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TRPV2 channel inhibitors attenuate fibroblast differentiation and contraction mediated by keratinocyte-derived TGF-β1 in an *in vitro* wound healing model of rats

Taro Ishii[a], Kunitoshi Uchida[b], Shozaburo Hata[a], Mitsutoki Hatta[b], Tomo Kita[b], Yuki Miyake[a], Kazuhiko Okamura[c], Sachio Tamaoki[a], Hiroyuki Ishikawa[a], Jun Yamazaki[b,*]

[a] Department of Oral Growth & Development, Fukuoka 814-0193, Japan
[b] Department of Physiological Science & Molecular Biology, Fukuoka 814-0193, Japan
[c] Department of Morphological Biology, Fukuoka Dental College, Fukuoka 814-0193, Japan

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ABSTRACT

Background: Keratinocytes release several factors that are involved in wound contracture and scar formation. We previously reported that a three-dimensional reconstruction model derived from rat skin represents a good wound healing model.
Objective: We characterized the role of transient receptor potential (TRP) channels in the release of transforming growth factor (TGF)-β1 from keratinocytes and the differentiation of fibroblasts to identify possible promising pharmacological approaches to prevent scar formation and contractures.
Methods: The three-dimensional culture model was made from rat keratinocytes seeded on a collagen gel in which dermal fibroblasts had been embedded.
Results: Among the TRP channel inhibitors tested, the TRPV2 inhibitors SKF96365 and tranilast attenuated most potently keratinocyte-dependent and − independent collagen gel contraction due to TGF-β signaling as well as TGF-β1 release from keratinocytes and α-smooth muscle actin production in myofibroblasts. Besides the low amounts detected in normal dermis, TRPV2 mRNA and protein levels were increased after fibroblasts were embedded in the gel. TRPV2 was also expressed in the epidermis and keratinocyte layers of the model. Both inhibitors and TRPV2 siRNA attenuated the intracellular increase of Ca^{2+} induced by the TRPV agonist 2-aminoethoxydiphenyl borate in TGF-β1-pretreated fibroblasts.
Conclusion: This is the first study to show that compounds targeting TRPV2 channels ameliorate wound contraction through the inhibition of TGF-β1 release and the differentiation of dermal fibroblasts in a culture model.

1. Introduction

The wound healing process in skin proceeds sequentially in three phases, namely, inflammation, proliferation, and scar maturation, and involves sequential interactions of different cell types [1]. In granulation tissue, fibroblasts called myofibroblasts [1,2] obtain a contractile phenotype through the expression of α-smooth muscle actin (α-SMA) [3,4]. Such differentiation of dermal fibroblasts is believed to be involved in pathogenic scarring and fibrosis [5]. Thus, pharmacological intervention in the differentiation of fibroblasts may be beneficial for the prevention of hypertrophic scar formation and contractures.

Keratinocytes at the wound edge migrate over granulation tissue and then produce new stratified layers (re-epithelialization) through the production of many growth factors and cytokines, including transforming growth factor (TGF)-βs. TGF-βs are secreted by platelets, fibroblasts, macrophages, and keratinocytes within injured tissue and stimulate granulation tissue formation and myofibroblast production [2,6–8]. Previously, we demonstrated that keratinocytes secrete the latent form of TGF-β1, which upregulates the expression of α-SMA in fibroblasts after the activation of latent TGF-β1 [9]. Thus, we proposed that inhibitors

* Corresponding author at: Department of Physiological Science & Molecular Biology, Fukuoka Dental College, 2-15-1 Tamura, Sawara-ku, Fukuoka 814-0193, Japan.
E-mail address: junyama@college.fdcnet.ac.jp (J. Yamazaki).

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of metalloproteinases and integrin α_V, which have a role in the activation of TGF-β1, could be therapeutic targets for the prevention of hypertrophic scar formation.

Possible other approaches are to target the different ion channels underlying hypertrophic scar formation. Accumulating evidence suggests that the activation of transient receptor potential (TRP) channels, which are nonselective ion channels, contributes to wound healing responses. In corneal fibroblasts, TGF-β1-induced myofibroblast development is highly dependent on TRPV1 [10]. TRPV4 is required for the TGF-β1-induced differentiation of cardiac fibroblasts into myofibroblasts [11]. TRPV4 activity results in the differentiation of lung myofibroblasts and pulmonary fibrogenesis through the TGF-β1 signaling pathway [12]. TRPA1 is required for TGF-β1 signaling and post-alkali burn inflammation and fibrosis in mouse corneal stroma [13]. TRPC3 is highly expressed in human hypertrophic scar tissue and its overexpression promotes wound constriction [14]. TRPC6-mediated Ca^{2+} influx and calcineurin activity are required for myofibroblast transdifferentiation and wound healing [15]. TRPC6 facilitates stress fiber formation, and its inhibition suppresses TGF-β1-mediated excessive intestinal fibrosis [16]. TRPM7 is a major Ca^{2+}-permeable channel with an essential role in TGF-β1-elicited fibrogenesis in human atrial fibrillation [17]. All of these studies focused on the roles of ion channels and downstream intracellular signaling pathways in fibroblast differentiation, although it remains uncertain whether the ion channel-mediated wound healing process is based on interactions between keratinocytes and fibroblasts during re-epithelialization, where keratinocytes migrate over the damaged area of the skin.

Keratinocytes are considered to affect the expression of several factors involved in wound contracture and skin homeostasis. For instance, TRPV3 is involved in epidermal wound healing [18], while TRPV4 contributes to epidermal barrier function [19]. The use of skin equivalents effectively enables the expression of growth factors and interactions between keratinocytes and fibroblasts [20]. We previously reported that a three-dimensional (3D) reconstruction model consisting of keratinocytes and fibroblasts/myofibroblasts derived from rat skin represents a good wound healing model [9]. Animal models have attempted to reflect human wound healing impairment, such as scarring, yet there seems to be considerable differences in wound contraction among species. In rat or mouse models, rapid wound contraction is observed predominantly, leading to wound contracture associated with the differentiation of myofibroblasts [21]. The present *in vitro* rat model, which can be generated in a short period of time, can offer reproducibility and quantified interpretation of wound contraction. Furthermore, this model can be used to clarify the interactions between keratinocytes and fibroblasts/myofibroblasts, and effectively removes the influence of complicated inflammatory processes. Using such a model, we aimed to demonstrate the mechanism underlying the release of TGF-β1 from keratinocytes and the contraction of myofibroblasts to obtain possible promising pharmacological approaches to prevent scar formation and contractures.

We found that the TRPV2 channel inhibitors SKF96365 [22] and tranilast [23–25] were effective at inhibiting the differentiation of dermal fibroblasts and contraction. Tranilast has been used clinically for the prevention of hypertrophic scar formation in Japan [26,27]. The TRPV2 channel functions as a noxious heat sensor that can be activated by high temperature with a threshold above 52 °C and has relatively high Ca^{2+} permeability [28]. TRPV2 was shown to be expressed not only in sensory neurons but also in non-neuronal tissues, suggesting its involvement in many physiological inputs besides nociception, such as mechanosensing and lipid sensing [29–31]. This is the first study to show that compounds targeting TRPV2 channels ameliorate wound contraction during TGF-β1 release and α-SMA production in a culture model.

2. Materials and Methods

2.1. Antibodies and reagents

The following primary antibodies were used: mouse monoclonal anti-α-SMA (clone 1A4; Dako, Glostrup, Denmark) and rabbit polyclonal anti-TRPV2 (generously gifted by Dr. Tominaga, National Institute for Physiological Sciences, Okazaki, Japan). Recombinant human TGF-β1 was purchased from PeproTech (Rocky Hill, NJ). LY364947 (Cayman Chemical, Ann Arbor, MI) and other water-insoluble compounds were dissolved in dimethyl sulfoxide to make stock solutions that were diluted more than 1,000-fold before use. TRP channel inhibitors and activators were purchased from commercial sources: SKF96365, AMG9810, and HC067047 (Wako Pure Chemical Industries Ltd., Tokyo, Japan), tranilast (Tokyo Chemical Industry Co., Tokyo, Japan), and 2-aminoethoxydiphenyl borate (2-APB), capsaicin, GSK1016790A, and RN1734 (Sigma-Aldrich Co., St. Louis, MO). The other compounds were obtained from Wako Pure Chemicals.

2.2. Dissociation of keratinocytes and dermal fibroblasts

Dorsal skin was obtained from 2-day-old Wistar rats; permission for the procedures used was granted by the Animal Research Committee of Fukuoka Dental College. The skin was incubated overnight at 4 °C in modified Eagle's medium containing dispase (Godo Shusei, Tokyo, Japan). To obtain keratinocytes, epidermal tissue was digested at 37 °C for 1 min in phosphate-buffered saline containing a 0.1% trypsin solution and 0.65 mM ethylene diamine tetraacetic acid (Gibco Life Technologies, Carlsbad, CA). Fetal bovine serum (FBS) was added to the suspension, which was then filtered (Cell Strainer; Becton Dickinson, Franklin Lakes, NJ) and resuspended in Ham's F-12 medium (Gibco), before use in the reconstruction culture.

Dermal tissue was minced into pieces and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 15% FBS. Fibroblasts that sprouted from the tissue were detached and cultured in new medium containing 10% FBS for 5–6 days. Collagen gels were prepared on ice by mixing type-I collagen (0.725 volume; Nitta Gelatin, Osaka, Japan) with a reconstitution buffer (0.1 volume; 2.2 g $NaHCO_3$ and 4.77 g HEPES in 100 mL of a 50 mmol/L NaOH solution, pH 7.0), 5-times concentrated DMEM (0.15 volume), and 10-times concentrated Ham's F-12 medium (0.025 volume). The gel (2.5 mL) was mixed with 2.0×10^6 or 4.0×10^5 dermal fibroblasts and poured into a 2.4-cm-diameter culture insert (Millicell CM culture plate inserts; Millipore Corp., Temecula, CA) or 1.05-cm-diameter culture insert (Falcon cell culture inserts; Corning, Inc., Corning, NY), respectively. The gel was solidified by warming at 37 °C. A culture insert without fibroblasts was also prepared.

2.3. Construction of the rat skin model using collagen gel matrix culture

Keratinocytes were overlaid on a collagen gel containing dermal fibroblasts that had been prepared 2 days earlier (KC[+]) (day 0, Fig. 1A). A fibroblast-embedded collagen gel without keratinocytes was also prepared (KC[−]). The culture insert was placed in an outer dish containing DMEM and Ham's F-12 medium (3:1 volume) supplemented with 10% FBS and a growth factor cocktail (0.4% v/v bovine pituitary extract, 0.2 ng/mL human epidermal growth factor, 5 μg/mL transferrin, 5 μg/mL insulin, and 0.18 μg/mL hydrocortisone; HKGS Kit; Gibco), 250 μM ascorbic acid