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Umbilical cord–derived mesenchymal stromal cell–conditioned medium exerts *in vitro* antiaging effects in human fibroblasts

MEIRONG LI[1,2,*], YALI ZHAO[1,2,*], HAOJIE HAO[1], LIANG DONG[1], JIEJIE LIU[1], WEIDONG HAN[1] & XIAOBING FU[1]

[1]*Wound Healing and Cell Biology Laboratory, Institute of Basic Medical Science, Chinese PLA General Hospital, Beijing, China, and* [2]*Trauma Treatment Center, Central Laboratory, Hainan Branch, Chinese PLA General Hospital, Sanya, China*

Abstract

Background aims. Chronic wounds are a common complication of diabetes. Fibroblast-myofibroblast differentiation is important for wound repair, which is commonly impaired in non-healing wounds, and the underlying mechanisms need to be further elucidated. *Methods.* We used high glucose (HG) to simulated the diabetes microenvironment and explored its effects on the biological features of fibroblasts *in vitro*. *Results.* The results showed that prolonged HG induced senescence in fibroblasts through activation of p21 and p16 in a reactive oxygen species (ROS)-dependent manner, further delayed the viability and migration in fibroblasts and also depressed fibroblast differentiation through the TGF-β/Smad signaling pathway. However, mesenchymal stromal cell–conditioned medium (MSC-CM) counteracts the effects of HG. Treatment of fibroblasts with MSC-CM decreased HG-induced ROS overproduction, ameliorated HG-induced senescence in fibroblasts and reversed the defects in myofibroblast formation. Our results may provide clues for the pathogenesis of chronic wounds and a theoretical basis to develop MSC-CM as an alternative therapeutic method to treatment of chronic wounds.

Key Words: *chronic wounds, high glucose, myofibroblast, mesenchymal stromal cell-conditioned medium, senescence, oxidative stress*

Introduction

With the expansion of the diabetes mellitus (DM) epidemic, the incidence of chronic wounds is dramatically increasing. Chronic wounds are important complications of diabetes that can lead to amputation and even death [1].

Fibroblasts, cells that are involved in wound healing, participate in granulation tissue formation and provides a scaffold for repopulating cells through the secreting of extracellular matrix (ECM) [2]. More importantly, fibroblasts differentiate into myofibroblasts, enhancing wound contraction, and then promote wound closure [2]. Transforming growth factor beta (TGF-β1), a pleiotropic factor, participates in the whole process of skin repair, and it is also considered to be the major growth factor inducing fibroblast differentiation [3]. The TGF-β signaling pathway activates and inhibits target genes, mainly through canonical Smad-dependent signaling. Deregulation of the canonical Smad-dependent signaling pathway of TGF-β impairs myofibroblast formation. Reduced myofibroblast development has been discovered in chronic wounds in diabetic patients [4], and the mechanisms underlying this developmental deficiency are not fully understood.

Impairment in regulating glucose metabolism and elevated glucose levels are the main etiology of DM [5]. Substantial evidence suggests that high-glucose-induced oxidative stress formation is closely related with senescence in a variety of cells, such as keratinocytes, endothelial cells and fibroblasts [6–8]. For example, hyperglycaemia-induced generation of reactive oxygen species (ROS) accelerates the shortening of telomere length of endothelial cells [9–11]. The replicative life span of skin fibroblasts derived from diabetic subjects is reduced compared with controls [12]. Therefore, we speculated that oxidative stress and senescence may be pathogenically linked with the development of chronic wounds.

Effective strategies for treating non-healing wounds are still lacking. Using stem cells, especially mesenchymal stromal cells (MSCs), may be a promising means for chronic wound therapy. A large body of

*These authors contributed equally to this work.

Correspondence: **Weidong Han**, MD, PhD, and **Xiaobing Fu**, MD, PhD, Institute of Basic Medical Science, Chinese PLA General Hospital, 28 Fuxing Road, HaiDian District, Beijing 100853, P. R. China. E-mail: hanwdrsw69@yahoo.com; fuxiaobing@vip.sina.com

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evidence indicates that MSCs can promote wound closure of chronic wounds in animal models and in preclinical studies [13,14]. Umbilical cord–derived MSCs (UC-MSCs), which are required with non-invasive harvesting procedures, meet the criteria for MSCs and exhibit a more prominent cytokine secretion profile than MSCs from other sources [15]. Additionally, increasing evidence suggests that the mechanism underlying UC-MSCs efficacy depends mostly on their paracrine activity [16]. All the bioactive factors and cytokines in MSCs secretions constitute can be collected in the conditioned medium. Accumulating evidence suggests that MSC–conditioned medium has similar therapeutic effects to MSCs. MSC-CM can enhance the repair of myocardial infarction [17], enhance wound healing [16] and reduce fibrotic kidney injury [18,19]. In addition, research reporting the ability of MSC-CM to repair dysfunctional cells in a diabetic environment is just emerging [20,21]. Whether MSC-CM can ameliorate the biological function of fibroblasts exposed to a diabetic microenvironment is not fully understood, and the potential mechanisms need to be further elucidated.

We hypothesized that the pathological stress of high-glucose conditions increased senescence markers in human fibroblasts that were correlated with functional impairment. Thus, in this study, we provide important evidence for the mechanism underlying the pathogenesis of non-healing wounds under the pathological state of diabetes. Additionally, we further demonstrate that MSC-CM exposure recovers the function of fibroblasts through regulation of the canonical Smad-dependent signaling pathway. These results provide a theoretical basis for the clinical application of MSC-CM in diabetic wound healing.

Methods

Fibroblast culture and treatment

Fibroblasts were isolated from human foreskin through routine circumcision. All patients provided written informed consent. Collected foreskin specimens were washed with a phosphate-buffered saline (PBS) supplement with 1% penicillin-streptomycin solution (PS). All samples were then cut into small pieces and incubated in collagenase type I (Sigma) for 1 h at 37°C. The digestion was terminated, and the supernatant was collected for centrifugation. The cell pellets were gently resuspended in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS), and the culture medium was released every 2 days.

Thomas *et al.* [22] have shown that the glucose concentration in subcutaneous tissue is similar to that in plasma (~6 mmol/L). Therefore, fibroblasts were cultured in normal glucose (6 mmol/L) as the control. Many previous studies have adopted 26 mmol/L glucose to simulate a high-glucose condition. In the study, fibroblasts are cultured in a glucose concentration of 26 mmol/L (HG1), and 30 mmol/L (HG2) were used to mimic hyperglycemic conditions as in other studies [6,8].

For investigating the effect of high glucose on foreskin fibroblasts (FFs) differentiation, FFs were incubated with glucose at the final concentration of 6 mmol/L (control), 26 mmol/L (HG1) and 30 mmol/L (HG2) for 1, 3 and 5 days, and then the cells further incubated with recombinant human TGF-β1 (5 ng/mL, 48 h) for inducing differentiation. To explore the regulatory role of MSC-CM in high glucose treated fibroblasts, fibroblasts were pretreated with UC-MSC-CM (at two concentrations, 1% [CM1] or 5% [CM2], respectively) and then incubated with high glucose (HG1 and HG2) for corresponding times. All experiments were performed three times, and results are reported as means ± SE.

Cell proliferation assay

Cell viability was assessed by the CCK8 assay. Fibroblasts were plated in 96-well plates (Costar), and 12 h later, the concentration of glucose in the culture medium was adjusted to 26 mmol/L and 30 mmol/L in the two high-glucose experimental groups. After incubation in high-glucose medium for 1, 3 or 5 days, CCK-8 was added to the media, and the cells were incubated for 4 h. The plates were read on an enzyme immunoassay analyzer at 450 nm. The CCK8 assays were performed in triplicate, and the mean values were used ($n = 5$).

Scratch assay

The motility properties of fibroblasts after exposure to high-glucose treatment for three different durations was evaluated by scratch wound assay. Fibroblasts were inoculate in six-well plates and cultured in DMEM containing 10% FBS for 12 h. The culture medium was then changed to DMEM containing 0.5% FBS, and the concentration of glucose in the culture system was modulated. When they reached 80% confluence, the cells were scraped with a p200 pipette tip. After washing the cells with PBS three times, serum-free DMEM was immediately added. To monitor wound closure, photographs were taken under a phase-contrast microscope (0 and 24 h). The distance of fibroblast migration was evaluated by a migration index. The scratch assays were performed in triplicate, and the mean values were used.