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Regulation of Keratinocyte Proliferation in Rats With Deep, Partial-Thickness Scald: Modulation of Cyclin D1-Cyclin-Dependent Kinase 4 and Histone H1 Kinase Activity of M-Phase Promoting Factor

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Background. **Keratinocyte proliferation, which is undergone by its cell cycle transition, is considered a major event during re-epithelialization over the wound size. Cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors interact to regulate the cell cycle. We investigated proliferative events associated with cell-cycle control in keratinocytes during wound healing in rats with deep, partial scald injuries.**

Materials and methods. **Male Sprague Dawley rats with starting weights of 200 to 220 g were inflicted with standardized deep partial-thickness burns by scalding 10% of the skin surface. The full thickness skin biopsies were harvested for histological evaluation at following time points: 0 d, post-burn day 3, post-burn day 7, and post-burn day 14. Keratinocytes from wound edge were isolated for cell cycle examination. The cell cycle regulators and their activity were detected.**

Results. **Keratinocytes tended to proliferate and had enlarged nuclei and nucleoli from day 3 after injury. Morphological features became evident on day 14, with an increase in keratinocytes. The percentage of S-phase keratinocytes tended to increase on day 14. The percentage G2/M-phase keratinocytes increased from day 3 and significantly increased on days 7 and 14. Cyclin D1 expression markedly increased from day 3, with down-regulation of cyclin-dependent kinase 4, which re-elevated on day 14. Cyclin B1 expression did not dramatically vary. Histone H1 kinase activity of mitosis phase promoting factor markedly increased on day 14.**

Conclusions. **These findings suggested early, active DNA synthesis and mitosis in keratinocytes, with marked proliferation on day 14, that depended on the modulation of cyclin D1-cyclin-dependent kinase 4 and histone H1 kinase activity of mitosis phase promoting factor. During wound healing, patterns of cell-cycle control expression differed from those previously known.**

Key Words: **keratinocyte; wound healing; cell-cycle; cyclin; cyclin-dependent kinase.**

INTRODUCTION

Wound healing is characterized by a sequence of pathophysiologic proliferative and regenerative events that can be described as a highly ordered and controlled biological process. Successful wound healing requires re-epithelialization over the wound size. Keratinocyte proliferation is a major event during re-epithelialization and contributes to the process and quality of healing. Therefore, study of the mechanisms of keratinocyte proliferation and its control seems especially important to thoroughly understand wound healing.

Keratinocyte proliferation is a universal process by which the cell cycle is executed in a timely and orderly manner [1]. Cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs) work together to regulate progression of the cell cycle. The formation of cyclins and CDK complexes play an important role in ensuring the onset and progression of the cell cycle, whereas CKIs have an inhibitory role against CDKs [2–4]. Kinase complexes formed from a catalytic subunit (CDK) and a regulatory subunit (cyclin) drive the cell cycle [2–5]. The CDK4 complex, which consists of a protein kinase catalytic subunit CDK4 and an activating cyclin D1 subunit, is necessary for S-phase transition [2–4, 6]. The M-phase promoting factor (MPF), which consists of a catalytic subunit p34cdc2 and an activating subunit cyclin B1, is required for G2-M phase transi-

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tion [2–7]. Members of the CKI family oppose progression of the cell cycle in various ways, such as binding to the CDK-cyclin complex to abrogate catalytic kinase activity [8].

Recent findings reflect confusion about cell-cycle regulatory events during wound healing. Down-modulation of several factors in the G1-S-phase, including cyclin D1 and CDK4, and a relative excess of CKIs, may coordinate to ensure the quiescence of migrating keratinocytes during re-epithelialization of wounds in human oral mucosa [9]. However, in the rat model, dramatic variations in the expression of cyclin D1, CDK2, and CDK4 were not observed during wound healing [10]. Little is known about these cell-cycle events during the healing of burns. Therefore, the purpose of this study was to examine the expression and activity of several cell-cycle regulatory proteins in keratinocyte in rat model of deep, partial scald injury.

METHODS

Animal Model and Experimental Design

Twenty-four male Sprague Dawley rats obtained from Shanghai Laboratory Center, China Medical Academy (Shanghai, China) with starting weights of 200 to 220 g were acclimated in a single cage under controlled conditions for 10 to 14 d after their arrival at the laboratory. The Committee on the Use and Care of Animals of Rui Jin Hospital, Shanghai Jiao Tong University, School of Medicine, approved all procedures.

An insulation mold (corresponds to 10% of the total body surface area) was specially prepared. Standardized, deep, partial-thickness burns were created in 18 rats by scalding 10% of the skin surface on the back for 6 s with hot water (80°C) under intraperitoneally delivered anesthesia with 100 mg/kg ketamine (Jiang Su Hengrui Medicine Co. Ltd, Jiang Su, China). The 24 rats were equally divided into four groups for evaluation on day 0 and on days 3, 7, and 14 after the burn, where six rats (0 d) served as unburned control group. The rats of the control group were immersed for 6 s with water of room temperature under anesthesia named above. Different groups of rats were biopsied, and then animals were killed at each time point via CO_2 euthanasia. Each rat only underwent one series of biopsies at the time of sacrifice. Skin was excised, and samples for analysis were treated as described.

Histological Evaluation of Skin Samples

Full-thickness skin samples for skin biopsy were harvested at each time point from the back of the rats at sacrifice. Skin samples were obtained from the border of the wound and from topographically corresponding sites of normal skin. They were stained with hematoxylin and eosin, and morphological characteristics of the epithelium were observed by using an image-analysis system (Zeiss, Oberkochen, Germany).

Cell Isolation

The skin specimens were trimmed of excess subcutaneous tissue and incubated in 0.5% dispase (Roche, Mannheim, Germany) at 4°C over night. Surface epithelium was stripped from submucosal connective tissue and treated with a trypsinization mixture (1:1 ratio mixture of 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid) for 5 min at 37°C to dissociate the cells. Phosphate-buffered saline (PBS) containing 10% fetal bovine serum was then added to inactivate the trypsin and ethylenediaminetetraacetic acid. After cell dissociation, the cell suspension was centrifuged for 5 min at 1000 *g* to harvest keratinocytes.

Flow-Cytometric Analysis of the Cell Cycle

The cell pellet was resuspended in GM/0.5-mM ethylenediaminetetraacetic acid solution (1.1 g/L glucose, 8 g/L NaCl, 4 g/L KCl, 0.39 g/L $Na_2HPO_4 \cdot 12H_2O$, 0.15 g/L KH_2PO_4, 0.5 mM ethylenediamine tetraacetic acid). It was fixed by adding three volumes of cold 95% ethanol. After 30 min of fixation, the cells were centrifuged, washed once in PBS, and stained for 20 min with 40 μg/mL of propidium iodide in PBS (10^6 cells/mL). For each sample, the cell cycle of 10^5 events were examined by means of flow cytometry and analyzed by using lysis software, (EPICS-XL; Beckman Coulter, Fullerton, CA).

Western Blot Analysis

Keratinocytes were lysed in lysis buffer (20 mmol/L HEPES buffer, 50 mmol/L NaF, 20 mmol/L ethyleneglycotetraacetic acid, 15 mmol/L $MgCl_2 \cdot 6H_2O$, 1 mmol/L dithiothreitol, 3 μg/mL leupeptin, 80 mmol/L β-glycerophosphate, 300 μmol/L phenylmethylsulfonyl fluoride) at 4°C for 15 min. The lysates were clarified by centrifuging them at 12,000 *g* for 15 min at 4°C.

The supernatant was transferred to another tube, and 5 μL was used to measure the protein concentration with a protein assay kit (BCA; Pierce, Rockford, IL). The protein concentration of each solution was determined according to the manufacturer's instructions. Added to the supernatant was 5 × sample buffer (50% glycerol, 0.1 M dithiothreitol, 10% sodium dodecyl sulfate, 0.01% bromphenol blue, 0.25 M Tris/HCl pH 6.8). The samples were boiled for 5 min, and 50 μg of each sample was separated in 10% sodium dodecyl sulfate-polyacrylamide gels and transferred into polyvinylidene difluoride membranes by following standard procedures.

Immunodetection was performed at room temperature by first blocking the membrane for 1 h in Tris-buffered saline containing 0.05% Tween 20 and 2% bovine serum albumin. This step was followed by incubation with diluted antiserum in the washing buffer (Tris-buffered saline and Tween 20) at 4°C for overnight. The membranes were washed three times and incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG antibody

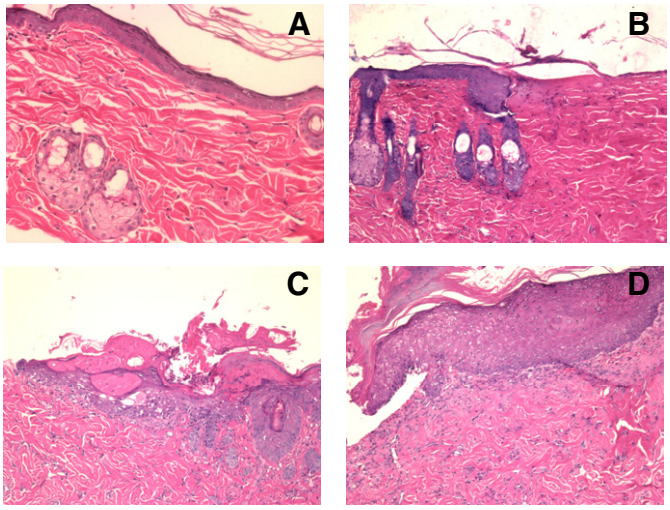

FIG. 1. Histological appearance of the epidermis during wound healing. Photomicrographs show normal, intact rat skin (A) and rat skin on days 3 (B), 7 (C), and 14 (D) after the burn (hematoxylin and eosin stain, original magnification ×100). (Color version of figure is available online.)