Journal of Dermatological Science 70 (2013) 196–203

Contents lists available at SciVerse ScienceDirect

Journal of Dermatological Science

journal homepage: www.elsevier.com/jds

Negative pressure accelerated monolayer keratinocyte healing involves Cdc42 mediated cell podia formation

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ARTICLE INFO

Article history:
Received 8 August 2012
Received in revised form 6 March 2013
Accepted 12 March 2013

Keywords:
Negative-pressure wound therapy
Wound healing
Cell movement
Actins

ABSTRACT

Background: Negative-pressure wound therapy (NPWT) is developed to facilitate wound healing at controlled subatmospheric pressures in modern medicine. Molecular mechanism for this therapy is still undefined.
Objective: This study highlights the localization and time-course of the cell division control protein 42 (Cdc42) in the cell membrane at ambient pressure (AP) and negative pressures of 75 mmHg (NP_{75}), 125 mmHg (NP_{125}) and 175 mmHg (NP_{175}).
Methods: The prepared cells were cultured in a negative pressure incubator with the same O_2 and CO_2 tensions at the four different pressures. The effective time, complete wound closure time, cell volume, cell viability, and the fluorescence of proliferating cell nuclear antigens (PCNA) and actins were evaluated in cells at different pressures. Wound-healing process and Cdc42 fluorescence were examined in cells with the knockdown of Cdc42. Cdc42 pathway proteins in cell membranes were analyzed after incubation at different pressures for 6 and 12 h.
Results: The cells at NP_{125} had less wound closure time and obvious cell podia. Similar PCNA fluorescent intensity was observed in cells at different pressures. The Cdc42, neural Wiskott–Aldrich syndrome protein, and actin expression increased significantly ($p < 0.05$) in plasma membranes of cells at NP_{125} for 12 h. The knockdown of active Cdc42 resulted in the absence of Cdc42 expression at the cell leading edge.
Conclusions: The activation and localization of Cdc42 pathway proteins in the cell membrane are involved in the cell podia formation in keratinocytes at NP_{125}. NPWT may facilitate cell migration to accelerate wound healing.

1. Introduction

An estimated 6.5 million persons suffer from chronic skin ulcers caused by pressure, venous stasis, or diabetes mellitus [1]. Negative-pressure wound therapy (NPWT), a treatment to facilitate wound healing at controlled subatmospheric pressures has gained popularity in modern wound cares [2]. Some of the advantages of using NPWT in wound management include reducing the time to wound closure and increasing wound closure incidence [3–5]. The proposed mechanisms underlying the effect of NPWT on wound healing include creating a moist wound-healing environment [6], relieving an imbalance of matrix metalloproteinase proteins and their inhibitors [7], enhancing angiogenesis at the wound bed [2,8], reducing bacterial loads [2], and translating physical stress to cell proliferation [9,10]. These observations provide valuable information in illustrating NPWT effects on wound healing at the tissue level. However, effects of this therapy on wound healing at the molecular level are still undefined.

Negative-pressure incubators (NPIs) have been constructed to simulate wound healing in NPWT at the cell level. Cell viability and migration results have been reported using an NPI [11–13]. To create negative pressure conditions for cell cultures, air must be removed from the incubator. Ideally, a NPI should have a capability of keeping a dynamic balance between the removal and the supply of the O_2 at different atmospheric pressures. However, the O_2

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0923-1811/$36.00
http://dx.doi.org/10.1016/j.jdermsci.2013.03.007

pressure settings in the NPI at different negative pressures were not mentioned in the previous reports. Hypoxia is a micro-environmental stress in wounded skin and this disturbance can facilitate wound healing by promoting cell motility [14]. In order to eliminate the hypoxic effects on wound healing at different negative pressures, we developed an NPI with air regulation to maintain O_2 and CO_2 tensions in the same value at different pressure states. This design could be a new model to determine the pure negative pressure effects on wound healing and may be helpful for an in-depth analysis of the biological behavior at various negative pressures.

The cell division control protein 42 (Cdc42) acts as an intracellular molecular switch between the activated GTP-bound and the inactivated GDP-bound states in the cell membrane. This protein is a major regulator of cell polarity and controls the direction of cell migration in wound healing. Activated GTP-bound Cdc42 accumulates at the leading edge of the plasma membrane in a migrating cell [15]. The neural Wiskott–Aldrich syndrome protein (N-WASp) functions downstream of Cdc42 and is involved in cell lamellipodia and filopodia formation [16]. N-WASp is ordinarily found in the auto-inhibited conformation. Active Cdc42 binds to N-WASp to relieve the auto-inhibition. Activated N-WASp stimulates actin polymerization and promotes the actin-related proteins 2 and 3 (Arp2/3) complex [17], to form branched actin filaments [18]. Membrane bound Ras-related C3 botulinum toxin substrate (Rac) has also been proposed to reorganize cytoskeletons during cell migration [19]. Actin assembly is critical in wound-induced cell movement [20] and can be activated by the Cdc42 pathway proteins [21]. The regulation of Cdc42 for actin reorganization in cell migration has been extensively reviewed [15,20,21], nevertheless, investigations for the effects of negative pressure on Cdc42 and its related proteins are still absent.

Shear stress-induced high Cdc42 activity at the leading edge of the migrating endothelial cell has been reported in a previous study [22]. Correlations between the Cdc42 transcription/translation and the mechanical stress are still unexplored in most of the studies. The basal area of the keratinocytes cultured in an NPI was increased approximately 11% at NP_{125} and could induce rapid wound closure in wounded monolayer cells [12]. The study also reported that the NP_{125} could disassemble the cell junction to promote cell motility and results in cell spreading. The membrane-bound proteins involved in cell movement, including Cdc42, N-WASp, the Arp2/3 complex, and membrane-bound actin [19], may be activated after the disassembly of the cell junction. Therefore, we hypothesized that the negative pressure stimulated the Cdc42 and its downstream proteins localization on the leading edge plasma membrane in the healing process of wounded monolayer keratinocytes. This activation further promoted cell migration and resulted in quick wound closure.

2. Materials and methods

2.1. Negative-pressure incubator (NPI)

A NPI (NPI1500, Linston Advanced Technology Corporation, Longtan, Taoyuan, Taiwan) consisting of an airtight chamber, a vacuum pump, an ultrasound mist generator, a heating system surrounding the chamber wall, gas (O_2 and CO_2) tanks and a gas analyzer was used to incubate cells at different pressures. The electric cell-substrate impedance sensing (ECIS) technique (1600R, Applied Biophysics, Troy, NY, USA) was integrated into the incubator. An adequate amount of air was introduced into the chamber to maintain O_2 and CO_2 pressure in the incubator at 150 and 38 mmHg at AP, NP_{75}, NP_{125} and NP_{175}. The relative humidity was greater than 60% and the temperature was 37 °C inside the NPI at different pressures.

2.2. Cell culture procedures

Human skin keratinocytes (HaCaT cell line), kindly provided by Dr. Weng-Hung Chung (Department of Dermatology, Chang Gung Memorial Hospital, Keelung, Taiwan), were cultured in DMEM/F12 (Sigma-Aldrich Corporation, St. Louis, MO, USA) containing 10% fetal bovine serum and 100 μg/mL streptomycin–penicillin.

2.3. Cell viability

Keratinocytes (3×10^4) were seeded in eight wells of a 96-well cell culture cluster (Corning Incorporated, Corning, NY, USA) and incubated overnight at AP and the three different negative pressures for 6 h. A 20 μL of sterile filtered (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) stock solution (Sigma-Aldrich Corporation, St. Louis, MO, USA) in phosphate buffered saline pH 7.4 (5 mg/ml) was added to each well reaching a final concentration of 0.5 mg MTT/ml. The insoluble formazan crystals in each well were dissolved by 200 ml of dimethylsulfoxide (Merck, Darmstadt, Germany) and the colored solution was measured spectrophotometrically in an ELISA reader (Multiskan GO, Thermo Fisher Scientific Inc., Vantaa, Finland) at a wavelength of 570 nm (test) and 690 nm (reference). The relative cell viability (%) related to that in cells incubated at AP calculated [23]. The cell viability in cells incubated at different pressures for 12 h was analyzed as described above.

2.4. Preparation of electrode arrays for electric cell-substrate impedance sensing (ECIS)

Culture medium of 200 μL was added to each well of an 8W1E electrode array (Applied Biophysics, Troy, NY, USA) and equilibrated at AP for 30 min. A 200 μL cell suspension containing 10^5 cells was added to each well. Each well contained a final concentration of 1.25×10^5 cells/cm^2 in 400 μL medium volume. Each array was subsequently incubated overnight at AP for cell spread and attachment. Resistance in each well was continuously measured by applying a 4 kHz alternative current (AC) of 1 μA in normal mode.

An AC of 1 mA at 60 kHz with duration of 100 ms was applied to create a wound of 5×10^{-4} cm^2 in the confluent monolayer of cells. The resistance dropped immediately after wounding and then increased gradually during the healing process. The wounded monolayer cells in each array were cultured at AP, NP_{75}, NP_{125}, or NP_{175}. When the wounded monolayer became confluent, the resistance plateaued at the maximum value. The resistance level of each well during the wound-healing process was normalized by the maximum value to avoid variations in cell–cell contact in different wells [24] and to compare resistance changes between different wells. Therefore, the effective time (ET) and the complete wound closure time (T_{max}) were estimated from normalized healing curves obtained at the four different pressures. The ET, representing the sensitivity of wounded cells to the environment, was defined as the time between the immediate post-wounded point and 50% of the normalized maximum resistance value. The time from initial wounding to the normalized maximum resistance was defined as T_{max}. The long- and short-axis diameters of 100 randomly selected cells in each well in the electrode area before injuries (D_0) and after complete wound closure (D_{end}) at the four different pressures were measured using a phase-contrast microscope (Leica DMIRB, Leica Microsystems, Wetzlar, Germany). The degree of cell spreading in long- and short-axis in each well at the four different pressures was estimated from the average D_0 ($\bar{D}_0$) and D_{end} ($\bar{D}_{end}$) and determined as $(\bar{D}_{end} - \bar{D}_0)/\bar{D}_0 \times 100\%$ [12].