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Pharmacological activation of cannabinoid 2 receptor attenuates inflammation, fibrogenesis, and promotes re-epithelialization during skin wound healing

Lin-Lin Wang [a,1], Rui Zhao [a,1], Jiao-Yong Li [a], Shan-Shan Li [a], Min Liu [a], Meng Wang [a], Meng-Zhou Zhang [a], Wen-Wen Dong [a], Shu-Kun Jiang [a], Miao Zhang [a], Zhi-Ling Tian [a], Chang-Sheng Liu [b], Da-Wei Guan [a,*]

[a] Department of Forensic Pathology, China Medical University School of Forensic Medicine, Shenyang, China
[b] Institute of Forensic Science, Anshan Municipal People's Procuratorate, Anshan, China

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ABSTRACT

Previous studies showed that cannabinoid 2 (CB_2) receptor is expressed in multiple effector cells during skin wound healing. Meanwhile, its functional involvement in inflammation, fibrosis, and cell proliferation in other organs and skin diseases implied CB_2 receptor might also regulate skin wound healing. To verify this hypothesis, mice excisional wounds were created and treated with highly selective CB_2 receptor agonist GP1a (1-(2,4-dichlorophenyl)-6-methyl- N-piperidin-1-yl-4H-indeno[1,2-c]pyrazole-3-carboxamide) and antagonist AM630 ([6-iodo-2- methyl-1-(2-morpholin-4-ylethyl)indol-3-yl]-(4-methoxyphenyl)methanone) respectively. The inflammatory infiltration, cytokine expression, fibrogenesis, and wound re-epithelialization were analyzed. After CB_2 receptor activation, neutrophil and macrophage infiltrations were reduced, and expressions of monocyte chemotactic protein (MCP)-1, stromal cell-derived factor (SDF)-1, Interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF)-A were decreased. Keratinocyte proliferation and migration were enhanced. Wound re-epithelialization was accelerated. Fibroblast accumulation and fibroblast-to-myofibroblast transformation were attenuated, and expression of pro-collagen I was decreased. Furthermore, HaCaT cells in vitro were treated with GP1a or AM630, which revealed that CB_2 receptor activation promoted keratinocyte migration by inducing the epithelial to mesenchymal transition. These results, taken together, indicate that activating CB_2 receptor could ameliorate wound healing by reducing inflammation, accelerating re-epithelialization, and attenuating scar formation. Thus, CB_2 receptor agonist might be a novel perspective for skin wound therapy.

1. Introduction

Skin wound healing is a complicated process proceeding by three stages that overlap in time and space: inflammation, new tissue formation, and remodeling. During this process, a series of cytokines, growth factors, extracellular matrix molecules, and various cells including leukocytes, fibroblasts, and keratinocytes coordinately interacted with each other to reform the epidermal barrier and dermal structure (Gurtner et al., 2008; Martin, 1997; Martin and Nunan, 2015).

The endocannabinoid system (ECS; comprising the endocannabinoids, G-protein-coupled cannabinoid receptors, biosynthetic and metabolism enzymes) in the mammals has attracted great attention in the last decades for its multiple functions both in health and diseases, and the ECS of the skin is recognized as a novel therapeutic target for cutaneous pathologies (Biro et al., 2009; Maccarrone et al., 2015). Cannabinoid 2 (CB_2) receptor is an important constituent of the ECS, which has been increasingly emphasized in recent years because it doesn't arouse psychotropic and cardiovascular side-effects (Yrjölä et al., 2015). CB_2 receptor is mainly distributed in peripheral and immune cells, including lymphocytes, NK cells, monocytes, macrophages, fibroblasts, keratinocytes, etc., and functionally involved in a series of pathologies referring to these cells (Battista et al., 2012; Kupczyk et al., 2009; Munro et al., 1993; Witkamp and Meijerink, 2014). Previous

* Corresponding author.
E-mail addresses: dwguan@mail.cmu.edu.cn, dwguan@aliyun.com (D.-W. Guan).
[1] These authors have contributed equally to the work.

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studies have revealed that CB_2 receptor interruption results in enhanced allergic inflammation in cutaneous contact hypersensitivity (Karsak et al., 2007), while activating CB_2 receptor reduces inflammation in murine pancreatitis (Michler et al., 2013), cystitis (Wang et al., 2014), traumatic brain injury (Amenta et al., 2014), and sepsis (Tschop et al., 2009). CB_2 receptor activation also attenuates pathological liver fibrosis in mice (Guillot et al., 2014; Mahmoud et al., 2014; Munoz-Luque et al., 2008), and exerts antifibrotic effect in bleomycin-induced dermal fibrosis (Akhmetshina et al., 2009). In addition, absence of CB_2 receptor improves keratinocyte differentiation and decreases proliferation in a tape-stripping-induced epidermal barrier abrogation model (Roelandt et al., 2012).

As aforementioned, CB_2 receptor is involved in the regulation of inflammation, fibrogenesis, and epidermal growth, which are the key biological processes during skin wound healing. Taking into account our previous finding that expression of CB_2 receptor was up-regulated during skin wound healing in mice (Zheng et al., 2012), we presumed CB_2 receptor might regulate skin wound healing, and pharmacomodulation of its activity might be a novel perspective for wound therapy. In the present study, we verified this hypothesis using Gp1a (highly selective CB_2 receptor agonist) and AM630 (highly selective CB_2 receptor antagonist) in mice skin excisional wounds in vivo and HaCaT keratinocytes in vitro. Our results indicate that activating CB_2 receptor could ameliorate skin wound healing by attenuating inflammation and fibrogenesis, as well as promoting re-epithelialization.

2. Materials and methods

2.1. Reagents and Abs

See details in Supplementary materials and methods.

2.2. Wound model and experimental grouping

Eight-weeks-old male BALB/c mice, each weighing 20–25 g, were used in this study. Wound model was established according to the instruction of Birch et al. (2005) with slight modification (see details in Supplementary materials and methods). Two 6 mm-diameter full-thickness dermal excisional wounds were created symmetrically on the dorsal skin of each mouse.

After surgery, mice were randomly divided into three groups and daily intraperitoneal injected with vehicle (5% DMSO/2% tween-80/physiological saline, 2.5 μl/g), Gp1a (3 mg/kg, dissolved in vehicle), or AM630 (3 mg/kg, dissolved in vehicle) respectively. Mice were euthanized at 12 h, 1 d, 3 d, 5 d, 7 d, 10 d, 13 d, 17 d and 21 d post-injury (6 mice at each time point). The original wound sizes (measured by tracing the dermal border of the wound) were analyzed. Six mice without surgery were used as control. Two 1 cm × 1 cm skin specimens centered on the wound were collected from each mouse. One specimen was used for morphometric, and another for Western blotting and real-time quantitative PCR (qRT-PCR) analyses.

Experiments were conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health published no.86-23, revised 1985), and the Guidelines for the Care and Use of Laboratory Animals of China Medical University.

2.3. Morphological analyses of wound re-epithelialization, inflammatory infiltration, fibrogenesis, and qRT-PCR and Western blotting assays

For morphometrical analyses, wound sections were per formed with H&E-staining; Masson's trichrome-staining; immunohistochemical staining of myeloperoxidase (MPO), F4/80, von Willebrand factor (vWF), proliferating cell nuclear antigen (PCNA), E-cadherin, and vimentin; as well as triple immunofluorescent staining of CD45/pro-collagen I/α-smooth muscle actin (SMA). mRNA expressions of monocyte chemotactic protein (MCP)-1, stromal cell-derived factor (SDF)-1, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β1, epidermal growth factor (EGF), and keratinocyte growth factor (KGF) were quantified by qRT-PCR. Protein expressions of interleukin (IL)-6, IL-1β, interferon (IFN)-γ, vascular endothelial growth factor (VEGF)-A, and pro-collagen I were assayed by Western blotting. See details in Supplementary materials and methods.

2.4. Cell culture and in vitro wound healing study

HaCaT cells were cultured as described previously (Zhao et al., 2013). Cells were treated with vehicle (0.1% DMSO), Gp1a (40 nM to 25 μM, dissolved in vehicle), and AM630 (40 nM to 25 μM, dissolved in vehicle) respectively. Wound scratch assay was performed. Cell viability was measured by cell counting kit (CCK)-8 assay. Protein expressions of involucrin, E-cadherin, vimentin, PCNA, and cleaved caspase 3 were measured by Western blotting. Co-localization of E-cadherin and vimentin was performed by immunofluorescent staining. See details in Supplementary materials and methods.

2.5. Statistical analysis

Data were expressed as means ± standard deviation and analyzed using SPSS for Windows 13.0. The one-way ANOVA with a Bonferroni *post hoc* test was used for data analysis. Difference associated with $P < 0.05$ was considered statistically significant.

3. Results

3.1. The general morphology changes after CB_2 receptor modulation

To investigate the regulatory function of CB_2 receptor during skin wound healing, we firstly detected the general morphology changes after the agonist/antagonist treatment. Macroscopically, CB_2 receptor activation by GP1a inhibited wound contraction after decrustation (Fig. 1A, B). The original wound sizes (measured by tracing the dermal border of the wound) in GP1a group were larger than vehicle group at 10–21 d post-injury. No significant difference was observed between AM630 and vehicle group. Microscopically, wound re-epithelialization was accelerated in GP1a group (Fig. 1C, D). The epithelial sheets were longer at 3–10 d, with larger cross-sectional areas at 5 d and 7 d post-injury. The inflammatory infiltration and granulation formation in the wound cavity were apparently decreased as compared with vehicle group. In AM630 group, wound re-epithelialization was delayed. The epithelial sheet length and cross-sectional area were both declined at 5 d and 7 d post-injury.

Keratinocyte proliferation continues for many days after complete re-epithelialization, resulting in hypertrophic epidermis that is about four times as thick as unwounded skin (Gallant-Behm et al., 2011). Similar appearances were observed in the present study. At 21 d post-injury, the epidermal thicknesses in three groups were all elevated as compared with the normal skin. However, the epidermal hypertrophy in GP1a group was greatly ameliorated when compared with vehicle group (Fig. 2A, C). In addition, the dermal scar in GP1a group was thinner and the collagen fibers were much slenderer (Fig. 2B, D). AM630 group showed similar epidermal and dermal morphologies with vehicle group.