Expression of β-catenin and cyclin D1 in epidermal stem cells of diabetic rats

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Abstract. The healing of diabetic wounds represents a formidable clinical challenge, and the molecular mechanisms invovled in diabetic wound healing are far from clear. In this study, we investigated the expression of β-catenin and cyclin D1 in the epidermal stem cells (ESCs) of diabetic rats, and explored whether the reduction of β-catenin and its downstream target in ESCs, cyclin D1, lead to poor wound healing in diabetes mellitus (DM). We found that, compared to the controls, the ESCs of diabetic rats were markedly reduced, the clone formation efficiency of the ESCs was markedly lower, and the mRNA and protein expression of β-catenin and cyclin D1 was significantly decreased. These findings suggest that the low expression of β-catenin and cyclin D1 may reduce the activity of ESCs from diabetic rats, which might be one of the important mechanisms of delayed wound healing in DM.

Introduction

Diabetes mellitus (DM) affects approximately 170 million people worldwide, and by 2030 the number of diabetics is expected to double (1,2). Diabetic status impairs cutaneous wound healing, resulting in chronic lesions and ulcers that lead to high morbidity and mortality, and increased treatment costs (3). The healing of the diabetic skin wounds is a challenge in current clinical therapy. The mechanism of how DM impairs cutaneous wound healing is not completely understood. Epidermal stem cells (ESCs), the specific stem cells of the skin, have powerful potential for proliferation and multiple differentiation, and play a critical role in repairing and rebuilding the skin. The Wnt/β-catenin signal transduction pathway is the main pathway regulating the growth, development and differentiation of these cells. β-catenin plays a key role in Wnt signal transduction. Cyclin D1, a downstream target gene of β-catenin, is the main factor regulating the entry of cells into the S phase of the cell cycle. Recent studies have identified β-catenin as an important component of cutaneous wound healing; nevertheless, its expression in the ESCs of diabetic rats remains unknown (1,12). In this study, we tested the expression of β-catenin and cyclin D1 in the ESCs of diabetic rats, and explored the possible mechanisms of delayed wound healing in diabetic patients.

Materials and methods

Materials. Twenty male Sprague-Dawley (SD) rats, weighing 250-300 g, were provided by the central animal care facility of Nanchang University Medical College. All animals received humane care according to the criteria outlined in the 'Guide for Care and Use of Laboratory Animals'. Streptozocin (STZ; Sigma, USA), keratin19 Antibody (K19; Lifespan Biosciences, USA), β1-integrin (Abcam, UK) β-catenin and Cyclin D1 (Santa Cruz, USA) were used in this study.

Construction of diabetic rat model. Twenty SD rats were fed at the central animal care facility of Nanchang University for a week, then randomly divided into two groups of ten: the DM group and the control group. After fasting for 16 h, the DM group rats were weighed and their blood was collected for serum glucose measurement by a glucose analyzer (Johnson, USA). Then, the DM group rats were given an intraperitoneal injection of a dose of streptozocin (65 mg/kg body weight, in 0.1 M citrate buffer filtered through a 0.22 μm sterilized filter, pH 4.5). After 72 h, the serum glucose of the DM group rats was measured again. For serum glucose measurement, blood was collected from the rat tail vein on the 7th, 14th and 21st day after STZ injection. Rats were considered diabetic when their blood glucose was >16.7 mmol/l at all three times, and when they had the following typical DM symptoms: polyphagia, polydipsia, polyuria and weight loss (4,5). Nine rats were diagnosed as diabetic in this study (n=9).

Isolation and culture of ESCs. Under sterile conditions, full-thickness skins were taken from the dorsa of the SD rats and rinsed with normal saline. The subcutaneous tissue and connective tissue of the samples were removed with a blade,

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leaving only the epidermis and dermis. The remaining skin was rinsed once with normal saline containing gentamicin (1,600 U/ml) and twice with D-Hank's solution, then cut into ~0.3x0.5 cm with eye scissors and placed in a culture bottle. Trypsinase and EDTA at a final concentration of 0.25 and 0.02%, respectively, were added to the bottle. The remaining skin in the bottle was digested at 4˚C overnight. Culture plates were evenly coated with type IV collagen diluted with phosphate-buffered saline (100 mg/l), then dried in an incubator. The type IV collagen rapid adhering method was successfully used to select the epidermal stem cells of the rats (6). On the 2nd day, the specimens were removed and the digestion process was terminated by the addition of a little FBS. The epidermis was separated with eye tweezers after the specimen was rinsed with D-Hank's solution, then repeatedly rinsed with D-Hank's solution. The rinsed solution was collected, then filtered with a 200-mesh filter. The filtrate was centrifugated for 5 min at 1,000 r/min, then the supernatant was discarded, leaving only the ESC cells. K19 and β-integrin, which are markers of ESCs, were used to identify the ESCs (7-10). Cells were suspended with keratinocyte serum-free medium (K-SFM). The cell suspension was adjusted to a concentration of $1x10^7$ cells/l, and was cultured in the prepared culture plate and incubated at 37˚C in an incubator with 5% CO_2 for 20 min. Then, the culture medium and non-adhesive cells were sucked out from the plate, and K-SFM containing 10% fetal calf serum and 10 μg/l epidermal growth factor was added to the plate. The cells were continuously cultured in the incubator for 7-10 days, and observed through an inverted phase contrast microscope. The medium was changed every second day.

Colony-formation rate. ESCs in the logarithmic phase of growth were seeded in 6-well plates at a density of 200 cells/well and cultivated in 2 ml of K-SFM with 10% FCS. Triplicate cultures were maintained for 7 days at 37˚C in a humidified atmosphere of 5% CO_2. Cloned cells were observed and counted through an inverted phase contrast microscope. The colony-forming rate was calculated according to the following formula: colony-forming rate (%) = number of cell clones/inoculated cell number x 100%.

Immunocytochemical staining. ESCs were harvested from subconflunent primary cultures, and the cell suspension was counted to prepare cells for cytospin after washing. The slides were air-dried and fixed in 4% paraformaldehyde for 10 min. Immunocytochemistry was performed to determine the expression of K19, β1 integrin, β-catenin and cyclin D1 according to the manufacturer's instructions. Finally, immunostaining was visualized using diaminobenzidine counterstained with hematoxylin. All the washings and dilutions were performed with PBS. Image analysis was accomplished to measure the average integral optical density of the positive cells using Image-Pro Plus 6.0 software.

Reverse transcription-polymerase chain reaction. Total RNA was extracted from the ESCs using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions, and its concentration and purity were detected. Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the mRNA expression of β-catenin and cyclin D1. The following primers were used for amplification: β-catenin, 5'-ACAAGATGATGGTGTGCCAA-3' (sense) and 5'-TGCACAAACAGTGGAATGGT-3' (antisense), product of 502 bp; cyclinD1,5'-ATTGAAGCCCTTCTGGAGTCAAGCC-3'(sense) and 5'-TCTATTTTTGTAGCACCCCCCCGTC-3' (antisense), product of 415 bp; β-actin was amplified as an internal control using 5'-GAGACCTTCAACACC CCAGCC-3' (sense) and 5'-GACTTCGAGCAAGAGATGGCC-3' (antisense), product of 312 bp. PCR reaction conditions were: 95˚C predegeneration for 5 min, 95˚C denaturation for 30 sec, 54˚C annealing for 30 sec, 72˚C extension for 10 min, for a total of 35 cycles. PCR products were examined using semi-quantitative analysis with a gel scanning analyzer (BioRad, USA).

Western blot analysis. To prepare the protein sample, an ultrasonic cell crusher (NinBo, P.R. China) was used to break down the ESCs ($1x10^7$/l) harvested from subconflunent primary cultures. A centrifuge (Beckman, USA) was used to separate the protein sample. Protein lysates were resolved on a 6% sodium dodecyl sulfate (SDS)-polyacrylamide gel, electrotransferred onto polyvinylidene fluoride membranes and blocked in 5% nonfat dry milk in Tris-buffer saline, pH 7.5 (100 mM NaCl, 50 mM Tris and 0.1% Tween-20). The membranes were immunoblotted overnight at 4˚C with anti-β-catenin monoclonal antibody, cyclin D1 monoclonal antibody and anti-β-actin antibody, followed by their respective horseradish peroxidase-conjugated secondary antibodies. Signals were detected by enhanced chemiluminescence (Pierce, USA). The densitometry of the target protein bands was captured, and the densities of the bands were recorded by QuantityOne 462 software.

Statistical analysis. SPSS 13.0 software was used for the statistical analyses. Numerical values were expressed as the mean ± SD. Statistical significance was calculated using the Student's t-test for paired samples. Statistically significant differences were set at $P<0.05$.

Results

Morphological characteristics of ESCs. ESCs examined by inverted phase contrast microscopy were found to be circular in shape and small in size, with strong refractivity. After 24 h, most of the ESCs in the normal rats were slightly extended into a flat polyangular shape with a round nucleus near the center of the plasma, and gradually formed tiny clones. After 6 days, the ESCs of the normal rats were fused into patches in close proximity, linked to each other, and had the appearence of paving stones. By contrast, ESCs in the diabetic rats grew very slowly at first, then after 8 days began to grow slightly faster. In morphology, The ESCs in the control rats appeared to be bigger in the clones connecting closely, whereas those in the diabetic rats appeared to be smaller in the clones that were loose or disperse. The number of ESCs in the diabetic rats was less than that in the controls in each clone.

Colony formation rate. The proliferation of 200 ESCs was observed 7 days after the start of the experiment. The ESCs in the control group were increased by 22.75±3.24, while

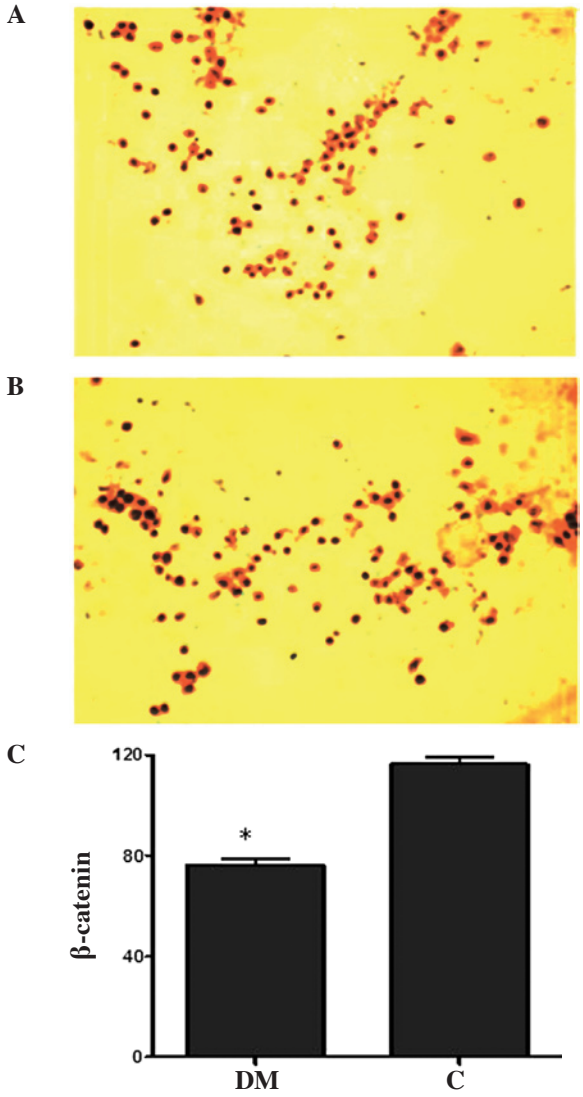

Figure 1. Expression of (A) β-catenin in ESCs of DM SD rats and (B) β-catenin in ESCs of normal SD rats; x400 magnification. (C) ESCs of diabetic rats revealed significant difference in the average integral optical density of positive cells of β-catenin compared to the controls. Values are the mean ± SEM; n=9 for each group; *P<0.01.

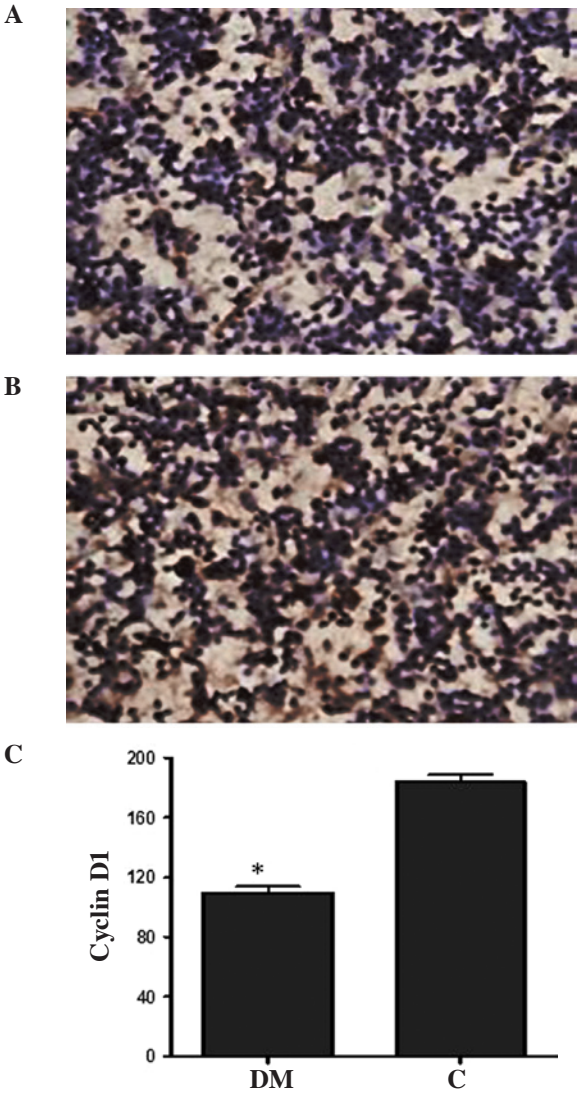

Figure 2. Expression of (A) cyclinD1 in ESCs of DM SD rats and (B) cyclin D1 in ESCs of normal SD rats; x400 magnification. (C) ESCs of diabetic rats revealed significant difference in the average integral optical density of positive cells of Cyclin D1 compared to the controls. Values are the mean ±SEM; n=9 for each group; *P<0.01.

those in the DM group were only increased by 12.87±2.03. This difference was statistically significant (P<0.01). The clone formation rates of the ESCs in the DM group and the control group were 6.43 and 11.37%, respectively. The clone formation rate of the ESCs in the DM group was markedly reduced compared to that of the controls. The results indicate that the ESCs in the DM group possessed a lower self-renewal capacity than those of the control group *in vitro*.

Immunocytochemical staining. Under a microscope, β-catenin and cyclin D1 were weakly expressed in the sorted ESCs of the DM group, whereas both were highly expressed in the sorted ESCs of the control group. β-catenin was only expressed in the membrane and cyclin D1 was expressed in the nucleus. The results of immunocytochemistry were used to measure the average integral optical density of the positive cells by image analysis. The ESCs of the diabetic rats were significantly different in terms of the average integral optical density of β-catenin (76.49±6.58 and 116.39±9.26; P<0.01) and cyclin D1 (109.70±11.99 and 183.95±14.51; P<0.01) in the positive cells compared to the controls (Figs. 1 and 2).

mRNA expression of β-catenin and cyclin D1. The mRNA expression of β-catenin (0.850±0.059 and 0.556±0.017; P<0.01) and cyclin D1 (0.887±0.038 adn 0.682±0.023; P<0.01) was lower in the DM group than in the control group (Fig.3).

Western blot analysis. Western blot analysis revealed that β-catenin and cyclin D1 were expressed in both groups, although the DM group exhibited weaker expression of β-catenin (0.583±0.054 and 1.128±0.077; P<0.01) and cyclin D1 (0.843±0.037 and 1.307±0.072; P<0.01) compared to the control group (Fig.4).

Discussion

Currently, the treatment of chronic wounds in diabetic patients remains a clinical challenge. Skin wounds occur easily in

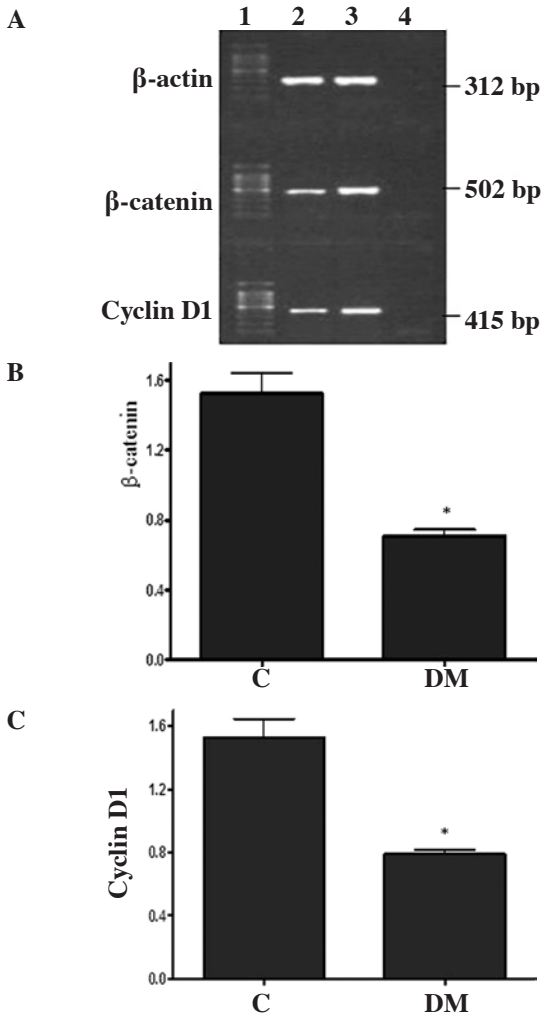

Figure 3. (A) Detection of β-catenin and cyclin D1 mRNA expression in ESCs of DM skin and normal skin. Lane 1, DNA marker DL 2000; Lane 2, ESCs of DM skin; Lane 3, ESCs of normal skin; Lane 4, blank control. (B) mRNA expression level of β-catenin and (C) cyclin D1 were lower in the DM group compared to the control group. Values are the mean ± SEM; n=9 for each group; *P<0.01.

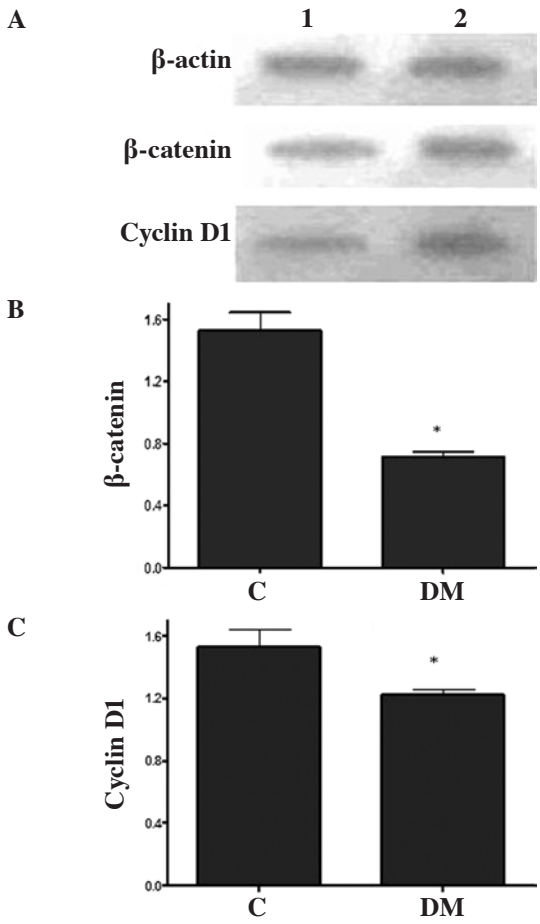

Figure 4. (A) Western blot analysis of β-catenin and cyclin D1 protein expression in ESCs of DM skin and normal skin. Lane 1, ESCs of DM skin; Lane 2, ESCs of normal skin. The DM group revealed weaker expression of (B) β-catenin and (C) cyclin D1 compared to the control group. Values are the mean ± SEM; n=9 for each group; *P< 0.01.

diabetic patients, and the healing process takes much longer than in the non-DM population; indeed the wounds may never heal completely (11). The mechanisms involved in the delay of the healing process are complicated (12). Specific studies (13,14) indicate that the re-epithelialisation of skin wounds in the healing process is dependant on the normal proliferation of epithelial cells. Li *et al* (15) reported that ESCs are actively involved in the healing process, and that the main function of ESCs on the wound edge is to promote the re-epithelialisation of skin wounds. Huang *et al* (16) found that the glycation of type IV collagen inhibits the adhesion and proliferation of ESCs. However, the mechanisms of the proliferation and differentiation of ESCs have yet to be clearly determined in the skin of diabetics. The effect of β-catenin in the ESCs of diabetic rats remains unclear.

β-catenin is a protein with multiple functions that binds with cadherin in epithelial cells and plays a crucial role in cell-cell adhesion. β-catenin is also involved in the regulation of cell growth, proliferation and differentiation, and has a critical function in Wnt signal transduction (17). The level of β-catenin may also play a role in determining the fate of ESCs. Specific studies have revealed that Wnt and β-catenin may increase the proliferation, differentiation and migration of skin epithelial cells, and may therefore improve skin wound healing (18,19). Activation of the canonical Wnt pathway causes an increase in β-catenin levels in cell plasma. β-catenin translocates to the cell nucleus and activates downstream genes, such as cyclin D1; as a result of this activation, epithelial cells proliferate and differentiate. In this study, we tested the expression of β-catenin and cyclin D1 in the ESCs of diabetic rats, and explored their relationship with the delay of skin wound healing in DM.

Whether ESCs can be applied to repair the diabetic wound remains to be determined. As the specific stem cells in skin tissues, ESCs, which are multipotent and posses high proliferative potential, play a key role in repairing and rebuilding the skin. In this study, we found that the mRNA and protein levels of β-catenin and cyclin D1 were significantly lower in ESCs (P<0.01) than in a normal control group, using various methods. The clone formation efficiency of ESCs in DM rats was markedly lower than that in normal rats. The ESCs of the diabetic rats were markedly reduced compared to those of the controls. These results suggest that the expression of cyclin D1 in the ESCs of diabetic patients is decreased when the expression of β-catenin is low, which may reduce the proliferation

of epidermal cells. Our previous study (20) revealed that the proliferation and differentiation of ESCs in diabetic rats were significantly inhibited compared to normal control rats. This suggests that the biological behavior of the ESCs in diabetic patients is already changed, even when the skin is intact. As a result, the skin of diabetic patients is more susceptible to injury, and once injured, skin wound healing is delayed and complete healing may sometimes never be achieved.

Whether skin wounds in diabetic patients are successfully treated or not depends on close control of blood sugar, as well as the epithelialisation of the skin wounds. Studies by Oberley *et al* (21) revealed that ESCs are capable of a large proliferative potential when stimulated, and are responsible for the long-term maintenance of the epidermis. In this study, we demonstrated that the expression of β-catenin and cyclin D1 was reduced in the ESCs of diabetic rats compared to the normal rats. As a result of the reduction of β-catenin and cyclin D1, implicit damage to the skin of diabetic rats may occur (22), resulting in the depletion of epidermal stem cells. This may be one of the key mechanisms in delayed wound healing in diabetic patients. Our results provide experimental evidence for the clinical application of ESCs to treat delayed wound healing in diabetic patients.

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