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Activation of RhoA, Smad2, c-Src, PKC-bII/d and JNK in atopic dermatitis

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ABSTRACT

Atopic dermatitis is a multifactorial skin disease characterized by chronic and relapsing inflammation whose pathogenesis is incompletely understood. We found that the expression of TGFβR1 and the activation of SMAD2, RhoA, JNK, PKC-bII/d and c-Src were upregulated in the infiltrated inflammatory cells, fibroblasts and vasculatures in the dermis and epidermis. In addition, increases in the expression of TGFβR1 and phosphorylation levels of JNK and c-Src were positively correlated with the inflammatory progression of atopic dermatitis severity.

INTRODUCTION

Atopic dermatitis is a skin disease associated with chronic and relapsing inflammation and heterogeneous clinical symptoms that affects 10–20% of children and 1–3% of adults worldwide.1 Many factors contribute to the development of atopic dermatitis, including immune dysregulation, skin barrier impairment, microbial infection, genetic mutations and pharmacological, psychological and environmental factors.[2] The main pathological phenotype of atopic dermatitis is the proliferation and infiltration of inflammatory cells, including mast cells, basophils, eosinophils, T cells and dendritic cells, which result in epidermal hyperplasia and homeostasis abnormalities.[2] Both innate and adaptive immune systems are dysregulated in atopic dermatitis, among which T helper (Th) 2 cells mediate a predominant inflammatory response in atopic dermatitis.[2] Th2 cytokines, such as interleukin-5, 13, 10 and 31, and chemokines are elevated in acute and chronic atopic dermatitis.[2] Th2 hyperproliferation also leads to the increase of immunoglobulin E levels in atopic dermatitis, which results in the degranulation of mast cells upon exposure to allergens.[2] The present management of atopic dermatitis includes corticosteroids, antihistamines, UV therapy and immunosuppressants. However, treatment outcomes for moderate and severe atopic dermatitis are unsatisfactory due to poor efficacy and adverse effects, and there is a medical need for more specific targeted approaches.

In this study we attempted to investigate the key signaling molecules reflecting the progression of inflammation in atopic dermatitis.

METHODS

The protocol for this clinical investigation was approved by the Ethics Committee of the Third XiangYa Hospital, Central South University. Standard criteria and guidelines were used to diagnose atopic dermatitis.[1] Altogether 11 patients with moderate to severe atopic dermatitis were enrolled in this study after providing their informed consents (see Table 1). The severity of the atopic dermatitis was determined for each patient using the scoring atopic dermatitis calculator (SCORAD). Ten normal skin biopsy specimens retrieved from the skin biopsy tissue bank at our department served as normal controls. All biopsy samples were de-identified during the study. Punch biopsies (4 mm) were generally obtained from the most active eczematous skin lesion, fixed in buffered 4% formalin and embedded in paraffin. Paraffin sections with 6-μm thickness were cut and collected. Antigen retrieval was performed in 10 mmol/L sodium citrate buffer (pH 6.0) at 97°C for 3 h. The sections were then incubated in a humidified chamber overnight at 4°C with the following primary antibodies and dilutions: anti-TGFβR1 (Abclonal, Cambridge, MA, USA; 1:500), anti-p-SMAD2 (Abclonal, 1:500), anti-p-RhoA (Santa Cruz Biotech, Dallas, TX, USA; 1:100), anti-p-PKC-bII/d (Santa Cruz Biotech; 1:50), anti-p-JNK (Santa Cruz Biotech; 1:500) and anti-p-c-Src (Y418) (Abclonal; 1:500). Biotinylated anti-mouse and anti-rabbit secondary antibodies were incubated with the sections for 1 h at room temperature after the reaction to the primary antibodies. An indirect biotin avidin diaminobenzidine system (Dako, Glostrup, Denmark) was used for detection.

The immunohistochemical score was evaluated by two independent blinded researchers from a different research group in the same institute, using a system previously reported, with minor modifications.[3] Briefly, six scales were used to evaluate the immunoreactive intensity scores: 0 (-), 1 (±), 2 (+), 3 (++), 4 (+++) and 5 (++++ and above). Four scales were used to assess the percentage scores of immunoreactive cells: 1 (<25%), 2 (25–50%), 3 (51–75%) and 4 (>75%). The final immunoreactivity scores for each antibody were estimated by multiplying the immunoreactive intensity and percentage scores. Spearman's correlation analysis was used to evaluate the relationship between the immunohistological scores and SCORAD. The immunohistological scores in the atopic dermatitis lesions were compared with normal skin using a paired t-test. A P value < 0.05 was considered to be significant.

Table 1 Clinical description of patients with atopic dermatitis (AD) and biopsy samples

Gender	Age	AD diagnosis	Biopsy sites	SCORAD	Serum IgE (IU/mL)
M	63	Moderate	Trunk	41	1280
F	22	Severe	Elbow	59.6	n.a.
M	33	Moderate	Upper middle back	45	n.a.
M	13	Severe	Lower extremity	63.1	1742
M	28	Severe	Abdomen	72.1	1849
F	21	Severe	Popliteal fossa	67	1457
M	23	Severe	Upper extremity	65.2	417
F	28	Severe	Lower extremity	64.2	561
M	16	Severe	Lower extremity	71	1729
M	72	Severe	Lower extremity	66	2007
M	67	Severe	Neck	74	1572

F, female; IgE, immunoglobulin E; M, male; SCORAD, scoring atopic dermatitis.

RESULTS

In normal skin, the expression level of TGFβR1 and phosphorylation levels of RhoA, Smad2, c-Src, PKC-bII/d and JNK were negative or mild in the epidermis and dermal blood vessels (Fig. 1a,c,e,g,i,k). In the atopic dermatitis lesions, epidermal hyperplasia and mononuclear cell infiltrate, mainly lymphocytes, were prominently observed (Fig. 1b,d,f,h,j,l). Perivascular inflammatory cell infiltrate was also frequently observed in the hyperplastic epidermis (Fig. 1b,d,f,h). TGFβR1 had a strong immunoreactive signal in the hyperplastic epidermis, dermal blood vessels, fibroblasts and infiltrate (Fig. 1b). Smad2 and RhoA displayed ubiquitous activation in keratinocytes and infiltrated inflammatory cells, fibroblasts and dermal blood vessels (Fig. 1d,f). The activation of JNK, PKC-bII/d and c-Src was mainly found in the infiltrated inflammatory cells, fibroblasts and dermal blood vessels, but it was less evident in keratinocytes (Fig. 1h,j,l). In addition, PKC-bII/d was strongly activated in Langerhans cells in the atopic dermatitis epidermis (Fig. 1j).

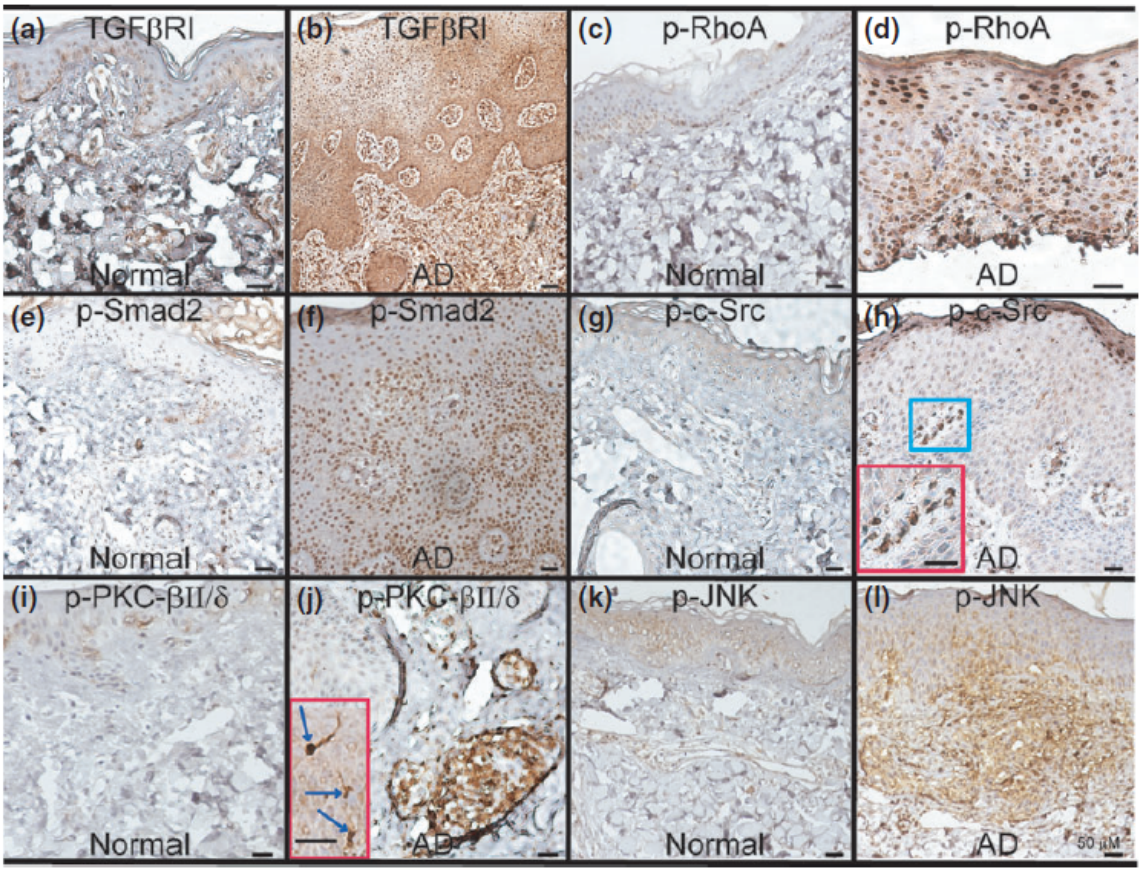

Figure 1 Upregulation of TGFβR1, p-RhoA, p-Smad2, p-c-Src, p-PKC-bII/d and p-JNK in atopic dermatitis lesions. The expression of (a,b) TGFβR1, (c,d) levels of p-RhoA (e,f), p-Smad2 (g,h), p-c-Src, (i,j) p-PKC-bII/d and (k,l) JNK were determined by immunohistological scores in atopic dermatitis lesions compared with normal skin. (h) The red inset shows a higher magnification from the blue boxed area in (h). (j) The red inset shows p-PKC-bII/d-immunoreactive Langerhans cells (indicated by purple arrows) in the epidermis. Scar bar: 50 lm.

The immunohistological scores of TGFβR1, p-Smad2, p-RhoA and p-c-Src increased in the atopic dermatitis epidermis and dermal infiltrates compared with normal skin (Fig. 2a, $P < 0.05$). The immunohistological scores of p-PKC-bII/d and p-JNK were upregulated in atopic dermatitis dermal infiltrates compared with normal skin (Fig. 2a, $P < 0.05$). In addition, the upregulation of TGFβR1 in the atopic dermatitis lesional epidermis and dermal infiltrate were positively correlated with SCORAD scores (Fig. 2b,c). The phosphorylation levels of c-Src and JNK in infiltrated inflammatory cells, fibroblasts and dermal blood vessels also showed positive correlations with SCORAD scores (Fig. 2d,e). We found no significant correlation between the immunohistological scores intensity of p-RhoA, p-Smad2 and p-PKC-bII/d and SCORAD scores.

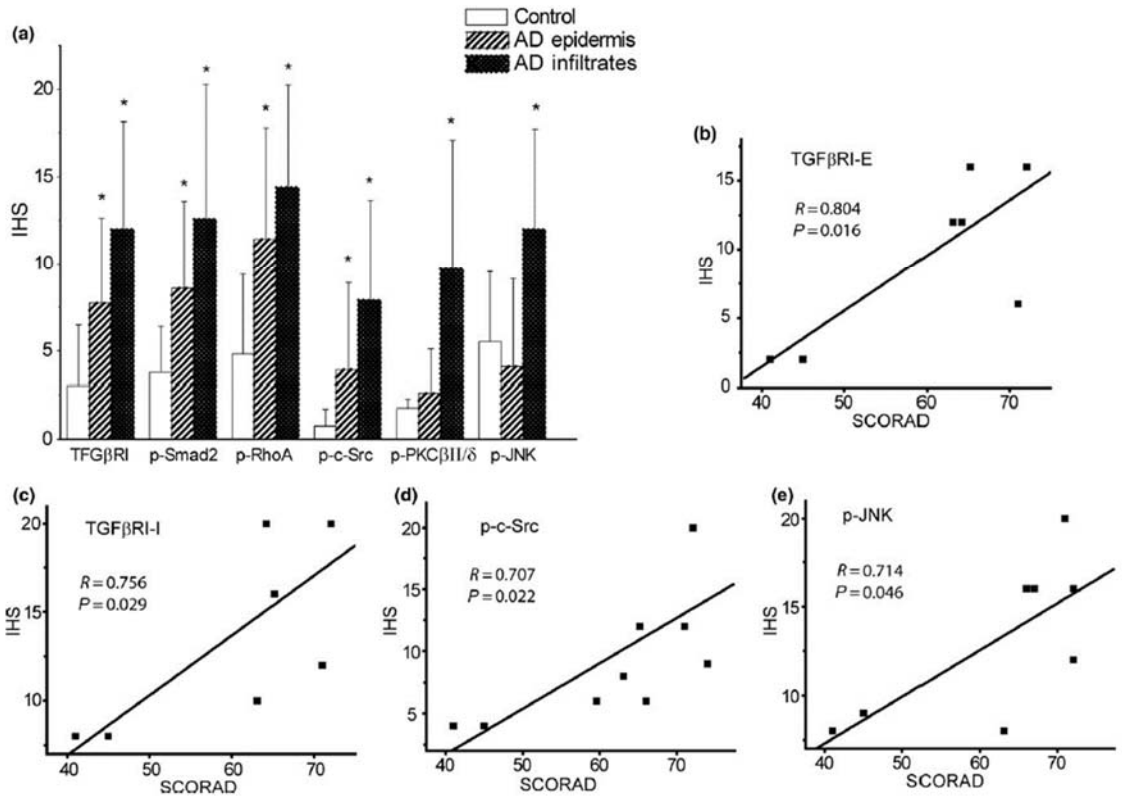

Figure 2 (a) Relative immunohistological scores (IHS) of TGFβR1, p-RhoA, p-Smad2, p-c-Src, p-PKC-bII/d and p-JNK in atopic dermatitis epidermis and dermal infiltrates. (b–e) *P < 0.05 compared with normal skin. The correlations of immunohistological scores of TGFβR1 in the (b) epidermis and (c) inflammatory infiltrate (d) p-c-Src and (e) p-JNK in dermal infiltrated cells with patients' scoring atopic dermatitis. SCORAD, scoring atopic dermatitis.

DISCUSSION

Immune dysregulation is a major cause of the development of atopic dermatitis.2 The proliferation and infiltrate of inflammatory cells, including mast cells, basophils, eosinophils, T cells and dendritic cells, result in epidermal hyperplasia, homeostasis abnormalities and skin fibrosis in atopic dermatitis lesions.[2] TGFβ signalling, in interaction with many other signalling systems, has dichotomous roles in immune suppression or proinflammation in the pathogenesis of atopic dermatitis.4,5 On one hand, it seems to facilitate progression of this disease. For example, TGFβ is very potent in stimulating the migration and infiltration of immune cells and fibroblasts that favour the development of atopic dermatitis.[5] In addition, it is required for the development and activation of Langerhans cells in the skin, the crucial immune cells mediating inflammation in the epidermis.[5] On the other hand, TGFβ may exhibit an inhibitory effect on a variety of immune cells during the development of atopic dermatitis. For example, TGFβ signalling can prevent mast cell granulation, thus alleviating atopic dermatitis.[4] TGFβ can regulate the development of T-regulatory cells, which will subsequently suppress the proliferation of effector T cells.[5] Th cells play critical roles in the

pathogenesis of atopic dermatitis. TGFβ can inhibit the differentiation of Th1 and Th2 cells through the suppression of T-bet/Stat4 and GATA-3/NFAT mechanisms.[6] In addition, TGFβ signalling is a crucial mediator of skin fibrosis.[4,5] The mRNA level of TGFβ in atopic dermatitis lesions does not show a significant change, compared with normal skin.[7] However, its protein level seems more abundant in epidermal keratinocytes and dermal infiltrates in atopic dermatitis lesions than in normal skin,[8] indicating that translational rather than transcriptional mechanisms underlie the alteration of TGFβ production in atopic dermatitis. Our data also show that TGFβR1 is upregulated in atopic dermatitis lesions. Together, these data suggest that TGFβ signalling is enhanced in atopic dermatitis lesions, which may play a pivotal role in immune dysregulation and fibrosis in atopic dermatitis.

TGFβ-induced activation of TGFβR1 can result in phosphorylation of SMAD 2/3, which subsequently leads to binding SMAD 4 and translocates to the nucleus to initiate the transcription of target genes.[6,9] In addition, the activation of TGFβR1 can also stimulate SMAD-independent pathways, including major intracellular signal transducers such as ERK, P38 and JNK.[6,9] In our study we found that the upregulation of TGFβR1 and p-Smad2 was likely to be a direct result of the dysregulation of TGFβ signalling in atopic dermatitis. JNK, PKC-bII/d and c-Src are proinflammatory kinases that can be directly activated by or cross-talk with TGFβ signalling. The activation of these kinases leads to the active inflammatory processes observed in atopic dermatitis lesions, which are probably associated with, or independent of, TGFβ signalling. TGFβ signalling can rapidly activate RhoA in a variety of cell types, which is critical for the reorganisation of the actin cytoskeleton and focal adhesion formation during cell migration.[10] TGFβ-stimulated activation of RhoA requires the kinase activity of TGFβR1 but appears to be independent of Smad signalling.[10] The upregulation of p-RhoA may directly facilitate the infiltration of inflammatory cells as well as result in skin fibrosis remodelling in atopic dermatitis.

In summary, our data demonstrate that TGFβR1, p-Smad2, p-RhoA, p-JNK, p-PKC-bII/d and p-c-Src were upregulated in atopic dermatitis lesions. The expression of TGFβR1, p-JNK and p-c-Src was positively correlated with the inflammatory progression of atopic dermatitis. Our results suggest that TGFβR1, JNK and c-Src may be potential therapeutic targets for treating atopic dermatitis.

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