Differential Regulation of Integrin α5 and β4 in Normal and Psoriatic Epidermal Keratinocytes

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Psoriasis is a chronic skin inflammation, characterized by impaired differentiation, hyperproliferation of keratinocytes involving pro-inflammatory factors interleukin (IL)-13/17A, tumor necrosis factor (TNF)-α, interferon (IFN)-γ. Among the integrin family, α5 is important for blood vessel formation, and β4 for proliferation, differentiation of keratinocytes. To investigate the expression and regulation of integrin α5 and β4 in psoriatic keratinocytes. Skin biopsies were obtained from 14 psoriatic patients and 12 normal volunteers. We compared the immunolocalization and regulation of α5 and β4 between the psoriatic and normal ones, before and after incubation with MEK/ERK pathway inhibitor U0126 by immunohistochemistry and western blot separately. Immunohistochemistry showed psoriatic keratinocytes had higher α5 than normal ones. According to western blot, IL-17A and IL-13 increased normal keratinocytes' α5 and β4 respectively, but psoriatic keratinocytes were the exact opposite. Incubated with U0126, normal keratinocytes' α5 was enhanced by the 5 cytokines; while IL-13/17A, IFN-γ suppressed β4. Psoriatic keratinocytes' α5 was increased by IL-13/17A, decreased by IFN-γ; but β4 increased by IL-17A, IFN-γ. IL-13/17A, TNF-α, IFN-γ regulate α5 and β4 through ERK pathway whether normal or psoriasis. The normal and psoriatic keratinocytes respond to the same cytokines differently.

Keywords: Integrin. Integrin α5. Integrin β4. Psoriasis. Erk pathway. same cytokines differently.

INTRODUCTION

Integrins, a large group of adhesion molecules distributed in many tissues, are heterodimers composing of a regulatory α-subunit and a signal transducing β-subunit.(Takada, Ye, Simon, 2007) These specific but diverse heterodimers play critical roles in cell growth and function.(Cox, Brennan, Moran, 2010) It has been shown that integrins are involved in health and certain diseases, such as inflammation, cancer, autoimmune disorders and thrombosis.(Iannone et al., 2005) Till now, 18 α-subunits and 10 β-subunits have been identified, which formed at least 24 integrins.(Cox, Brennan, Moran, 2010, Papusheva, Heisenberg, 2010) β4 integrin plays multiple roles in cellular polarity, proliferation, differentiation, migration, senescence, macroautophagy and survival of keratinocytes. (Nikolopoulos et al., 2005, Raymond et al., 2005, Wang et al., 2012) α5 integrin, a fibronectin-binding receptor, is important for blood vessel formation (Bhaskar et al., 2007) and cellular proliferation (Deng, Wan, Yan, 2019). In normal epidermis, staining for α5 is weaker and more diffuse than for the other subunits.(Rippa et al., 2013)

Psoriasis, a common, chronic, inflammatory and proliferative skin disease, is characterized by impaired differentiation, hyperproliferation of epidermal keratinocytes, disturbed keratinization and aberrant activation of T lymphocytes.(Nestle, Kaplan, Barker, 2009, Schon, Boehncke, 2005) Previous studies have shown that integrin α5 is highly increased(Koivisto et al., 2014), while β4 remained basally restricted(Malakou et al., 2018) in psoriasis determined by flow cytometric analysis and, immunofluorescence separately. Here, we further defined the regulation of integrin α5 and β4 in normal and psoriatic epidermal keratinocytes.
MATERIAL AND METHODS

Material

Human subjects

Volunteers enrolled were divided as normal volunteers and psoriatic patients.

The inclusion criteria of psoriatic patients were the following: (1) patients with moderate to severe plaque psoriasis; (2) patients between 18-65 years old, diagnosed with plaque psoriasis for at least 6 months before the beginning of the study; (3) patients with PASI > 12, and skin lesion area takes up > 10% of total body surface area; (4) patients with no history of biologic agents treatment.

Exclusion standards were: (1) patients with erythrodermic psoriasis or psoriatic arthritis or psoriasis pustulosa or psoriasis guttate; (2) patients with severe and uncontrollable local or systematic acute/chronic infections; (3) patients with active or potential tuberculosis or asthma; (4) patients with malignancy history; (5) patients with other severe systematic disease; (6) patients who were prescribed with drugs or biologic agent; (7) patients who received immunosuppressor within 1 month or (8) psoriasis systematic treatment or phototherapy within 1 month or (9) psoriasis topical agents treatment.

Volunteers applying for normal volunteers accepted medical examination. Only those confirmed without any acute or chronic diseases were qualified.

Skin biopsies were taken from normal volunteers (n=12, including males and females, aged 19-71, mean age 42.3±17.2) and lesional skin of psoriasis patients (n=14, including males and females, aged 21-73, mean age 40.8±13.2) with moderate to severe chronic plaques. A medication-free period of 1 month was required in psoriasis group before the procedures. Informed consents were obtained from all donors. After skin biopsy, the specimen was divided into two parts. One part was fixed overnight in 10% buffered formalin at room temperature, after which they were cleared and embedded into paraffin. The other was immersed in 0.5% dispase (Gibco, Invitrogen, USA) immediately. This study was performed under the guidelines of Zhejiang University Institutional Review Board.

Methods

Immunohistochemical staining

Immunohistochemical staining was performed using a standard procedure. Sections from normal and psoriatic skin specimens were formalin-fixed and paraffin-embedded. Then cut into 4-5µm sections and placed on clean electrostatically charged glass slides. After deparaffinization and rehydration of the sections, all slides were boiled for antigen unmasking in 10mM sodium citrate buffer pH 6.0 for 20 minutes at a subboiling temperature. Then, the slides were incubated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity and were immersed with 10% fetal bovine serum for 1 hour at room temperature in a humidified chamber. The sections were applied overnight with primary antibody and then secondary antibody (antibodies against β-actin, integrin α5, integrin β4 were from Santa Cruz, CA, U.S.A; antibodies against interleukin-1β, interleukin-13, interleukin -17A, tumor necrosis factor-α, transforming growth factor-β1, endothelin-1, interferon- γ and vascular endothelial growth factor-165 were from PeproTech, London, UK) for 2 hours. DAB kit (Vector Laboratories, Burlingame, CA, U.S.A.) were used for staining, then sections were dehydrate and mounted by coverslips.

Primary cell culture

Skin specimens were immersed in 0.5% dispase (Gibco BRL, Rockville, MD, U.S.A.) overnight at 4ºC, and then separated into epidermis and dermis. The epidermis was incubated in 0.25% trypsin (Gibco BRL, Rockville, MD, U.S.A.) for 10 minutes at 37ºC. After digestion, fetal bovine serum (Gibco BRL, Rockville, MD, U.S.A.) were used to neutralize the enzyme activity. The cell suspension was centrifuged at 500g for 5 minutes and pour off the supernatant. We applied
defined keratinocyte serum free medium (KSFM) supplemented with keratinocyte growth factor (KGF) to plant keratinocytes in a 75 cm² flask in humidified incubator with 5% CO₂ at 37°C. Keratinocytes of 2-3 generation were used in our study. Protein extraction and western blot was performed following a standard procedure.(Man et al., 2013) The mouse monoclonal antibody against integrin α5, β4, and β-actin were obtained from Santa Cruz technology (Santa Cruz, CA, USA).

**Inhibitor and cytokine treatment**

Keratinocytes were seeded into 6-well plates and grew until confluent, then incubated for 24 hours in serum-free, non-supplemented medium prior to treatment of 25ng/ml interleukin-1β (IL-1β), IL-13, IL-17A, tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), endothelin-1 (ET-1), interferon-γ (IFN-γ), or vascular endothelial growth factor-165 (VEGF₁₆₅) (PeproTech, London, UK) for a further 24 hours. For the inhibitor study, the cells were pretreated with 20μmol/L U0126 (from Sigma-Aldrich, USA) for 2 hours, then stimulated with 25ng/ml IL-1β, IL-13, IL-17A, TNF-α, TGF-β1, ET-1, IFN-γ, or VEGF₁₆₅ for 24 hours or not. Control cells received no inhibitors or cytokines.

**Western blot**

Protein extraction and preparation were performed following a standard procedure.(Man et al., 2013) The protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels and transferred to a nitrocellulose membrane. The blots were incubated overnight at 4°C with mouse monoclonal antibody against integrin α5, β4 and β-actin (Santa Cruz technology, Santa Cruz, CA, USA). Proper HRP-conjugated secondary antibodies (West Grove, PA, USA) were applied to the blots for 2 hours at room temperature and signals were detected by enhanced chemiluminescence (Millipore, Billerica, U.S.A) on X-ray films.

**RESULTS**

**Immunolocalization and expression of integrin α5 and β4 in normal and psoriatic skin**

The immunohistochemical analysis revealed immunolocalization and expression of integrin α5 and β4 protein in normal and psoriatic skin (Figure 1). Integrin α5 was shown to be localized in the whole skin, except for squamous layer in both normal and psoriatic epidermis. In normal epidermis, the expression of α5 was very weak, but showed a relative concentration at the basal layer. In psoriasis, the distribution of α5 was almost homogenous in basal and prickle keratinocytes, and the immunoreaction was much stronger than that in normal epidermis (Figure 1a). Integrin β4 was detected in the membrane of normal epidermal keratinocytes from basal to granular layers, while β4 being absent in the basal and parakeratosis layer of psoriatic epidermis (Figure 1b).

To further confirm the expression of integrin α5 and β4, normal and psoriatic keratinocytes were cultured in vitro and western blot was performed, which showed bands corresponding to integrin α5 and β4 respectively (Figure 1c). Furthermore, expression of α5 in normal keratinocytes was less than that in psoriatic keratinocytes significantly (P<0.01), while no significant difference of β4 being observed (*, p<0.05, Figure 1c).
Regulation of integrin α5 and β4 in normal and psoriatic keratinocytes by IL-13, IL-17A, ET-1, TNF-α, IFN-γ, IL-1β, TGF-β1 and VEGF165

Western blot was conducted to compare how IL-13, IL-17A, ET-1, TNF-α, IFN-γ, IL-1β, TGF-β1 and VEGF165 regulated integrins α5 and β4 in normal and psoriatic keratinocytes. In normal keratinocytes, α5 was upregulated by IL-13, ET-1, especially by IL-17A, TNF-α and IFN-γ (Figure 2a), not altered by TGF-β1 and VEGF165, but decreased by IL-1β (Figure 3a). β4 was increased by IL-17A, ET-1, TNF-α, particularly by IL-13, IFN-γ, IL-1β, TGF-β1 and VEGF165 in normal keratinocytes (Figure 2a & 3a). However, in psoriatic keratinocytes, α5 was decreased by IL-13, ET-1, TNF-α, IFN-γ, VEGF165, especially IL-17A, not being altered by IL-1β and TGF-β1 (Figure 2b & 3b). β4 was decreased by IL-17A and TNF-α, not being altered by IL-13, ET-1, IFN-γ, IL-1β, TGF-β1 and VEGF165 (Figure 2b & 3b).
FIGURE 2 - Western blot determining regulation of integrin α5 and β4 by 25ng/ml IL-13, IL-17A, ET-1, TNF-α and IFN-γ in normal and psoriatic keratinocytes. (a) regulation in normal keratinocytes. (b) regulation in psoriatic keratinocytes. Columns under corresponding bands show relative quantitation of optical intensity of integrin α5 or β4 to β-actin. The number of experiments: 2.

FIGURE 3 - Western blot determining regulation of integrin α5 and β4 by 25ng/ml IL-1β, VEGF_{165} and TGF-β1 in normal and psoriatic keratinocytes. (a) regulation in normal keratinocytes. (b) regulation in psoriatic keratinocytes. Columns under corresponding bands show relative quantitation of optical intensity of integrin α5 or β4 to β-actin. The number of experiments: 2.
**DISCUSSION**

Previous studies have shown that integrin α5 is weakly or not expressed by epidermal keratinocytes under homeostatic conditions. (Rippa et al., 2013) However, in psoriasis, its expression was highly upregulated and showed a focal cytoplasmic positivity in the spinous layer. (Filoni et al., 2018) Our immunohistochemical data confirmed that α5 was weakly expressed in normal epidermis but strongly in psoriatic epidermis. This was further confirmed by Western blot, which is consistent with the immunohistochemical results. The increased expression of α5 may contribute to the hyperproliferative state. (Koria, Andreadis, 2007) which was confirmed in the transgenic mice expressing α5β1, there were keratinocyte hyperproliferation and abnormal terminal differentiation and an immune response in the skin, all of which are features of psoriasis. (Li et al., 2017)

Being a composition of the hemidesmosome and intermediary filaments, integrin β4 may play a
functional role in keratinocyte attachment. There are also controversial results of integrin β4 in normal and psoriatic epidermis. In normal adult human epidermis, β4 showed a linear staining pattern polarizing to the basal surface juxtaposed to the dermal-epidermal basement membrane. In contrast, in fetal skin, the staining patterns demonstrated the presence of β4 surrounding the entire cell surface of both basal and suprabasal keratinocytes. (Levy et al., 2000) In psoriatic epidermis, β4 subunit was distributed on the basal and lateral surface of basal keratinocytes, and around contiguous suprabasal keratinocytes. (Roberson, Bowcock, 2010) However, in our study, β4 was not localized to dermal-epidermal basement membrane, but distributed in the membrane of normal epidermal keratinocytes from basal to granular layers. Furthermore, in psoriatic epidermis, β4 was only detected in prickle keratinocytes, not in the basal and parakeratosis layers. This difference might be due to recognition variance between epitopes and antibodies. As the exact location of β4 in psoriatic skin and the underlying significance of its absence in psoriatic basal layer remains unclear, further investigation is expected.

There are dozens of cytokines mediated in onset and development of psoriasis. Among the complicated cytokine networks, the importance of IL-23/Th17 axis is well ahead. (Boehncke, Schon, 2015) TNF-α, IFN-γ are elevated in psoriatic skin. TNF-α promotes infiltration of T cells, and also acts as a regulator of the IL-23/Th17 axis. (Deng, Chang, Lu, 2016) IFN-γ activates antigen-presenting cells early in the psoriatic cascade, regulates polarization of regulatory T cells by a combination with TGF-β and IL-4. IFN-γ also promotes the release of IL-1β, chemokines, and adhesion molecules from dendritic cells (DCs). (Deng, Chang, Lu, 2016) IL-17A stimulates the production of chemokines and antimicrobial peptides by keratinocytes. Keratinocytes in turn promote Th17 cell recruitment and produce more IL-17, resulting in a positive feedback loop that perpetuates the inflammatory response of psoriasis. (Deng, Chang, Lu, 2016) ET-1 is produced by psoriatic keratinocytes and suppresses apoptosis. (Borska et al., 2017) Plasma levels of ET-1 are elevated in psoriasis. ET-1 polarizes the DCs response toward Th17 differentiation and may augment the persistent course of psoriasis. (Nakahara et al., 2018) VEGF is a potent endothelial cell mitogen playing a key role in angiogenesis in psoriasis, and it has been shown that VEGF expression is increased in lesion psoriatic skin. (Theoharides et al., 2010) IL-13 has been found to be a risk allele for psoriasis. It is also upregulated in psoriatic lesions. (Tsai, Tsai, 2017)

Our results revealed that normal and psoriatic keratinocytes had different responses to the same cytokines in producing integrin α5 and β4. IL-13, IL-17A, ET-1, TNF-α and IFN-γ upregulated α5 and β4 expression in normal keratinocytes; but decreased α5 and β4 in psoriatic keratinocytes, except for ET-1. In addition, IL-1β, TGF-β1 and VEGF did not show obvious effects on α5 but strongly increased β4 in normal keratinocytes. However, in psoriatic keratinocytes, VEGF decreased α5, and β4 was not altered by IL-1β, TGF-β1 and VEGF. Identical cells’ reactions to the same cytokine may vary depending on physiological or pathological conditions. In other words, the dysfunctional cells and normal cells may respond differently, sometimes even reversely, to the same regulator.

We further investigated how ERK1/2 interacts with these cytokines. The MAPK/ERK pathway regulates various cellular activities, including proliferation, differentiation, survival, and apoptosis. (Kim, Choi, 2010) The activity of the ERK1/2 MAPKs was demonstrated increased in lesional psoriatic skin compared with non-lesional skin. (Rodrigues et al., 2019) 10 µM of U0126 (1,4-diamino-2,3-dicyano-1,4-bis-(2-aminophenylthio)butadiene) selectively inhibits MEK1/2, blocking signal transduction from MEK1/2 to ERK1/2. (Ahnstedt et al., 2015) We found the blockade of ERK1/2 reversed the effects of several cytokines and also sustained others’. In normal keratinocytes, U0126 greatly decreased α5 but didn’t change the increasing effect of IL-13, IL-17A, ET-1, TNF-α, IFN-γ on α5. In psoriatic keratinocytes, with the addition of U0126, IL-13, IL-17A increased α5, while the downregulation of α5 by ET-1 and TNF-α was eliminated. IFN-γ decreased α5 whether ERK1/2 was inhibited. Adding U0126 solely, psoriatic keratinocytes’ expression of α5 wasn’t altered. Consequently, in normal keratinocytes, ERK1/2 pathway, IL-13, IL-17A, ET-1, TNF-α, IFN-γ can activate α5 expression, but the 5 cytokines exert their effects independently from ERK1/2.
In psoriatic keratinocytes, ERK1/2 inhibited IL-13, IL-17A, ET-1 and TNF-α from raising α5. IFN-γ depleted α5 without ERK1/2 involved. For β4, U0126 didn’t impact upon its level in normal keratinocytes but largely suppressed it in psoriatic ones. In normal keratinocytes, with U0126, IL-13, IL-17A, IFN-γ lowered β4 level, opposite to non-U0126 groups. Elevating effect of ET-1 and TNF-α maintained. In psoriatic keratinocytes, IL-13 and IL-17A enhanced β4 level in the background of ERK1/2 inhibitor. ET-1, TNF-α and IFN-γ showed no effect. Comparing the results of U0126 groups and non-U0126 groups, we inferred that ERK1/2 pathway participated in IL-13, IL-17A, IFN-γ’s increasing normal keratinocytes’ expression of β4, not in ET-1 or TNF-α’s. ERK1/2 may play an inhibitory role in IL-13-, IL-17A- and TNF-α-mediated production of β4 in psoriatic keratinocytes.

In summary, integrin α5 and β4 are both expressed in normal and psoriatic keratinocytes, and α5 being upregulated in psoriatic keratinocytes. Keratinocytes may respond differently, sometimes even reversely, to the same cytokines under physiological or pathological circumstances. Signals, such as ERK1/2, may also have a different role under pathological state.

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