Abnormal regulation of fibronectin production by fibroblasts in psoriasis

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Summary

Background Data indicate that in psoriasis, abnormalities are already present in nonlesional skin. Transforming growth factor-β and keratinocyte growth factor (KGF), together with fibronectin and α5β1 integrin, were suggested to play a crucial role in the pathogenesis of psoriasis by influencing inflammation and keratinocyte hyperproliferation.

Objectives To investigate the expression of KGF, fibroblast growth factor receptor (FGFR)2, fibronectin (FN) and extra domain A (EDA)-positive FN in healthy and nonlesional psoriatic skin, and to study the effect of KGF on the regulation of FN and EDA+FN production by fibroblasts.

Methods Healthy, nonlesional psoriatic skin and lesional psoriatic skin were immunostained for α5 integrin, KGF, FGFR2, EDA+FN and signal transducer and activator of transcription (STAT)1. KGF-treated cell cultures were analysed for FN and EDA+FN mRNA and protein by real-time reverse-transcriptase polymerase chain reaction and flow cytometry, respectively. The major downstream signalling of KGF was investigated by blocking experiments using inhibitors of mitogen-activated protein kinase (MAPK) kinase (MEK1), AKT1/2, STAT1 and STAT3.

Results The expression of α5 integrin, EDA+FN, KGF and its receptor FGFR2 is elevated in psoriatic nonlesional skin compared with healthy skin. KGF mildly induced EDA+FN, but not FN expression in healthy fibroblasts through MAPK signalling. Fibroblasts express the FGFR2-IIIc splice variant. STAT1 negatively regulates both FN and EDA+FN expression in healthy fibroblasts, and this regulation is compromised in fibroblasts derived from nonlesional psoriatic dermis. We detected active STAT1 in healthy and lesional skin, similarly to a previous report. However, in the nonlesional skin STAT1 activation was absent in tissues far away from lesions.

Conclusions The production of FN and EDA+FN by fibroblasts and the signalling of STAT1 are abnormally regulated in psoriatic nonlesional skin.

What’s already known about this topic?

- In psoriasis, nonlesional skin already contains abnormalities as a base for the manifestation of the disease.
- Fibronectin (FN) and its splice variant EDA+FN are essential extracellular matrix proteins influencing major cellular processes, and they are abnormally expressed in psoriatic skin.
- Keratinocyte growth factor (KGF) is overexpressed in psoriatic lesional skin and contributes to keratinocyte hyperproliferation.

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Conflicts of interest
None declared.

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It is well known that psoriasis is an inflammatory skin disease in which both innate and adaptive immune activation play an essential role in the maintenance of the abnormal skin phenotype. However, it is less well known how much resident cells of the tissue contribute to the pathology. A reasonable approach to seek alterations in tissue responses in psoriasis is to investigate the phenotypically normal-looking nonlesional psoriatic skin.

The main fibronectin (FN) receptor, α5β1 integrin was found to be overexpressed in nonlesional psoriatic epidermis compared with normal skin. There is evidence that proper regulation of integrin expression in keratinocytes is essential for normal epidermal homeostasis, as shown by the suprabasal overexpression of integrins in transgenic mice resulting in epidermal hyperproliferation and inflammatory reaction. In the steady state of normal human adult epidermis, α5 integrin expression of keratinocytes is downregulated. A possible explanation for α5 integrin overexpression in nonlesional skin is the presence of FN and one of its isoforms, extra domain A-containing fibronectin (EDA+FN), in the microenvironment of basal keratinocytes.

Keratinocyte growth factor (KGF/FGF7) is a member of the fibroblast growth factor (FGF) family produced by mesenchymal cells and considered to be a major growth factor for keratinocytes. Several reports suggest that KGF reduces the ability of keratinocytes to initiate terminal differentiation and undergo programmed cell death. Overexpression of KGF in the basal epidermal cell layer of transgenic mice results in epidermal hyperplasia. There is also evidence suggesting that fibroblasts from lesional and nonlesional skin of patients with psoriasis induce keratinocyte outgrowth by producing soluble signals. More recently, it has been reported that KGF can induce the expression of α5 integrin, and it delays the expression of keratin 10 and transglutaminase in keratinocytes. Indirect evidence, such as measurement of mitogenic activity, suggests that the specific receptor for KGF on epithelial cells is the IIb splice variant of fibroblast growth factor receptor (FGFR)2.

According to our observations, psoriatic nonlesional skin displays a marked overexpression of KGF, FGFR2, α5 integrin and EDA+FN compared with healthy skin. Therefore, in this study we focused on investigating regulatory mechanisms involving KGF and FN. Our results revealed that KGF regulates EDA+FN production in an autocrine manner through mitogen-activated protein kinase (MAPK) signalling in healthy fibroblasts. With these results we designed an in silico model to predict a possible regulatory network for the effect of KGF on EDA+FN production. We provide in vitro evidence that both FN and EDA+FN are negatively regulated by activated signal transducer and activator of transcription (STAT)1 in healthy fibroblasts, and this is in contrast to psoriatic nonlesional skin-derived fibroblasts. Active, phosphorylated STAT1 was found in healthy skin as well as in psoriatic lesions, but not in psoriatic nonlesional skin, suggesting a crucial mechanism for keeping nonlesional skin in a preactivated state to developing a chronic wound-healing phenotype.

Materials and methods

Skin biopsies

Patients with moderate-to-severe chronic plaque-type psoriasis and healthy volunteers (age 18–60 years) were enrolled into the study. Patients had a medication-free period of ≥4 weeks without systemic therapy and/or ≥2 weeks without local therapy. Participant-informed consent was obtained prior to surgical intervention. The Psoriasis Area and Severity Index (PASI) score was determined for all patients by dermatologists. Samples were collected from 16 patients and 25 healthy volunteers for the experiments. Mechanical stress was induced by a tape-stripping procedure, then punch biopsies were taken from uninvolved skin of patients with psoriasis (n = 6) and healthy subjects (n = 6) from a tape-stripped and nontreated (control) skin area from the buttock area at 24 and 48 h after treatment (Fig. S1). For all other experiments, tissues without the introduction of mechanical stress were used. All tissue collection complied with the guidelines of the Declaration of Helsinki and was approved by the Regional and Institutional Research Ethics Committee (2799, 3517).

Immunofluorescence staining

Human punch biopsies were frozen embedded, stored at −80 °C, then cut into 6-μm sections. Samples were fixed in Fixation/Permeabilization Concentrate and Diluent (eBioscience, San Diego, CA, U.S.A.) and resuspended in phosphate-buffered saline (PBS). The sections were incubated for 30 min in goat serum (Sigma-Aldrich, St Louis, MO, U.S.A.). The following primary antibodies were used, overnight at 4 °C: FGFR2 [1 : 100, Bek (C-17); Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.], KGF [1 : 500, FGF-7 (N-14); Santa Cruz Biotechnology], α5 integrin (1 : 200; Becton-Dickinson, Franklin Lakes, NJ, U.S.A.), EDA+FN (1 : 500, clone IST9; Abcam, Cambridge, U.K.) and p-STAT1 (Ser727) and p-STAT1 (Tyr701) (both 1 : 400; Cell Signaling Technology, Beverly, MA, U.S.A.). Goat antirabbit IgG-

What does this study add?

- KGF and its receptor FGFR2 are overexpressed in psoriatic nonlesional skin. KGF influences EDA+FN production in fibroblasts through MEK1 signalling.
- Fibroblasts express the FGFR2-IIIc splice variant of the FGFR2 receptor.
- STAT1 negatively regulates FN and EDA+FN production in normal cultured fibroblasts, but not in fibroblasts derived from psoriatic nonlesional skin.
- STAT1 is active in healthy skin and psoriatic lesional skin, but not in nonlesional psoriatic skin.
Alexa Fluor 488, donkey antigoat IgG-Alexa Fluor 546, goat antimouse IgG-Alexa Fluor 647 and Alexa Fluor 546 were used as secondary antibodies (all 1 : 500; Life Technologies, Carlsbad, CA, U.S.A.) for 2 h at room temperature. Sections were incubated without the primary antibody or with isotype control antibody for negative staining controls. Nuclei were stained with DAPI (4’,6-diamidino-2-phenylindole; Sigma-Aldrich).

Cultured human fibroblasts, keratinocytes and HaCaT cells were collected after brief trypsinization (0-25% trypsin solution; Sigma-Aldrich) and centrifuged onto a slide using a cytocentrifuge (Cytopro™; Wescor, Logan, UT, U.S.A.), then fixed in 2% paraformaldehyde (Sigma-Aldrich). Stainings were carried out as described above. Images were documented with a Zeiss AxioImager Z1 fluorescence microscope (Carl Zeiss Microscopy GmbH, Munich, Germany) equipped with an AxioCam MRm camera. Image processing and data analysis were conducted using ZEN 2012 Microscope and Imaging (Carl Zeiss) or ImageJ software (http://imagej.nih.gov/ij/).

Flow cytometry

Cells were harvested as described above, fixed in Fixation/Permeabilization Concentrate and Diluent (eBioscience) and resuspended in PBS. Primary antibodies anti-EDA+FN (1 : 500, clone IST9; Abcam) and anti-FN (1 : 1000, clone IST4; Sigma-Aldrich) were applied for 45 min. Mouse IgG1 isotype antibody (Sigma-Aldrich) was used as the negative control. Cells were then washed in PBS and incubated with secondary antibodies as described above. Samples were analysed on a FACSCalibur flow cytometer equipped with 488-mm and 633-nm lasers (Becton-Dickinson).

Statistical analysis

For statistical analysis, repeated-measures Friedman ANOVA, Wilcoxon test or one-way ANOVA were used. Multiple comparisons were carried out with SigmaPlot software (Systat Software Inc., Chicago, IL, U.S.A.). A probability test was used to test for normality; P ≤ 0.05 was considered significant.

Further methods

Detailed information of the materials and methods regarding cell culture experiments, real-time quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and the in silico model are presented in Appendix S1 (see Supporting Information).

Results

Altered protein expression of α5 integrin, extra domain A-positive fibronectin, keratinocyte growth factor and fibroblast growth factor receptor 2 in psoriatic nonlesional skin

Healthy skin and psoriatic nonlesional skin were investigated for differences in the expression of α5 integrin, EDA+FN, KGF and FGFR2 before and 24 and 48 h after tape stripping. Immunofluorescence staining revealed a striking difference in all protein expressions examined, even without tape stripping, between normal and psoriatic nonlesional skin (Fig. 1). Mild mechanical stress introduced by tape stripping of the skin did not lead to any obvious changes in α5 integrin, EDA+FN, KGF or FGFR2 expression or distribution at 24 and 48 h after treatment in nonlesional skin. In healthy control sections a slight increase in all protein expression was observed based on immunostaining (Fig. S1a–d).

Fig 1. Increased expression of α5 integrin, extra domain A-containing fibronectin (EDA+FN), keratinocyte growth factor (KGF) and fibroblast growth factor receptor (FGFR2) in nonlesional skin without mechanical stress stimuli. Immunofluorescence analysis of α5 integrin, EDA+FN, KGF and FGFR2 in healthy (left column) and nonlesional skin (right column) (n = 6). DAPI, 4’,6-diamidino-2-phenylindole. Bar: 50 μm.
Keratinocyte growth factor treatment of healthy human fibroblasts leads to the elevation of extra domain A-positive fibronectin production

EDA+FN and KGF are known to stimulate keratinocyte proliferation. KGF induces the expression of the major FN cell surface receptor α5β1 integrin. Given the differences in EDA+FN and KGF protein levels in healthy and nonlesional psoriatic skin, we set out to investigate a putative regulatory connection between these two molecules. Exogenous treatment of cultured healthy fibroblasts with KGF for 24 h increased the level of the EDA+FN splice variant, but not the total FN protein, based on immunostaining and flow cytometry measurements (Fig. 2a, b). A similar increase in the EDA+FN mRNA splice variant was detected, while the total FN mRNA remained unchanged (Fig. 2c).

We also investigated this effect of KGF on normal human keratinocytes and melanocytes and on the keratinocyte cell line, HaCaT (Figs S2, S3). As expected, keratinocytes and HaCaT cells expressed FN and EDA+FN at a very low level compared with fibroblasts, while melanocytes expressed both proteins at a comparable level with fibroblasts (Fig. S3). However, upon in vitro KGF treatment these cells did not display detectable changes in mRNA and protein expression of EDA+FN and total FN (Fig. S2).

Expression of fibroblast growth factor receptor 2 splice variants in fibroblasts, melanocytes and keratinocytes

Two splice variants have been identified for FGFR2, designated as FGFR2-IIIb and FGFR2-IIIc, which have different ligand-binding preferences. These results were based on mitotic activity measurements. KGF has been shown to act on the FGFR2-IIIb variant, increasing cellular proliferation. We determined the expression of the splice variants on healthy fibroblasts, melanocytes and keratinocytes using specific primers designed for RT-PCR, revealing that melanocytes and keratinocytes expressed FGFR2-IIIb only, while fibroblasts expressed mainly the FGFR2-IIIc variant (Fig. 3).

Mitogen-activated protein kinase signalling is involved in the regulation of extra domain A-positive fibronectin by keratinocyte growth factor receptor in healthy fibroblasts

Fibroblast growth factor signals (including KGF) are coordinated by four major pathways: Ras-Raf-MAPK, phos-

![Fig 2. The expression of extra domain A-containing fibronectin (EDA+FN) is induced by keratinocyte growth factor (KGF) in normal human fibroblasts. KGF-treated fibroblasts and the change in expression in fibronectin (FN) and EDA+FN at 24 h. (a) Immunofluorescent detection of EDA+FN. (b) Flow cytometry measurement of EDA+FN and FN levels following KGF treatment. RFI, relative fluorescence intensity; con, control. (c) FN and EDA+FN mRNA expression following KGF stimulation. Values are expressed in arbitrary units. Data represent the mean ± SEM (n = 6). DAPI, 4',6-diamidino-2-phenylindole. Bar: 50 μm.](image-url)
Changes upon STAT3 impairment were not significant; derived fibroblasts 24 h after STAT1 or STAT3 inhibition in culture. The KGF-induced FN elevation was abolished by the inhibition of MEK1. This suggested inhibitory effect of STAT3 on MEK1 may account for the increased EDA+FN protein levels. Our model also indicates that STAT3 negatively regulates the expression of MEK1. This suggested inhibitory effect of STAT3 on MEK1 may account for the increased EDA+FN production upon STAT inhibition. However, our model did not explain our data showing a significant increase in EDA+FN and FN following STAT1 inhibition in normal fibroblasts. The schematic summary model of KGF-induced changes on FN based on our results in normal skin (in vivo, in vitro and in silico) is presented in Figure 6.

**Discussion**

One of the major characteristics of psoriatic lesional skin is the hyperproliferation of keratinocytes, where KGF is known to play an important role. At the protein level both KGF and FGFR2 were demonstrated to be elevated in lesional skin compared with normal skin. At the mRNA level in both nonlesional and lesional tissue, increased expression of both KGF

Abnormal signal transducer and activator of transcription 1 activation in psoriatic skin plays a role in the regulation of both fibronectin and extra domain A-positive fibronectin

To investigate further, we compared the FN and EDA+FN expressions between healthy and psoriatic nonlesional skin-derived fibroblasts 24 h after STAT1 or STAT3 inhibition in culture. Changes upon STAT3 impairment were not significant; however, blockade of STAT1 did not lead to the elevation of total FN and EDA+FN in fibroblasts from nonlesional skin in contrast to healthy controls (Fig. 4c). Therefore, we investigated the phosphorylation pattern of STAT1 at Tyr701 and Ser727, which are known to be key amino acid modifications leading to dimerization and influencing activity.

Immunofluorescent staining for the phosphorylated Ser727 was highest in lesional psoriatic skin, and lower, but clearly detectable, in healthy skin, whereas nonlesional skin samples showed the lowest intensity. In two of four investigated nonlesional samples phosphorylation was undetectable (Fig. 4d). Staining for the phosphorylated Tyr701 showed a much less noticeable but similar pattern. In samples of patients where no serine phosphorylation was detected in the nonlesional skin, staining for the phosphorylated Tyr701 was also not visible in either nonlesional or lesional areas. To elucidate whether differences observed in STAT1 activity among patients correlated with the severity of the disease, PASI scores of donor patients were compared. The patients whose nonlesional skin was negative for phosphorylations of STAT1 had lower PASI scores (12-4 and 17-8), therefore we were able to take nonlesional samples further away from lesions, at least 10-cm distance. Patients with detectable but low STAT1 activity in nonlesional skin had higher PASI scores (19-6 and 20).

**In silico model construction**

We constructed an in silico model based on our in vitro results involving KGF and FN signalling and the resultant transcriptional regulation network (Fig. 5 and Tables S1, S2). Our data already suggested the participation of MAPK signalling in KGF-induced FN splicing. The generated coexpression matrix implied a potential role of peptidylprolyl cis-trans isomerase G (PPIG), a protein important in both protein folding and splicing, which is regulated by MEK1-induced activating protein (AP)-1. Changes in MEK1 activity could lead to modulation of FN splicing through PPIG, resulting in elevated EDA+FN levels.

Our model also indicates that STAT3 negatively regulates the expression of MEK1. This suggested inhibitory effect of STAT3 on MEK1 may account for the increased EDA+FN production upon STAT inhibition. However, our model did not explain our data showing a significant increase in EDA+FN and FN following STAT1 inhibition in normal fibroblasts. The schematic summary model of KGF-induced changes on FN based on our results in normal skin (in vivo, in vitro and in silico) is presented in Figure 6.
and FGFR2 transcripts was also described by others. KGF stimulates keratinocyte proliferation and influences terminal differentiation. The only known source of KGF in the skin is fibroblasts, and keratinocytes can mediate KGF production in fibroblasts through interleukin (IL)-1, establishing a double paracrine regulatory loop, controlled by AP-1 subunits c-Jun and JunB, and directing the regeneration of the epidermis and maintaining tissue homeostasis in the skin. It was reported that the microRNA miR-125 is downregulated in psoriatic lesional tissue. miR-125 is known to have a negative influence on keratinocyte proliferation, partially by downregulating FGFR2 expression and driving keratinocytes towards differentiation.

Integrins and their corresponding extracellular ligands also influence cell proliferation and differentiation. Overexpression of α5 integrin and EDA+FN has already been reported in connection with psoriasis, and our group demonstrated that one of the potential sources of EDA+FN in nonlesional psoriatic skin is activated keratinocytes. Another report on nonlesional skin has shown that EDA+FN was localized mainly at the dermoepidermal junction. It is well established that α5 integrin and EDA+FN could play an important regulatory role in the abnormal epidermal homeostasis of psoriatic skin, and more recent evidence suggests that EDA+FN could be crucial in activating immune cells. The EDA motif encompasses two nonclassical binding sites for α5β1 and α9β1 integrins, receptors present on the surface of T cells that could enhance T-cell migration and accumulation in EDA+FN-containing tissues, such as the skin.

EDA+FN promotes acute inflammation and brain injury following cerebral ischaemia in mice. EDA+FN is normally absent in human plasma; however, patients with chronic inflammation and ischaemic stroke have high plasma EDA+FN. The abnormally regulated overproduction of EDA+FN in psoriatic fibroblasts may not only be responsible for maintaining a local chronic inflammation in the skin, but may also influence plasma levels that could play a role in the systemic inflammation that has been reported recently in psoriasis.
crucial for maintaining the characteristic epidermal changes, as well as the chronic inflammation in psoriatic lesions.\textsuperscript{31,32}

It has been reported that KGF stimulates EDA inclusion in a mouse mammary epithelial cell line (SCp2).\textsuperscript{33} Although the exact regulation of EDA+FN splicing is not completely clear, it is known that TGF-\(\beta\) enhances EDA+FN production.\textsuperscript{34} Our \textit{in silico} model of KGF and FN signalling corroborates our experimental results in suggesting the participation of MEK1 in KGF-induced FN splicing through PPIG, a protein known to be important in protein folding and splicing\textsuperscript{14} and regulated by MEK1-induced AP-1.

Autocrine feedback of several different FGFs through FGFRs exists under both normal and pathological conditions.\textsuperscript{10,13,35} KGF exerts its effect mainly through FGFR2-IIIb, but also via FGFR2-IIIc and FGFR1.\textsuperscript{11} As both FGFR2 and FGFR1 can initiate MEK1 signalling, KGF could potentially modulate FN splicing in a similar autocrine manner to other FGFs, most likely either through FGFR2-IIIc or FGFR1\textsuperscript{10,36} in fibroblasts. However, the participation of other unknown receptors also cannot be excluded, and further research is needed to understand the exact mechanism through which KGF affects FN splicing.

Directly blocking EDA+FN seems less feasible as a therapeutic approach compared with blocking KGF or its receptors.\textsuperscript{37} STAT1 is the founding member of the STAT family and is activated by interferons (IFNs), and signalling from IL-27, IL-35 and Toll-like receptors.\textsuperscript{38} There is ample evidence...
implicating IFN-γ in the pathomechanism of psoriasis. Aber-
rant signalling and transcription have been reported for psori-
atic keratinocytes with regard to SOCS1 (suppressor of cyto-
kine signalling 1), a negative regulator of IFN-γ sig-
nalling, and also for STAT1 and interferon-regulated factor 1. The complex role of STAT1 in immune regulation is
apparent from animal studies showing that both STAT1−/−
and IFNγ−/− mice are highly susceptible to experimental
autoimmune encephalomyelitis (EAE). It has been dem-
onstrated in both multiple sclerosis and in the EAE model that α5 integrin and FN could be responsible for the extensive vas-
cular remodelling, characteristic for MS, that takes place
during the presymptomatic phase in EAE. The regulatory connection between STAT1 and FN described in this article may explain the more severe disease manifestation in STAT1−/− animals. STAT1 has diverse func-
tions: it inhibits certain genes and is a functional transcription
factor even in the absence of inducer-mediated activation.
Among its major targets of negative regulation are genes encoding proteins involved in regulating the extracellular
matrix: matrix metalloproteinases, perlecan and bullous pem-
phigoid antigen-1. As our results indicate, in normal home-
ostatic skin tissue, a steady state of STAT1 activation is
present, and this could be partially responsible for downregu-
lating FN and EDA/FN production, as blocking STAT1 in nor-
mal fibroblasts results in enhanced production of FN and EDA/FN. The absent suppressive function of STAT1 in psori-
atic fibroblasts could be crucial in the pathomechanism of psoriasis. Using a STAT1 decoy oligodeoxynucleotide-containing
olintment Wagner et al. were able to inhibit hapten-induced contact hypersensitivity reaction in animal models, indicating
that topical STAT1 blockade could be a novel anti-inflamma-
tory therapy in skin inflammation. Our data indicate that in
psoriasis STAT1, inactivation characterizes the uninvolved skin;
thus, this type of therapy may well work as a symptomatic
therapy for lesional skin.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:
Appendix S1. Supplementary materials and methods.

Fig S1. Mild mechanical stress induced by tape stripping has no effect on the expression of α5 integrin, extra domain A-containing fibronectin, keratinocyte growth factor or fibroblast growth factor receptor 2 in nonlesional psoriatic skin and only a mild effect on healthy skin.

Fig S2. Exogenous keratinocyte growth factor treatment has no effect on extra domain A-containing fibronectin expression of keratinocytes and HaCaT cells.

Fig S3. Fibroblasts and melanocytes show higher expression of extra domain A-containing fibronectin and fibronectin than keratinocytes.

Fig S4. Increase of extra domain A-containing fibronectin and fibronectin triggered by exogenous keratinocyte growth factor treatment is not influenced by Akt1/2 inhibition.

Table S1. List of genes applied for the construction of the coexpression matrix gene model.

Table S2. Coexpression matrix of genes in our model.