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Inhibition of indoleamine 2,3-dioxygenase activity accelerates skin wound healing

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ABSTRACT

Skin wound healing is a complex process involving several stages that include inflammation, proliferation, and remodeling. In the inflammatory phase, pro-inflammatory cytokines and chemokines are induced at the wound site and, they contribute to the development of wound healing. These cytokines also induce indoleamine 2,3-dioxygenase (IDO1) activity; this is the rate-limiting and first enzyme in the L-tryptophan (TRP)-L-kynurenine (KYN) pathway. This study examined the effect of IDO1 on the process of skin wound healing. The expression of the *Ido1* mRNA was enhanced after creating a wound in wild-type (WT) mice. TRP concentration was simultaneously reduced at the wound site. The rate of wound healing in IDO1 knockout (IDO-KO) mice was significantly higher than that in WT mice. 1-Methyl-DL-tryptophan (1-MT), a potent inhibitor of IDO1, increased the rate of wound healing in WT mice. The administration of TRP accelerated wound healing in vivo and in an in vitro experimental model, whereas the rate of wound healing was not affected by the administration of KYN. The present study identifies the role of IDO1 in skin wound healing, and indicates that the local administration of 1-MT or TRP may provide an effective strategy for accelerating wound healing.

1. Introduction

The biological process of skin wound healing is not yet fully understood. The process has three stages; the inflammatory stage, proliferative stage, and remodeling stage [1]. These stages are critical for the appropriate progression of wound healing. In the inflammatory stage, the expression of pro-inflammatory cytokines, in particular IL-1β, IL6 and TNF-α, is up-regulated at the wound site [1]. A previous study demonstrated that the rate of wound healing was reduced in IL-6 knockout (KO) mice [2]. IL-1β and IL-6 have mitogenic and proliferative effects on keratinocytes, and contribute to the process of wound healing [3]. Moreover, IL-1β and TNF-α can promote the induction of keratinocyte growth factor, FGF-7, which can in tum promote re-epithelialization at the wound site [4]. Thus, these pro-inflammatory cytokines are directly and/or indirectly involved in the wound healing process, and their up-regulation is required for optimal skin wound healing.

Indoleamine 2,3-dioxygenase (IDO)1 is the rate-limiting and first enzyme in the L-tryptophan (TRP)-L-kynurenine (KYN) pathway that converts the essential amino acid TRP to N-formylkynurenine [5,6]. IDO activity in several tissues is highly induced under various pathological conditions including tumor progression, allograft rejection, liver disease, and viral or bacterial infection [7–10]. In particular, pro-inflammatory cytokines, including type I and type II interferons, can markedly induce IDO1 in epithelial, macrophage, and dendritic cells [9,11]. Pro-inflammatory cytokines such as IL-1β and IL-6 also enhance the expression of IDO1 in several cell types [12]. Many studies have demonstrated that IDO1 has powerful immunomodulatory effects. Both animal and human studies indicated that IDO-expressing cells' function as immunosuppressors is achieved by increasing T-lymphocyte tolerance [13]. IDO1-expressing cells deplete TRP from the extracellular milieu and secrete TRP metabolites (including KYN, 3-hydroxy-kynurenine, 3-hydroxyanthranilic acid, and quinolonic acid), which induce T-cell apoptosis and suppress immune responses in vitro [14,15].

During the wound healing process, an increase in IDO1 expression is predicted at the wound site because the expression of

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pro-inflammatory cytokines is up-regulated here after the creation of a wound. However, whether IDO1 expression is indeed up-regulated at the wound site, and the role of IDO1 in the wound healing process, is unclear. In the present study, we evaluated the role of IDO1 in the wound healing process using IDO1 knockout (IDO-KO) mice. We report that the inhibition of IDO activity at the wound site accelerated wound healing in vivo. The increase in IDO1 expression after the occurrence of a skin wound may reduce the rate of wound healing.

2. Materials and methods

2.1. Mice

C57BL/6J (H-2b) wild-type (WT) mice (age, 8–10 weeks; weight; 25–30 g) were obtained from Japan SLC Inc. (Shizuoka, Japan). IDO-KO mice on a C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, ME). All procedures were conducted in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and with the guidelines for the care and use of animals established by the Animal Care and Use Committee of Gifu University (Gifu, Japan).

2.2. Reagents

Monoclonal antibodys (Ab) specific for murine IL-6 and TNF-α were purchased from Bio X Cell (West Lebanon, NH). These antibodies were intraperitoneally administered on days 0 and 3 after creating wound. 1-Methyl-DL-tryptophan (1-MT), TRP, and KYN were purchased from Sigma–Aldrich (St Louis, MO). In some studies, 1-MT (200 μg), TRP (200 μg), or KYN (200 μg) were added to 100 μl of white petrolatum, and the formulation was then heated to 65 °C and vigorously mixed to emulsify the components.

2.3. Murine in vivo wound repair model

Mice were anesthetized, and the skin on the back was cleaned, shaved, and sterilized with betadine solution followed by 70% ethanol. A 6-mm full-thickness (including the panniclulus carnosus) excisional biopsy was taken using a biopsy punch (Kai Industries Co., Gifu, Japan) from the right- and left-upper paravertebral region of each animal. The biopsy sites were covered with non-adhesive sterile gauze. In some experiments, individual biopsy sites were coated with 100 μl of white petrolatum containing 1-MT, TRP, or KYN, before being covered with non-adhesive sterile gauze. Mice were wrapped with a form-fitting bandage to further protect the biopsy sites. Wounds were checked daily for infection and at the same time, they were photographed. Changes in wound contraction over time were calculated using the Image J software (version 1.37; NIH, Bethesda, MD). For each treatment tested, results were averaged for a minimum of six animals/treatment group.

2.4. Measurements of tryptophan

Tryptophan levels were measured by using high-performance liquid chromatography (HPLC) with a fluorescence spectrometric detector (Hitachi, Tokyo, Japan) as described previously [16].

2.5. Western blot analysis

Protein (20 μg) from the lysate of wound sites was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking non-specific reaction with 5% skim milk, the membrane was incubated with anti-IDO and anti-GAPDH antibodies for 60 min at room temperature and subsequently incubated with peroxidase-labeled anti-mouse or -rabbit IgG antibody for 60 min at room temperature. Immunoreactive protein bands were visualized with ECL plus (GE Healthcare UK Ltd., England).

2.6. Real-time reverse transcription (RT)–PCR

Tissue from the biopsy site was excised, and total RNA was isolated using ISOGEN II reagent (Nippon Gene, Tokyo, Japan), and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The resulting cDNA was used as a template for real-time RT-PCR with primer-robe sets for IDO1, IDO2, TDO, IL-6, TNF-α, EGF, VEGF, TGF-α, and 18S rRNA, the latter as an internal control, according to the manufacturer's instructions (TaqMan® Gene Expression Assays and Universal PCR Master mix; Applied Biosystems). Real-time RT-PCR was carried out using the Light-Cycler® 480 system (Roche Diagnostic Systems, Basel, Switzerland). The expression of MMP2 and MMP9 was analyzed using the Light-Cycler® 480 system in conjunction with a KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Wilmington, MA).

2.7. In vitro scratch assay

Timed pregnant WT and IDO-KO mice were euthanized, and the embryos were harvested at embryonic day 14.5. Mouse embryonic fibroblast (MEF) cultures were prepared using standard techniques. Cells were maintained in complete DMEM medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine (Gibco®, Invitrogen, Life Technologies, Grand Island, NY). Scratch assays were carried out as previously described [17,18]. Cultured MEFs from WT and IDO-KO mice were grown in 12-well plates. When the cells had reached confluence, a scratch was made across the cell monolayer with a yellow pipette tip (approximately 0.5 mm width). The culture medium was changed to RPMI 1640 or RPMI 1640 without TRP (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) immediately after creating a scratch wound. After 16 h, five representative images of the scratched areas were photographed for each experimental condition. In each photographic field, the distances from the margin of the lesion to each of the 10 cells that had migrated the furthest away from it were measured, and the mean value of the distances was considered to represent the mobility of the cells in each culture dish.

2.8. Statistical analysis

Values are expressed as the mean ± SE. Differences between experimental and control groups were analyzed by the Kruskal–Wallis test followed by Scheffe's test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Up-regulation of IDO expression and activity in wound site

The first stage in the skin wound healing process is the inflammatory phase, in which various pro-inflammatory cytokines are up-regulated at the wound site. A previous study demonstrated that inflammatory cytokines such as IL-1β and IL-6 could induce IDO1 expression [12]. We measured the expression of IDO1 mRNA at the wound site by real-time RT-PCR (Fig. 1A). IDO1 mRNA expression was significantly increased at the wound site in WT mice, but it was not detected in IDO-KO mice. Western bolt analysis revealed that IDO protein level in wound sites were increased 1 d and 3 d after creating wound (Fig. 1B). Next, to evaluate the IDO activity in wound sites, we measured TRP levels in the skin tissue from the wound site (Fig. 1C). TRP levels in the skin tissue were significantly reduced after creating wound. Recent reports demonstrated that TRP to KYN metabolism is controlled by IDO1, IDO2, and tryptophan 2,3-dioxygenase (TDO). Therefore, we also measured the mRNA expression of IDO2 and TDO at the wound site (Fig. 1D). The expression of IDO2 mRNA in wound sites in WT and IDO-KO mice were similarly increased at 1 d after creating the wound. The expression of TDO mRNA was not affected by creating wound in the skin of WT and IDO-KO mice.

3.2. Acceleration of skin wound healing in IDO-KO mice

To evaluate the role of IDO1 in the skin wound healing process, we created a wound in WT and IDO-KO mice and took sequential photographs of the wound site over subsequent days (Fig. 2A). The rate of wound healing in IDO-KO mice was significantly higher than that in WT mice up to day 6 (Fig. 2B). We next evaluated the mRNA expression of pro-inflammatory cytokines and growth factors at wound sites of WT and IDO-KO mice. A previous study demonstrated that the expression of pro-inflammatory cytokines, including IL-6 and TNF-α was increased at wound sites, and these cytokines were involved in the wound healing process [1]. EGF, VEGF, and TGF-α are also implicated in wound healing process [19]. As shown in Fig. 2C, the expression of IL-6 and TNF-α is enhanced after creation of the wound in WT and IDO-KO mice. Moreover, the up-regulation of these cytokines expression in IDO-KO mice occurred at an earlier time point than in WT mice. The expression of EGF mRNA was also significantly increased in the wounded IDO-KO mice. A previous study demonstrated that MMPs are required in the normal wound healing process [20]. We measured the expression of MMP2 and MMP9 at the wound site and detected no difference in the expression of MMP2 between WT and IDO-KO mice (Fig. 2C). MMP9 expression was slightly enhanced in IDO-KO