Process Biochemistry 50 (2015) 1662–1668

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Short communication

The effects of negative pressure treatment on the extracellular matrix gene expression and protein production of fibroblasts

Pei-Yu Chen[a], Chih-Chin Hsu[b,c], Kai-Chiang Yang[d], Chang-Chin Wu[a,e], Chung-Li Wang[a,f,*]

[a] Department of Orthopedic Surgery, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei 10002, Taiwan
[b] Department of Physical Medicine and Rehabilitation, Chang Gung Memorial Hospital, Keelung 20401, Taiwan
[c] School of Traditional Chinese Medicine, College of Medicine, Chang Gung University, Taoyuan 33302, Taiwan
[d] School of Dental Technology, College of Oral Medicine, Taipei Medical University, Taipei 11031, Taiwan
[e] Department of Orthopedics, En Chu Kong Hospital, New Taipei City 237, Taiwan
[f] Department of Orthopedic Surgery, National Taiwan University Hospital Yun-Lin Branch, College of Medicine, National Taiwan University, Yunlin 64041, Taiwan

ARTICLE INFO

Article history:
Received 10 February 2015
Received in revised form 17 May 2015
Accepted 16 June 2015
Available online 20 June 2015

Keywords:
Wound healing
Negative pressure wound therapy
Fibroblast
Extracellular matrix

ABSTRACT

Although negative pressure wound therapy (NPWT) has been suggested for the management of acute and chronic wounds, the underlying mechanism of NPWT on the cellular level is not fully understood. Using a primary fibroblast culture model with controlled negative pressure, the cellular responses to the negative pressure was investigated. The present results showed that cell proliferation of fibroblasts was not affected under negative pressure. The mRNA expressions of collagen type I, collagen type III, and fibronectin were down-regulated when cells treated with negative pressure for once only. However, those mRNA expressions were up-regulated when cells subjected to periodical negative pressure consecutively for 5 days. The protein production of TGF-β1 was enhanced, while the TIMP-1, decorin, and IL-6 were not influenced. The pro-MMP-9 was not stimulated when fibroblasts subjected to negative pressure. The regulations of extracellular matrix production in fibroblasts provides an insight into the cellular mechanisms of NPWT.

1. Introduction

The negative pressure (or subatmospheric pressure) wound therapy (NPWT) has been suggested for virtually all kinds of acute and chronic wounds to accelerate healing, such as in pressure wounds, diabetic leg ulcers, lower leg wounds, surgical incision, traumatic wounds, burns, infected wounds, necrotizing fasciitis, infected sternal wounds and after skin grafting in all population [1–3]. In practice, the open wound is covered by a wound dressing and an air-tight film, and connected by suction tubes to a control unit to provide an evenly distributed negative pressure on the wound surface and remove excessive fluid. Generally, −80 to −125 mmHg of negative pressure is used, either continuously or in cycles. The duration of the therapy varies from a few days to months, depending on the treatment aim and the nature of the wound [4]. In comparison with conventional wound therapy, the average time for wound closure and hospital stay have been significantly reduced [5].

To explain how NPWT promotes wound healing, a fluid-based mechanism and a mechanical mechanism were proposed [6–8]. Application of a controlled vacuum to the wound interface facilitates removal of excess interstitial fluid because of increased pressure gradients. The removal of fluids decreases edema and interstitial pressure and reduces the soluble factors which inhibit fibroblasts and endothelial cell proliferation [6,9]. Besides, infection alters the normal healing process by disrupting and prolonging the inflammatory phase of wound healing [10]. It also inhibits the function of leukocytes and hinders the formation of granulation tissue. Morykwas et al. [11] demonstrated that the Vacuum Assisted Closure (VAC™) decreased bacterial count in a grossly infected wound to levels that would allow for spontaneous healing within 4–5 days, which may be attributable to the increased blood flow, decreased interstitial edema, and removal of harmful enzymes from the wound bed.

* Corresponding author at: National Taiwan University Hospital Yun-Lin Branch, Department of Orthopedic Surgery, No. 579, Sec. 2, Yunlin Road, Douliu, Yunlin 640, Taiwan.
E-mail addresses: f92548050@gmail.com (P.-Y. Chen), steele1@ms10.hinet.net (C.-C. Hsu), pumpkin@tmu.edu.tw (K.-C. Yang), dtorth65@yahoo.com.tw (C.-C. Wu), chungli@ntu.edu.tw (C.-L. Wang).

http://dx.doi.org/10.1016/j.procbio.2015.06.014
1359-5113/

The mechanical mechanism is believed to contribute to the increased formation of granulation tissue by the stress that the alternating pressure cycle exerted on the cells at the wound surface [12]. The applied forces induce microdeformations in the extracellular matrix (ECM) and thus, as cells are anchorage-dependent, deform the cells in the stretched tissues [13,14]. The ability of tissue to respond to mechanical stress with increased cellular proliferation has been described previously [15,16]. Using a swine model, Morykwas et al. [17] discovered that wounds treated with intermittent negative pressure had an increase in granulation tissue formation compared with control wounds. This increase was also significantly greater than the increase observed in wounds treated with continuous negative pressure. However, it is important to note that even the continuous technique of negative pressure results in a significantly greater rate of granulation tissue formation than is seen in wounds treated with more conventional techniques [8].

Regarding the cellular behavior in response to negative pressure, studies have identified certain molecular basis in the wound, such as up-regulation of basic fibroblast growth factor (bFGF) and extracellular signal-regulated kinase (ERK) 1/2 [18]. However, it is difficult to observe independently in animal models or in clinical settings without being confounded by the host factors. Some studies using specialized culture systems with negative pressure to demonstrate the cellular responses directly. McNulty et al. applied negative pressure on fibroblasts in fibrin matrix through different dressing materials to study the influences of different dressing. The results showed that cells treated with negative pressure applied through open-cell foam had better cell proliferation and less apoptosis than that through gauze. The differences in response were attributed to differences in strain fields created by the combination of the negative pressure and manifolding material [19]. The same group further reported that NPWT significantly increased the levels of transforming growth factor-beta (TGF-β) and platelet-derived growth factor (PDGF) over static control cultures [20]. Potter et al. used an endothelial cell culture model to assess the angiogenesis and biointegration into four different synthetic dermal replacements with different regimens of topical negative pressure. Intermittent regimen of topical negative pressure with open-celled spongy showed the best angiogenesis and biointegration [21]. Nevertheless, there were only a few studies discussing the influences of negative pressure on the cells.

In spite of some promising results are reported, the systematic reviews of clinical effectiveness showed divergent results among different patient groups. Moreover, lacking deep knowledge of the underlying mechanisms makes it difficult to identify the proper indications. Therefore, the studies focus on the effects of negative pressure on the cell may provide confident evidences and benefit further applications for NPWT. In the current study, by using a cell culture system with controlled negative pressure, the cellular responses to the NPWT were investigated.

2. Materials and methods

2.1. Negative pressure (subatmospheric pressure) culture system

A custom cell culture system with controllable air pressure was fabricated (NPI1000, Linston Advanced Technology, Longtan, Taoyuan, Taiwan) [22]. Air in the chamber was removed by a vacuum pump with a speed of 46 l/min and the rate of pressure drop of 12.5 mmHg/s. A manual valve was used to control the pressure in the sealed chamber. Relative humidity was kept greater than 90%. The O_2 and CO_2 tension were maintained at 20% and 5% respectively. The temperature inside the chamber was maintained at 37 °C.

2.2. Mouse fibroblast harvest and cultivation

Animal study was approved by the Institutional Animal Care and Use Committee at the College of Medicine, National Taiwan University. Newborn C57BL/6 mice less than 4 days old were obtained from Laboratory Animal Center. Primary dermal fibroblasts were obtained from skin explant tissue according to the previously established protocol with minor modifications [23]. Cells were cultured in DMEM (4.5 g/L glucose, 12100-061, Gibco, Carlsbad, CA) containing 0.02 mol/L of L-glutamine (21051-024, Gibco, Carlsbad, CA), 1% antibiotic (Penicillin 10,000 U/mL and Streptomycin 10 mg/mL, P4433, Sigma–Aldrich, USA), and 10% FBS (Penicillin-Streptomycin, 10,000 U/mL, 10099-141, Gibco, Carlsbad, CA) in the conventional CO_2 incubator.

2.3. Atmospheric and negative pressures cell cultivation

When 70–80% confluence was reached, the cells were trypsinized, collected, counted, and sub-cultured. The fibroblasts in passage 2–5 were used for the subsequent experiments. The cells were seeded at the density of 5000 cells/cm^2 and cultured in a 12-well culture plate in HEPES-buffered DMEM containing 10% FBS. The cells were cultured in a conventional incubator set at 37 °C under 5% CO_2, or in the negative pressure incubator. The negative pressure was set at −125 mmHg and applied to the cultured cells for once (6 h), or periodically (6 h/daily) for 1, 3, and 5 days.

2.4. DNA quantification for cell proliferation (PicoGreen dsDNA assay)

The cell morphology of each group was observed daily using an optical microscope. At the pre-determined intervals, the double-stranded DNA was quantified (Quant-iT™ PicoGreen® dsDNA Reagent and Kits, Invitrogen, USA) to assess cell proliferation. The cells on each dish were homogenized in TRIzol reagent (15596-026, Life Technologies, Invitrogen, CA, USA). The DNA sample was diluted in 1× TE to a desired volume. Equal volume of the 2× working solution of the PicoGreen® was then added into each sample, well mixed, and transferred the mixture to a proper cuvette. Finally, the samples were protected from light and incubated at room temperature for 5 min.

2.5. Cell viability

The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, M5655, Sigma, USA) reagent was used to examine the activity of fibroblasts cultured at different conditions. At the pre-determined intervals, the culture media were removed and MTT reagent was added. The cells were incubated at 37 °C for further 4 h to allow the formation of formazan crystal. The dark blue crystals were dissolved by acid-isopropanol, aspirated to a 96-well plate, and the optic density was determined by a spectrophotometer.

2.6. RNA extraction and real-time polymerase chain reaction

Total RNA of the cells were extracted using TRIzol reagent. The quantity and purity of the RNA was measured using a NanoDrop spectrophotometer (ND1000, Thermo, DE, USA). The 260/280 ratio was generally ranged between 1.9 and 2.0. First-strand cDNA was synthesized from 1 μg of total RNA using a SuperScript II RT kit (Invitrogen, Carlsbad, CA, USA). Minus-RT controls were also prepared for each sample. Upon quantification, the cDNA was stored at −80 °C until further analysis.

The reaction mixtures for amplification contained 100 ng of cDNA and Universal TaqMan 2X MasterMix (Applied Biosystems,