1:1000; CST, Beverly, MA, USA), and p53(diluted 1:1000; CST, Beverly, MA, USA) at 4 °C overnight. Then, the membranes were incubated with the appropriate secondary antibody, and immunoreactivity was detected using an enhanced chemiluminescence reagent. The relative intensities of the protein bands were analyzed by Alpha Image software (Alpha Innotech, Santa Clara, CA, USA).

Statistical analysis

Statistical analysis was carried out using either Student's t-test or one-way ANOVA for comparisons of more than two groups. Bonferroni's multiple comparisons test was used to conduct post hoc analysis. Results are represented as the mean ± SEM. All in vitro experiments were repeated at least three times. A value of $p < 0.05$ was considered significant.

Results

Keratinocyte apoptosis and MMP9 expression were increased in diabetic wounds

We evaluated keratinocytes apoptosis and MMP9 expression in biopsies of wounds in diabetic and non-diabetic patients by immunohistochemistry method. Cleaved caspase-3,

the executive in most apoptotic pathway, was measured to reflect the apoptosis of keratinocytes. As shown in Fig. 1, the accumulated data from immunohistochemistry revealed that cleaved caspase-3 was elevated in keratinocytes from diabetic specimens compared with those from non-diabetic specimens. IRS value were 4.93 ± 1.04 and 1.3 ± 0.86 in diabetic and non-diabetic group, respectively (Fig. 1a). MMP9 expression was also detected in these specimens and the profiles showed a similar tendency, with IRS value of 5.43 ± 1.59 and 1.16 ± 0.82, respectively for diabetic and non-diabetic groups (Fig. 1b). These results indicated the coexistence of increased keratinocyte apoptosis and MMP9 expression in diabetic wounds.

Keratinocyte apoptosis and MMP9 expression enhanced with AGE-BSA treatment

AGEs occur through non-enzymatic glycation of long-lived proteins in diabetes and play an important role in diabetic complications. AGE-BSA is a good substitute to simulate the pathophysiological role of AGEs [28]. In this study, keratinocytes were treated with BSA or AGE-BSA for 72 h. Cell apoptosis was investigated by flow cytometry analysis, TUNEL assay and western blotting. AGE-BSA treatment resulted in a dose-dependent apoptosis of keratinocyte compared to the control group (Fig. 2a–c). Meanwhile, AGE-BSA also up-regulated MMP9 expression. AGE-BSA 100 μg/ml increased MMP9 mRNA level to about fourfold and it was even higher to 6.81 ± 1.11 in AGE-BSA 200 μg/ml group when compared to the control (Fig. 3a). Elevated MMP9 protein expression and MMP9 activity detected by gelatin zymography were also found in AGE-BSA treatment groups (Fig. 3b, c). In addition to the previous results, the in vitro data directly demonstrated the increased keratinocyte apoptosis and elevated MMP9 under diabetic condition.

Enhanced MMP9 plays a pro-apoptotic role on *human* keratinocytes

We next investigated the role of MMP9 on keratinocyte apoptosis. To further clarify these, we adopted both extra-cellular rh-MMP9 and intra-cellular MMP9 using adenovirus expression system. Cells were treated with different concentrations of rh-MMP9 and results indicated that lower concentrations of rh-MMP9 were not sufficient to induce apoptosis but a high concentration of 40 ng/ml significantly promoted apoptosis, with about threefold apoptotic rate as indicated by flow cytometry (Fig. 4a). As expectedly, GM6001, the MMPs inhibitor, significantly reversed the induced-apoptotic property of

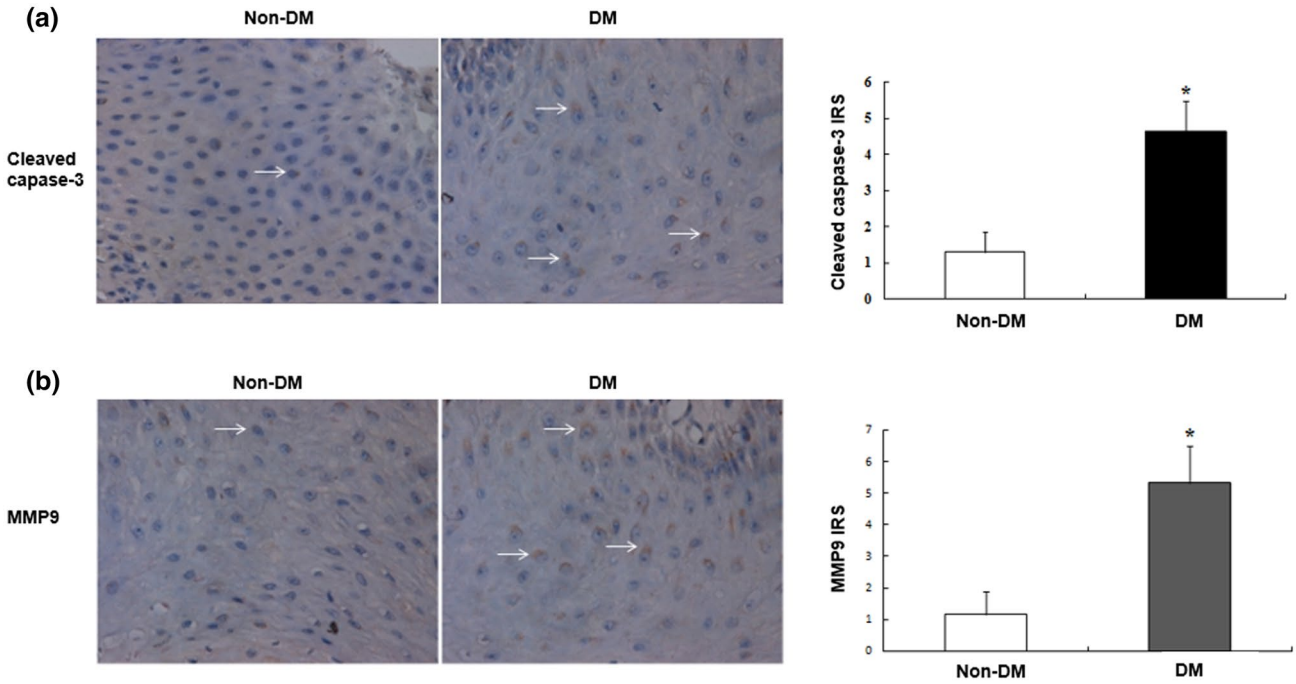

Fig. 1 Comparison of cleaved caspase-3 and MMP9 expression in keratinocytes from diabetic and non-diabetic foot wounds specimens (n = 15 per group) (400×). **a** Immunohistochemistry analysis of cleaved caspase-3 expression in keratinocytes from wound biopsy specimens and calculated IRS value. **b** Immunohistochemistry analysis of MMP9 expression in keratinocytes from wound biopsy specimens and calculated IRS value. *$p < 0.05$. *non-DM* non-diabetes mellitus, *DM* diabetes mellitus. Arrows indicate cleaved caspase-3 or MMP9 positive keratinocytes from wound biopsy specimens

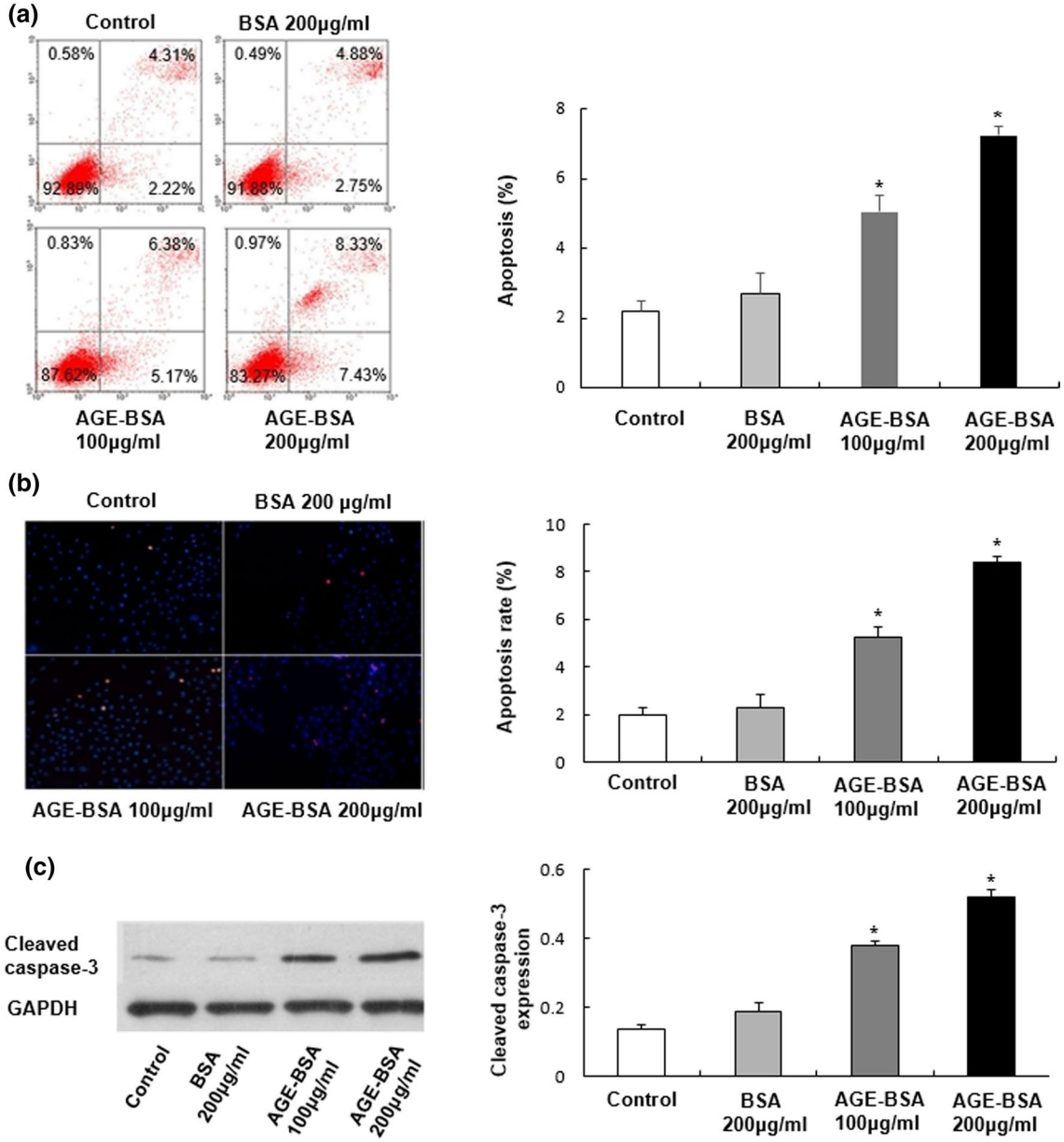

Fig. 2 AGE-BSA increases the apoptosis of *human* keratinocytes. Cultured *human* keratinocytes were treated with BSA 200 μg/ml and AGE-BSA 100 μg/ml, 200 μg/ml for 72 h. **a** Keratinocyte apoptosis was analyzed by flow cytometry and TUNEL assay (**b**, 400×). **c** Cleaved caspase-3 expression was detected by western blotting. (n = 6) $*p < 0.05$

MMP9 (Fig. 4a). Results from cleaved caspase-3 yielded similar results (Fig. 4b). Adenovirus carrying MMP9 coding region was used to induce intra-cellular expression of MMP9 and apoptosis was assessed. Similar to those results of extra-cellular MMP9, intra-cellular MMP9 induced apoptosis, as indicated both by flow cytometry and cleaved caspase-3 expression (Fig. 5), which was counteracted by GM6001.

Mechanisms of pro-apoptotic effect of MMP9 on keratinocytes

Further, we explored the underlying pro-apoptotic mechanism of MMP9 by analyze the apoptotic pathway. As shown in the figure (Fig. 6a), rh-MMP9 40 ng/ml treatment resulted in an increase of FasL protein expression compared with the control group. However, Fas or p53 expression remained no change. Meanwhile, the increase

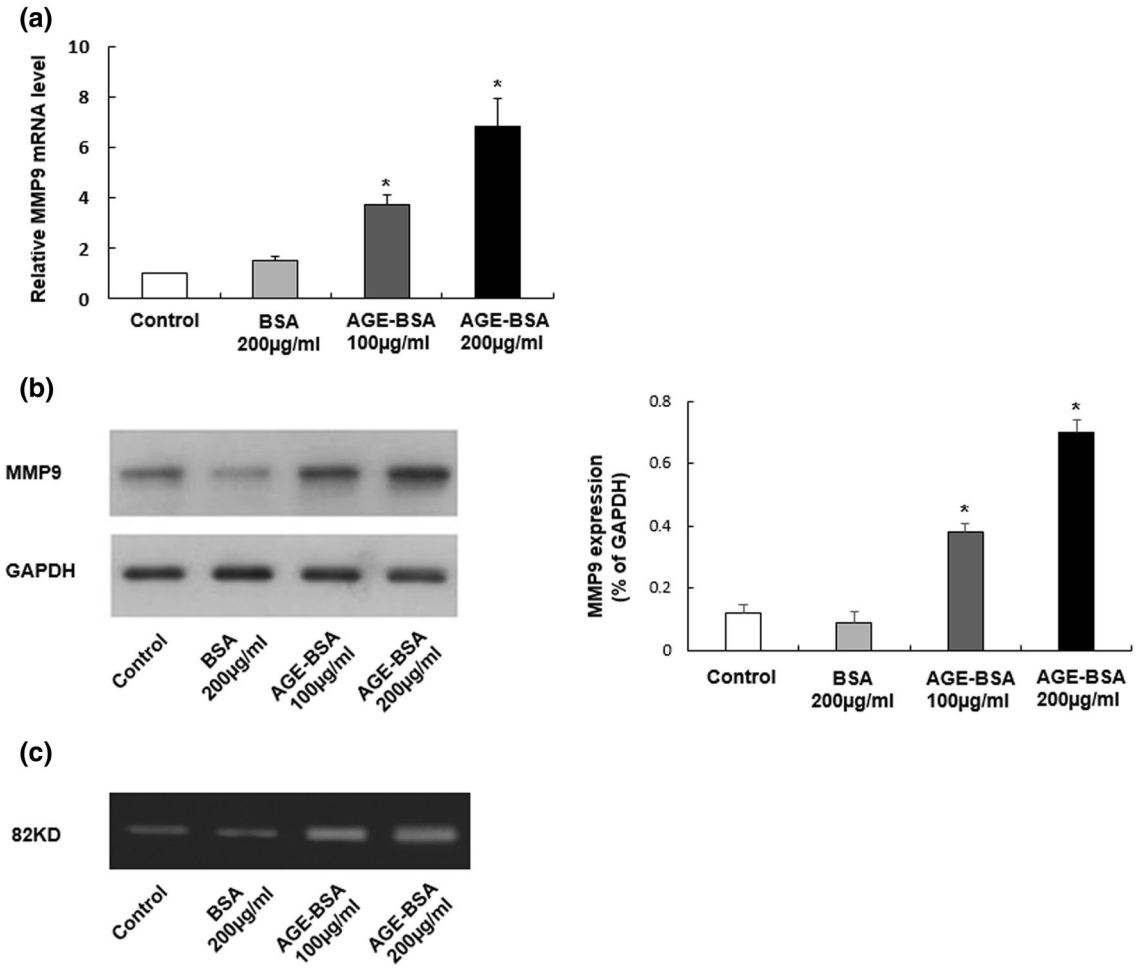

Fig. 3 AGE-BSA treatment enhances MMP9 expression and activity. Keratinocytes were serum starved and were then treated for 72 h with treated with BSA 200 μg/ml and AGE-BSA 100 μg/ml, 200 μg/ml. **a** MMP9 mRNA expression was analyzed by RT-PCR, normalized by GAPDH. **b** MMP9 protein expression was analyzed by western blotting. **c** The cell supernatants were assessed by gelatin zymography to display the activity of MMP9. (n = 6) $^*p < 0.05$

of FasL in rh-MMP9 group was abolished by GM6001 pre-treatment.

Endogenous expression of MMP9 using adenovirus resulted in similar findings (Fig. 6b). Taken together, these data implies that FasL/Fas signaling pathway, not p53 pathway, is involved in the pro-apoptotic effect of MMP9.

Discussion

The role of MMP9 and cell apoptosis in diabetic wound repair has been well established while the involvement of MMP9 in diabetic skin cell apoptosis is remain unclear. The present study clearly demonstrated that excessive MMP9 induced apoptosis in keratinocyte, which was mediated via up-regulation of FasL expression, rather than Fas or p53.

MMPs are necessary for wound repair but can negatively affect healing process if not present in the correct quantity [29]. A large number of both preclinical as well as clinical studies have demonstrated that MMP9 increased in poor wound healing of diabetes. In delayed healing wounds of diabetic mice, MMP9 level was 3–4 times increased when compared to non-diabetic mice during the whole wound healing period [30]. In diabetic patients with non-healed ulcers, serum MMP9 level was about 321 ng/ml while it was 203 ng/ml in diabetic patients with healed ulcers or with no ulcers [31]. The average concentration of MMP9 detected in skin biopsies was increased 14-fold in diabetic ulcers (35.2 ± 51 ng/ml for diabetic patients vs. 2.5 ± 2 ng/ml for non-diabetic patients) [32]. Wounds directly treated with MMP9 resulted in delayed closure in mice [33]. Numerous groups have correlated overexpression of MMP9 within chronic ulcers to poor wound healing outcomes in diabetic patients. It is believed that increased level of MMP9 could be a contributing factor to poor wound healing in DFUs [21]. In consistent with the previous studies, our study found that

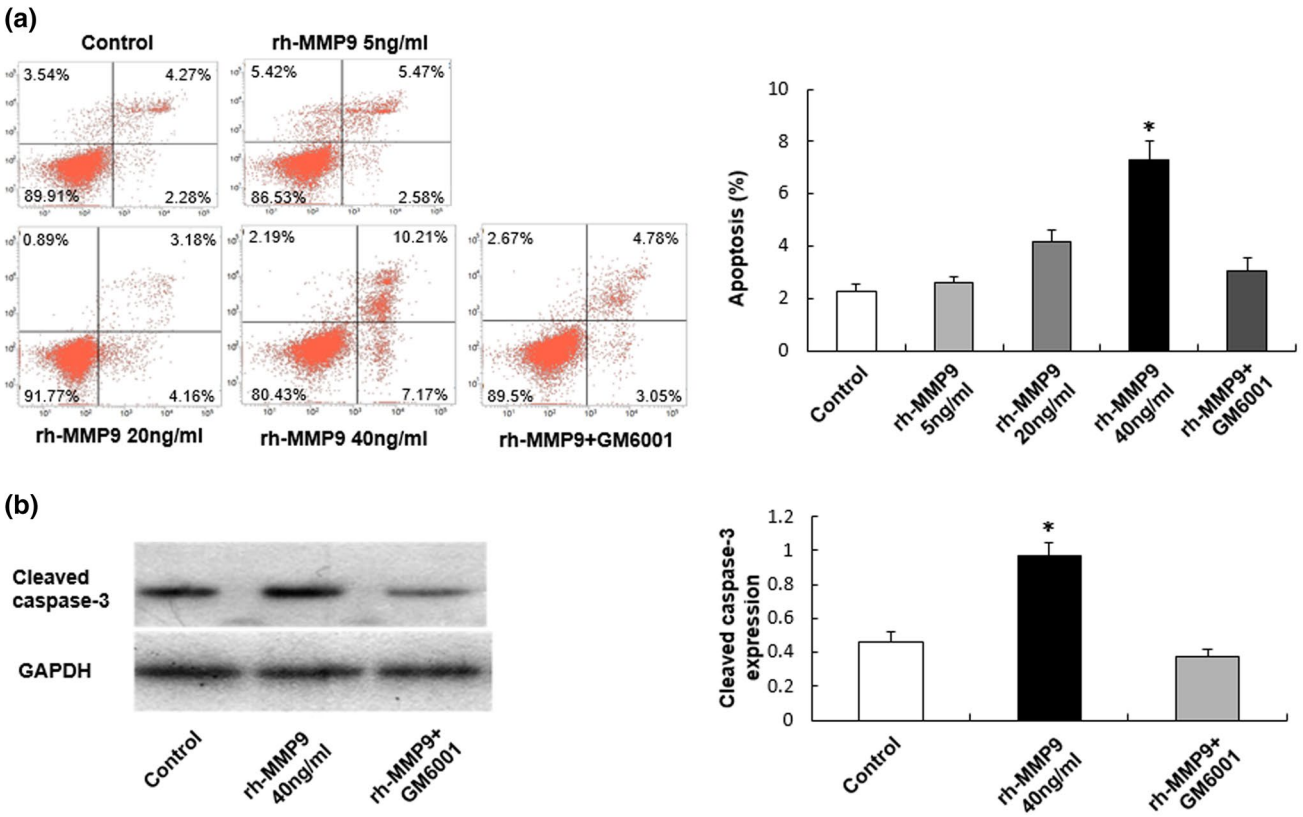

Fig. 4 Changes in keratinocytes apoptosis induced by rh-MMP9. Keratinocytes were serums starved and then cultured for 24 h with rh-MMP9 at a concentration of 5 ng/ml, 20 ng/ml, and 40 ng /ml. In addition, GM6001 was 1 h pre-treated in rh-MMP9 + GM6001 group. **a** Apoptosis rate was assessed by flow cytometry analysis in each group. **b** Cleaved caspase-3 protein expression was detected by western blotting. (n = 6) $*p < 0.05$

MMP9 increased in diabetic wound biopsy specimens and it could be triggered by AGEs.

Previous studies usually believed that the deleterious effect of MMP9 on delayed wound healing process owed to its classical function of ECM degradation [19–21]. However, recent studies suggested that MMP9 may exert more functions. Importantly, MMP9 may affect apoptosis, both pro-apoptotic and anti-apoptotic properties, depending on different cell types and pathological process [34]. Hoehna et al. exhibited that MMP9 contributed to cell death after pilocarpine-induced seizures in the developing brain [22]. In rats, MMP9 was activated by neuronal nitric oxide synthase and triggered an extracellular signaling cascade leading to retinal ganglion cell apoptosis [23]. Studies exerted MMP9 siRNA to transfect endothelial cells resulted in decreased cell apoptosis and ameliorated accelerated apoptosis in retinal endothelial cells [25, 35]. $tPA^{-/-}/MMP9^{-/-}$ double-knockout mice presented a protective effect on cell apoptosis and left ventricular remodeling [36]. The findings of our study indicated that in keratinocyte apoptosis increased, as well as MMP9 level. Therefore, although essential in every phase of the healing process, MMP9 for a successful healing process is dependent on a rigorous spatial and temporal pattern of expression. Excessive MMP9, as exhibited in our study, may induce keratinocyte apoptosis and insult wound healing in diabetes.

Despite the important role of MMP9 on apoptosis has been demonstrated, the underline mechanisms are largely unknown. A study in retinal endothelial cells showed that a high glucose level activated MMP9 resulting in apoptosis through caspase-3 activation [35]. However, the definite mechanism involving MMP9 and caspase-3 has not yet been elucidated. The established apoptosis signaling pathways at present comprises the mitochondrial pathway (intrinsic pathway), death receptor FasL/Fas pathway (extrinsic pathway) and p53 pathway [4]. MMP9 is secreted from cells as proMMP9 and activated extracellularly [37]. In the wound healing process, various expression patterns of apoptosis key regulators have been studied showing that the healing in mucosa takes place predominantly through the intrinsic pathway whereas skin healing is predominantly through the extrinsic pathway [38]. Additionally, p53 is involved in transferring the messages for proliferation and apoptosis [8]. The quantitative

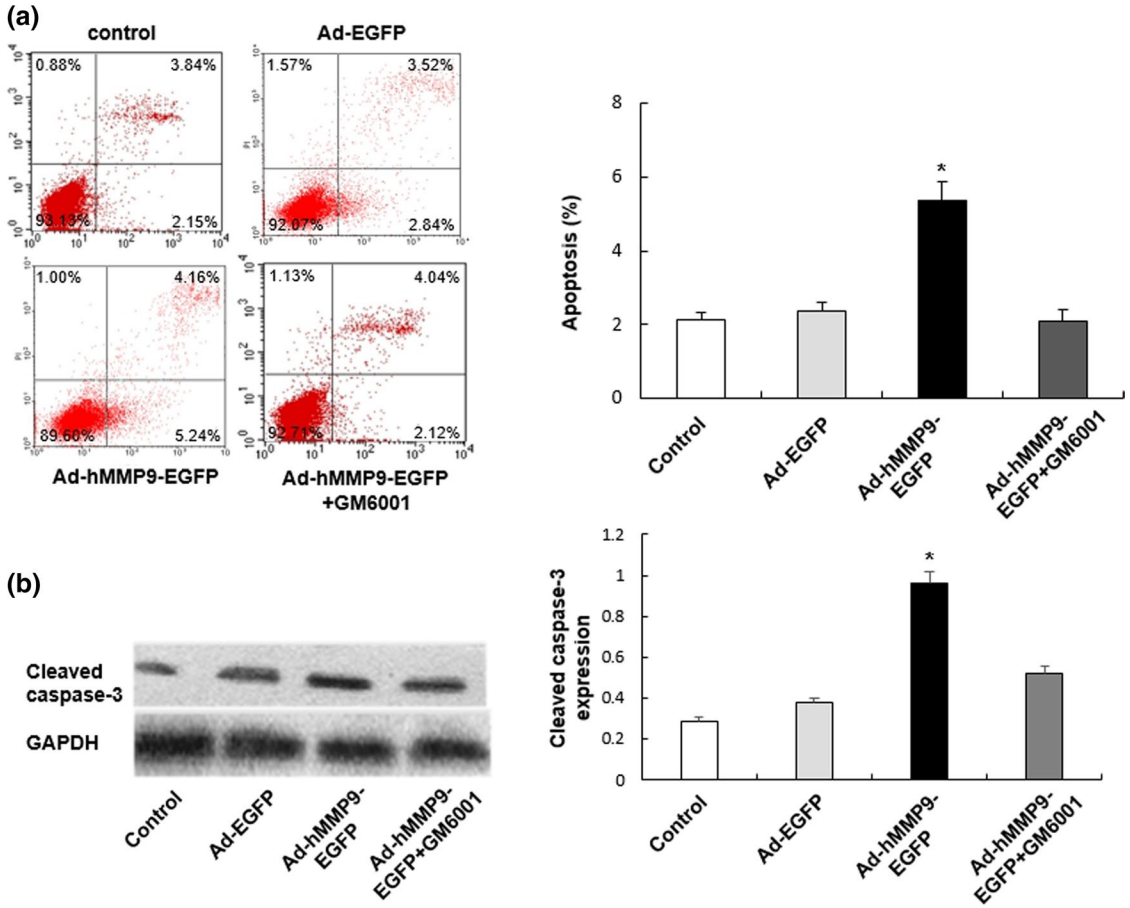

Fig. 5 Changes in keratinocytes apoptosis induced by Ad-hMMP9-EGFP MMP9. Transfection cells with Ad-hMMP9-EGFP were divided into control, Ad-EGFP, Ad-hMMP9-EGFP and with GM6001 pre-treated group (GM6001 was administered 1 h before Ad-hMMP9-EGFP treatment) and then incubated for 48 h. **a** Apoptosis rate was assessed by flow cytometry analysis in each group. **b** Cleaved caspase-3 protein expression was detected by western blotting. (n = 6) $^*p < 0.05$

proteomic assessment of selected signaling proteins of DFU revealed that p53 and caspase-3 significantly increased in non-healing DFU keratinocytes compared to normal tissue [39]. Therefore, we explored the FasL/Fas and p53 pathway. Results demonstrated that excessive MMP9 regulated keratinocyte apoptosis mainly through up-regulation of the death receptor, FasL, other than Fas protein or regulation of p53, and subsequently enhanced the extrinsic pathway of apoptosis. In vivo and in-vitro studies have demonstrated that keratinocytes can express FasL [40–44]. The FasL/Fas system is essential for maintaining the functional integrity of the epidermis. Although keratinocytes express Fas and FasL, keratinocyte apoptosis is a rare event in the epidermis under normal conditions. This is due to the cellular localization of Fas and FasL: Fas protein is expressed in keratinocytes in all subcorneal layers of the epidermis, whereas FasL is only expressed in keratinocytes in the basal and first suprabasal layers [45]. FasL level can increase and express on cell surface to induce apoptosis when necessary. Increased p53 protein was reported in diabetic skin tissue [8], while some studies showed that keratinocytes were p53 negative and caspase-3 positive in DFUs [46].

In summary, the present study validated the increase of keratinocyte apoptosis and MMP9 expression occurred in diabetic impaired wound healing process and investigated the pro-apoptotic effect of MMP9 through activated FasL/Fas pathway. In addition to the traditional role of MMP9 on ECM, a new mechanism by which MMP9 may act as a cytokine-like factor was identified. Therefore, increased understanding of MMP9 involved in wound healing will lead to development of new and more promising therapies. However, the mechanism underlying the regulation of FasL expression by MMP9 is unknown and requires further investigation.

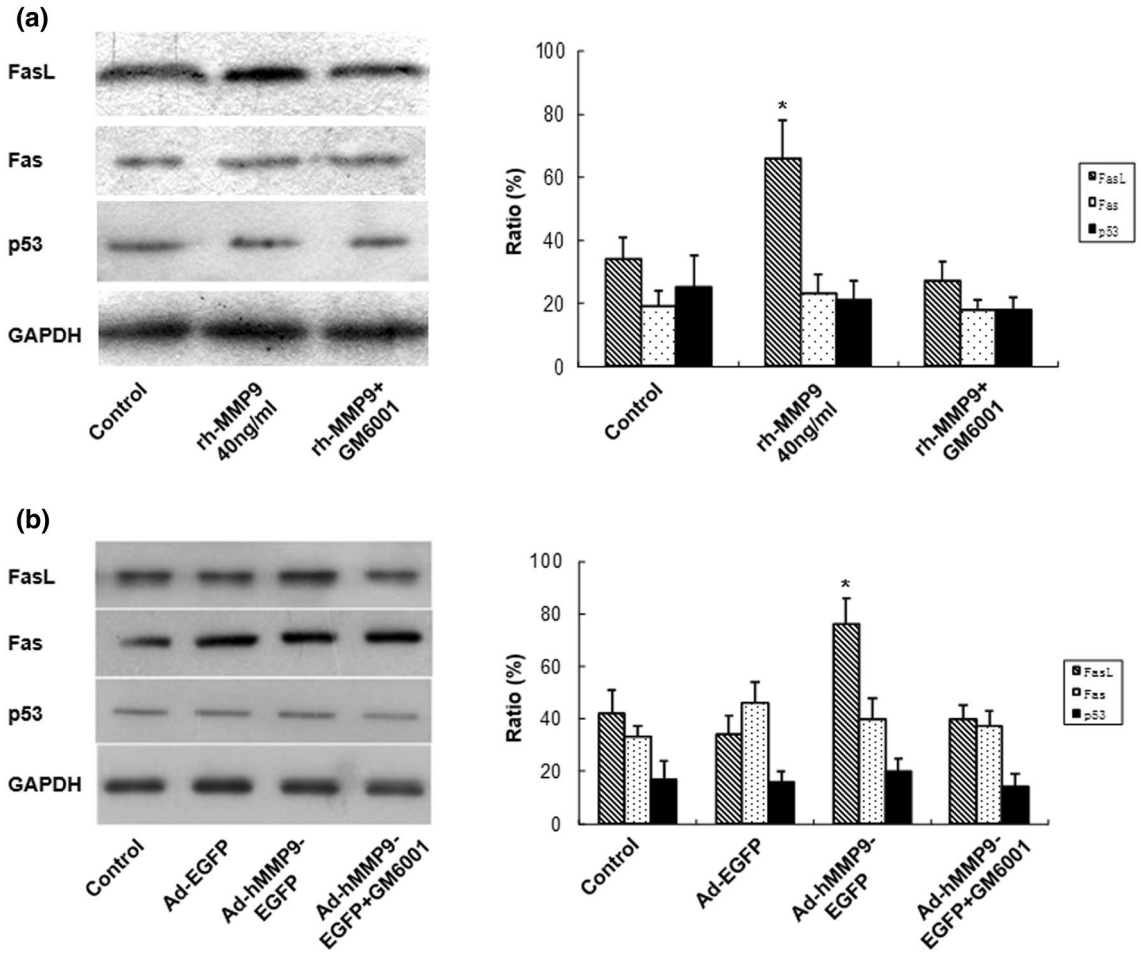

Fig. 6 The underlying mechanism of MMP9 pro-apoptotic effect. **a** Keratinocytes were treated with 40 ng/ml rh-MMP9 for 24 h with or without GM6001 1 h pre-treatment. FasL, Fas, and p53 protein expression was analyzed by western blotting. **b** Transfection cells with Ad-hMMP9-EGFP were cultured with or without GM6001 1 h pre-treatment. After 48 h, FasL, Fas, and p53 protein expression was detected by western blotting. (n = 6) $*p < 0.05$

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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