

Original Paper

The Antimicrobial Peptides Psoriasin (S100A7) and Koebnerisin (S100A15) Suppress Extracellular Matrix Production and Proliferation of Human Fibroblasts

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Key Words

Fibroblasts · Psoriasin (S100A7) · Koebnerisin (S100A15) · Wound healing · Excessive scarring

Abstract

Background/Aims: Keloids result from aberrations in the normal wound healing cascade and can lead to pruritus, contractures and pain. The underlying mechanisms of excessive scarring are not yet understood, and most therapeutic strategies remain unsatisfactory. Psoriasin (S100A7) and koebnerisin (S100A15) are released by keratinocytes during physiological wound healing. We found S100 production is markedly decreased in keloid scar tissue. The disturbed epidermal S100 expression might contribute to keloid formation; thus, we studied their effect on dermal fibroblasts and extracellular matrix (ECM) production. **Methods:** S100 peptides, ECM regulation and distribution were analysed in normal and keloid tissue by quantitative PCR (qPCR), immunoblotting and immunofluorescent staining. Isolated dermal fibroblasts were incubated with S100 proteins, and the regulation of ECM and transforming growth factor (TGF)- β was determined using qPCR. Fibroblast proliferation and viability were determined by the 5-bromo-2'-deoxyuridine assay and crystal violet assay. **Results:** Keloid tissue featured a pronounced expression of ECMs, such as collagen types 1

and 3, whereas the production of psoriasin and koebnerisin was markedly decreased in keloid-derived cells and keloid tissue. Both S100 proteins inhibited the expression of collagens, fibronectin-1, α -smooth-muscle actin and TGF- β by fibroblasts. Further, they also suppressed fibroblast proliferation. **Conclusion:** Psoriasin and koebnerisin show antifibrotic effects and may lead to novel preventive and therapeutic strategies for fibroproliferative diseases.

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Introduction

Excess scar formation occurs after dermal injury as a result of abnormal wound healing. Both hypertrophic scars and keloids are associated with significant morbidity by causing pruritus, pain and/or contractures, which significantly affect the patient's quality of life [1]. Despite the relatively high prevalence of keloids and hypertrophic scars in the general population, the molecular mechanisms underlying excessive scar formation are not fully understood. Most therapeutic strategies remain unsatisfactory due to poor knowledge of the complex mechanisms underlying the process of excessive scarring [2–4].

Excessive scar tissue formation results from increased fibroblast proliferation and disturbed extracellular ma-

trix (ECM) deposition in the dermis. Keloids and hypertrophic scars are densely populated by inflammatory cells that release fibrogenic factors, such as transforming growth factor- β (TGF- β). This environment leads to increased transcription and translation of collagen I and III, fibronectin and laminin, as well as deficient ECM degradation and remodelling [5]. Recent evidence suggests that both the severity of inflammation and the type of immune response predispose to excessive scar formation [5]. Development of a Th2 response promotes fibrogenesis, whereas a Th1 predominance attenuates tissue fibrosis [6, 7]. Current research in keloid pathophysiology further suggests that epidermal-dermal interactions [8] and alterations of the fibroblast phenotype through several (growth) factors and downstream signalling pathways [9] may be involved in excessive scarring.

Recently, high levels of psoriasin (S100A7) proteins have been detected in human wound exudate and granulation tissue [10]. Immunohistological studies suggest that psoriasin is produced by keratinocytes surrounding the wound and is released into the wound exudate to inhibit bacterial survival [11]. S100 proteins are effectors of calcium-dependent processes and intervene with the control of cell cycle, cell growth and cell differentiation as well as chemoattractants for inflammatory cells [12, 13]. Hence, the expression of S100 proteins has been associated with epidermal maturation, inflammation and wound healing [14–16].

Koebnerisin (S100A15) protein shares 93% overall identity with psoriasin, with differences mainly concerning the deduced N-terminal Ca^{2+} -binding site [17]. Koebnerisin expression is induced in cultured human keratinocytes upon treatment with tumour necrosis factor- α and interferon (IFN)- γ , and interleukin-1 β , suggesting that the pro-inflammatory environment in diseased skin contributes to koebnerisin expression in the epidermis. A similar regulation pattern through Th1 cytokines has been shown for psoriasin with coregulation of both psoriasin and koebnerisin in inflammation [18]. In fact, compared with normal skin, both psoriasin and koebnerisin proteins are upregulated in inflamed lesional skin, such as in psoriasis or chronic atopic eczema [18]. Since we observed that patients suffering from psoriasis rarely develop keloids upon dermal injury (observation of the authors), we hypothesized that psoriasin/koebnerisin proteins may have antifibrotic effects. Indeed, here, we could demonstrate that epidermal psoriasin and koebnerisin alone or in combination have suppressive effects on ECM production and fibroblast proliferation *in vitro*.

Materials and Methods

Patients and Skin Samples

Sample acquisition was approved by the local ethical committee (Faculty of Medicine, Ludwig-Maximilian University, Munich, Germany). The study was conducted according to the Declaration of Helsinki principles. For all procedures, informed written consent was obtained from the patients. Skin biopsies were taken from patients ($n = 9$) who underwent surgical removal of keloid tissue. Gender- and age-matched tissue specimens from patients ($n = 9$) with no history of excessive scarring served as normal controls.

Isolation and Cell Culture of Human Fibroblasts

Primary fibroblasts were isolated from normal skin. Subcutaneous fat was removed from the respective tissue specimens and the remaining tissue was washed in phosphate-buffered saline. In order to isolate the dermis, specimens were incubated overnight at 4°C in sterile Dispase II (Roche) solution (4 mg/ml). The isolated dermis was minced into small pieces, and explants placed on culture dishes were overlaid with Dulbecco's modified Eagle's medium that was supplemented with 10% fetal bovine serum and penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$; PAA Laboratories, Pasching, Austria), and incubated at 37°C and 5% CO_2 . Skin-derived fibroblasts were grown in culture for 3–4 weeks and then passaged after trypsinization. Early passages of fibroblasts were seeded at a density of 3×10^4 cells into 12-well plates and switched to serum-free medium at 70–80% confluence 24 h prior to exposure to IFN- γ (100 ng/ml; Biomol, Hamburg, Germany), psoriasin (S100A7) and koebnerisin (S100A15; 0.01–1 $\mu\text{g}/\text{ml}$) alone or in combination.

RNA Isolation and Quantitative Real-Time PCR

Cultured cells were harvested, and RNA was isolated using the RNA Miniprep Kit (Zymo Research, Irvine, Calif., USA). cDNA synthesis was performed with 1 μg of total RNA using the DyNAmo cDNA Synthesis Kit (Finnzymes, Espo, Finland). Expression of COL1A1, COL1A2 und COL3A1, TGF- β_1 , - β_2 , - β_3 , fibronectin-1, laminin- β_2 and α -smooth-muscle actin were normalized against β -actin using commercial gene-specific primers (Qiagen, Hilden, Germany). Quantitative PCR (qPCR) assays were run in the CFX96 Real Time System as specified by the manufacturer (Bio-Rad, Hercules, Calif., USA). All analyses were performed in triplicate from 2 to 3 independent cell stimulation experiments.

Cell Viability and Proliferation Assays

Fibroblast viability was determined by crystal violet assay. Cells were exposed to koebnerisin (S100A15) and psoriasin (S100A7) alone or in combination for 24 h and then stained with crystal violet (0.5 in 20% methanol) for 20 min at room temperature. Then, cells were decolorized with 0.1 M sodium citrate in 50% ethanol, and absorbance was read at 550 nm using a microplate reader (Spectra MR, Dynex Technology, Chantilly, Va., USA).

Fibroblast proliferation was determined by the 5-bromo-2'-deoxyuridine (BrdU) Assay (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Cells were exposed to IFN- γ (100 ng/ml), koebnerisin (S100A15) and psoriasin (S100A7) alone or in combination for 48 h. At 24 h, BrdU labelling medium was added, and incorporated BrdU during cell proliferation was detected by an anti-BrdU horseradish peroxidase-labelled antibody at 370 nm using a microplate reader (Spectra MR, Dynex Technology).

Immunofluorescence Staining and Immunoblotting

Immunofluorescence staining was performed on serial 5- μ m frozen sections of human normal and keloid skin and on primary fibroblasts derived from normal skin and keloids. Plated cells and skin sections were fixed in acetone and blocked in 10% normal goat serum. After overnight incubation with anti-S100A15 (5 μ g/ml, customized), anti-S100A7 (1 μ g/ml, Abcam, Cambridge, UK) and anti-vimentin (1:50, Sigma Aldrich, Steinheim, Germany) antibody, sections and cells were then incubated with an Alexa Fluor 647-labelled goat anti-rabbit antibody (Invitrogen, Carlsbad, Calif., USA) and with a tetramethylrhodamine-conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany) diluted in 10% normal goat serum and incubated for 1 h at room temperature in a dark humidified chamber. Staining with secondary antibodies only was performed as a control for unspecific antibody binding. Sections were overlaid with 4',6-diamidino-2-phenylindole (ProLong Gold antifade reagent, Invitrogen). Images of fluorescent stained tissues were recorded using a 12-bit CCD digital camera PCO PixelFly (PCO, Kelheim, Germany) on a fluorescent microscope Zeiss Imager Z1 (Zeiss, Jena, Germany). Immunoblotting was performed as previously described [19]. In brief, skin or fibroblast lysates (15 μ g/lane) were prepared using radioimmunoprecipitation assay buffer. Proteins were separated using a 12% sodium dodecyl sulphate polyacrylamide gel, transferred to polyvinylidene fluoride membranes, blocked and incubated with anti-hS100A15 (5 μ g/ml, Costum), anti-S100A7 antibody (1 μ g/ml, Abcam) and anti- β -actin (1 μ g/ml, Cell Signaling, Danvers, Mass., USA) overnight. After incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, Cell Signaling; anti-mouse, Pierce), proteins were detected by chemoluminescence.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc.). Student's t test was used to calculate statistical differences. Values of $p < 0.05$ were considered significant, and all data are displayed as means \pm SD.

Results

Psoriasin (S100A7) and Koebnerisin (S100A15) Are Decreased in Keloid Tissue and Differently Produced by Normal and Keloid Fibroblasts

Our data showed that psoriasin (S100A7) and koebnerisin (S100A15) are expressed in the upper differentiated epidermal layers of normal skin (fig. 1A, a, c). Compared to psoriasin, koebnerisin was also detectable at the epidermal-dermal junction. In keloid tissue sections, staining of either S100 protein was reduced within the epidermis or underlying dermis (fig. 1A, b, d). We further identified normal fibroblasts as an additional cellular source for koebnerisin (fig. 1B, a, c). Koebnerisin was distributed throughout the cellular cytoplasm and partially colocalized with the mesenchymal marker vimentin. Keloid-derived fibroblasts showed a similar expression pat-

tern, but stained more weakly for koebnerisin (fig. 1B, b, d), while psoriasin was not detectable in both normal and keloid-derived fibroblasts (fig. 1B, e, f). Immunoblot analysis in whole tissue showed psoriasin- and koebnerisin-reactive bands in normal skin (fig. 1C). The psoriasin and koebnerisin protein expression varied among the individual skin tissue samples, but was overall weaker in keloid tissue when normalized to β -actin (fig. 1D). Thus, the coregulation of psoriasin and koebnerisin and their distribution in different cellular compartments in the skin suggest their functional contribution in keloid tissues.

Psoriasin (S100A7) and Koebnerisin (S100A15) Are Associated with Reduced Collagen Expression in the Skin and Inhibit Collagen Expression by Fibroblasts

Keloids form after prolonged inflammation that leads to an increased proliferation and activation of fibroblasts with enhanced deposition of disorganized collagen fibres of types 1 and 3 in the dermis [20]. Whole tissue analysis demonstrated that COL1A1, COL1A2 and COL3A1 were produced in normal skin (fig. 2A). Skin biopsies from keloid tissue showed an increased production of the investigated collagen types, which participate in fibrotic scarring. In corresponding samples, both psoriasin (S100A7) and koebnerisin (S100A15) alternate transcripts (S100A15L and S100A15S) were present in normal skin. However, both S100 peptides were downregulated in the keloid tissue and revealed a reverse S100-collagen regulation pattern under fibrotic conditions (fig. 2B, compare fig. 1). Data implied that psoriasin and koebnerisin affect collagen production in fibroblasts. Cultured fibroblasts were then exposed to increasing S100 peptide concentrations, and their effect on collagen expression was measured. Data showed that recombinant psoriasin decreased expression of COL1A1, COL1A2 and COL3A1 at various concentrations and significantly reduced levels of COL3A1 compared to controls (fig. 2C). Also, recombinant koebnerisin significantly decreased expression of COL1A1 and COL3A1 at most of the concentrations utilized (fig. 2D). Exposure of cultured fibroblasts to psoriasin and koebnerisin at effective collagen-suppressing concentration did not influence the number of viable cells as determined by the crystal violet assay (data not shown).

Psoriasin (S100A7) and Koebnerisin (S100A15) Synergize to Attenuate Expression of ECM Proteins by Fibroblasts

Psoriasin (S100A7) and koebnerisin (S100A15) are coregulated under pathological conditions and show synergistic effects important for disease pathogenesis [21, 22]. As

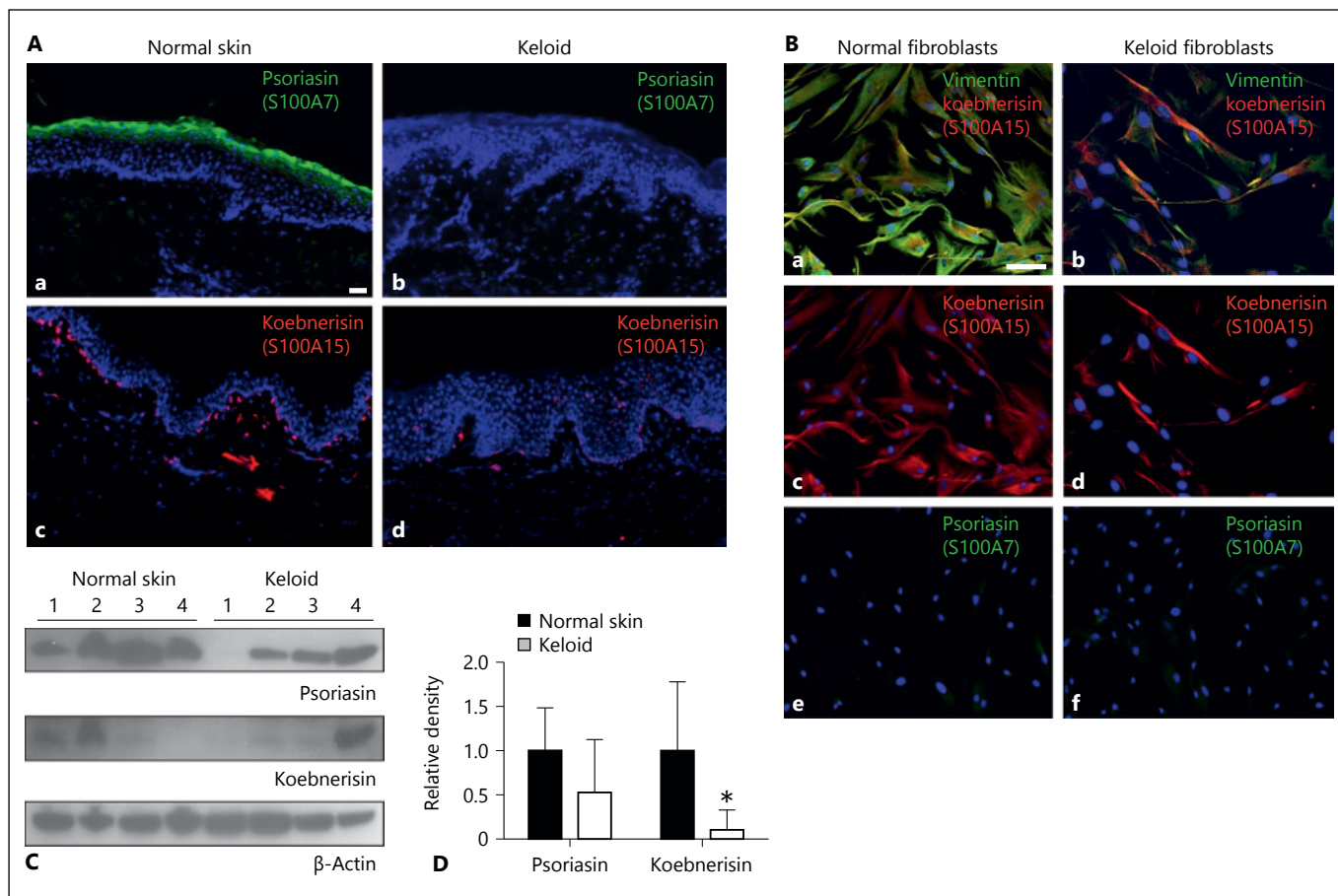


Fig. 1. Psoriasin (S100A7) and koebnerisin (S100A15) expression and distribution in keloid tissue and keloid fibroblasts. **A** Frozen skin tissue sections from normal subjects (**a, c**) and patients with keloids (**b, d**) immunostained for psoriasin (S100A7) and koebnerisin (S100A15). Scale bar = 100 μ m. **B** Cultured fibroblasts isolated from normal skin (**a, c, e**) and keloid biopsies (**b, d, f**) immunostained for vimentin, psoriasin and koebnerisin. Scale bar = 100 μ m. **C** Total protein lysates of normal skin and keloid scar tissue (15 μ g/lane) were probed with specific antibodies against psoriasin and koebnerisin. **D** Psoriasin and koebnerisin staining intensities were normalized against β -actin. * $p < 0.05$ determined by Student's t test.

both peptides individually regulate collagen expression in fibroblasts (fig. 2C, D), their synergistic effect on fibroblast activity was investigated. When used in combination, the suppressing effect of psoriasin and koebnerisin on fibroblast activity was further enhanced for all investigated collagen types (COL1A1, COL1A2, COL3A1; fig. 3A). Next, cultured fibroblasts were exposed to psoriasin and koebnerisin peptides at concentrations that were effective to suppress the regulation of collagens. Data showed that each recombinant psoriasin and koebnerisin peptide decreased the expression of fibronectin in fibroblasts (fig. 3B). Their suppressing effect was further enhanced when both peptides were used in combination and was more effective than IFN- γ (positive control, fig. 3B). The combination of psoriasin and koebnerisin also significantly inhibited the ex-

pression of laminin- β_2 and α -smooth-muscle actin in fibroblasts (fig. 3C, D). Cultured fibroblasts exposed to either recombinant psoriasin or koebnerisin peptides slightly affected the expression of TGF- β_1 , - β_2 and - β_3 , where koebnerisin had the most pronounced effect on TGF- β_3 (fig. 3E). When used in combination, psoriasin and koebnerisin significantly reduced expression of all investigated TGF- β isoforms. Compared to the S100 peptides, IFN- γ suppressed the expression of TGF- β_2 and - β_3 in cultured fibroblasts, but did not regulate TGF- β_1 expression.

Psoriasin (S100A7) and Koebnerisin (S100A15) Synergize to Suppress Fibroblast Proliferation

To gain insight into other antifibrotic effects of the S100 peptides, we investigated the functional importance of

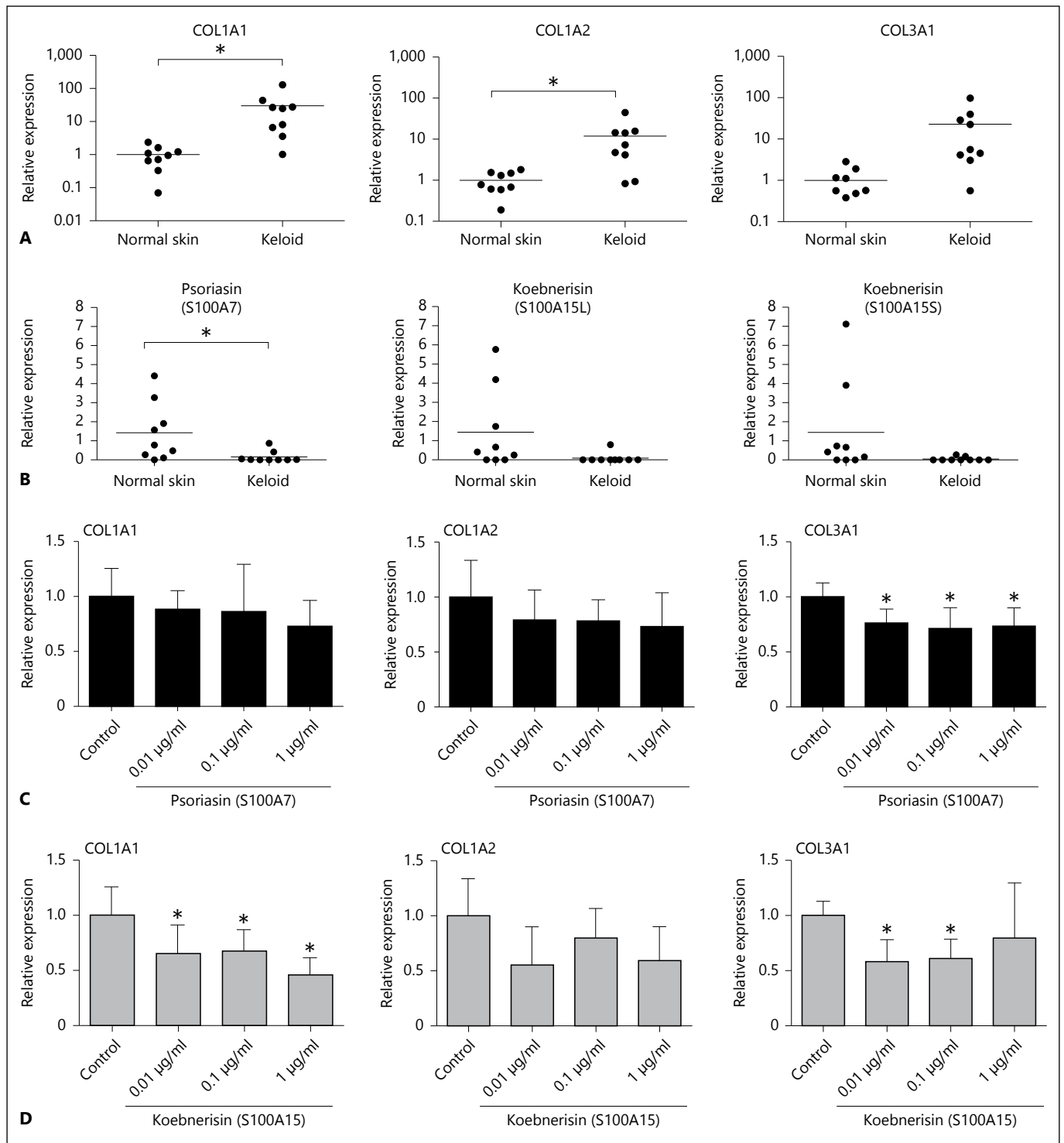


Fig. 2. Psoriasin (S100A7) and koebnerisin (S100A15) mark decreased collagen expression in the skin and inhibit collagen expression by fibroblasts. Total RNA was extracted from biopsies of normal skin (n = 9) and keloid scar tissue (n = 9), and real-time qPCR was performed to assay collagen types 1 and 3 (COL1A1, COL1A2, COL3A1; **A**) and psoriasin (S100A7) and koebnerisin (S100A15-S,

S100A15-L) transcripts (**B**) using gene-specific primers. Cultured fibroblasts were treated with indicated concentrations of psoriasin (S100A7; **C**) and koebnerisin (S100A15; **D**), and collagen type 1 and 3 expression was analysed by real-time qPCR. * p < 0.05 determined by Student's t test.

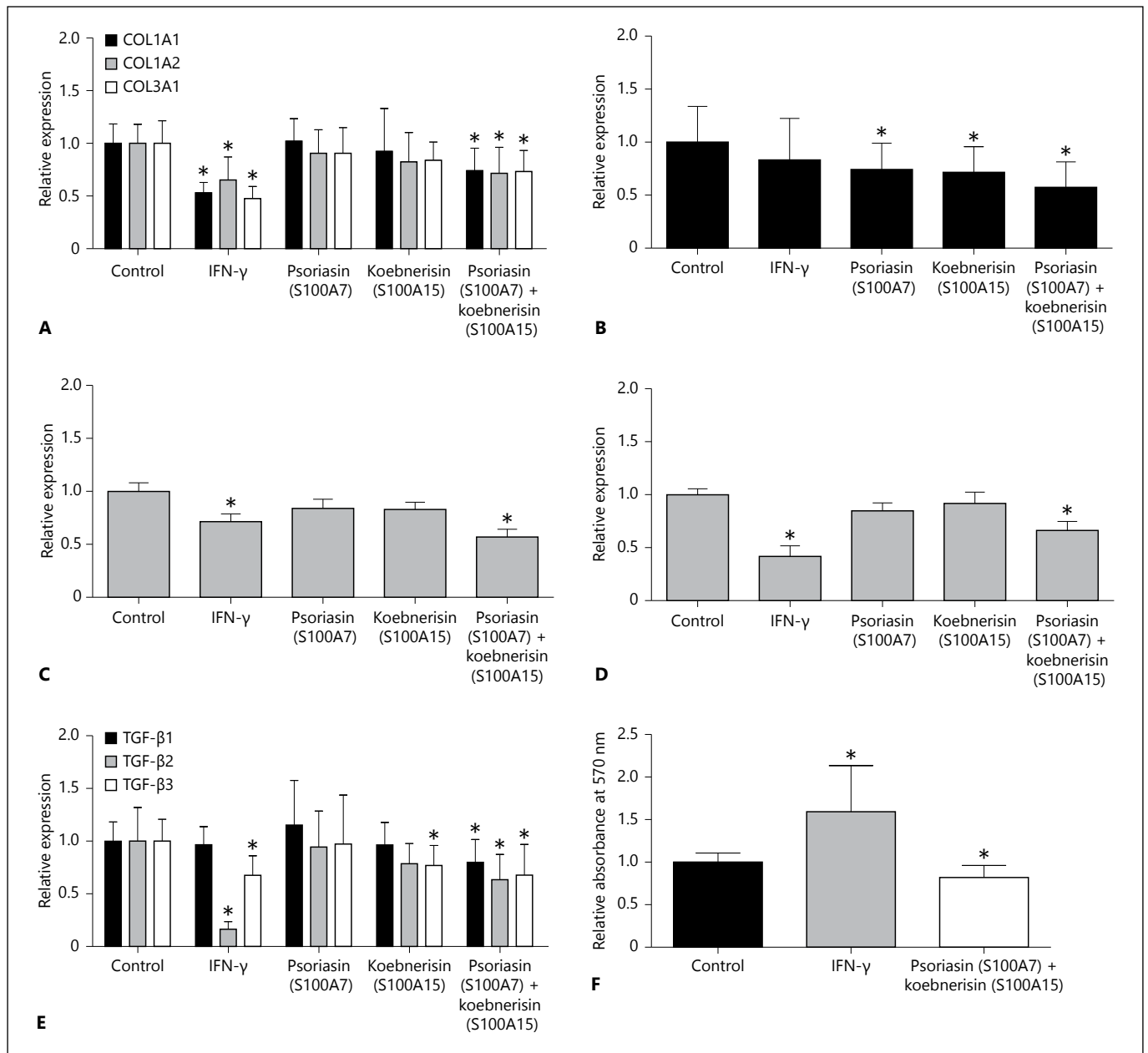


Fig. 3. Psoriasin (S100A7) and koebnerisin (S100A15) synergize to suppress ECM expression by fibroblasts and inhibit fibroblast proliferation. **A–F** Cultured fibroblasts were treated with IFN- γ (0.1 $\mu\text{g/ml}$), psoriasin (S100A7) and koebnerisin (S100A15) alone or in combination. Total RNA was extracted, and real-time qPCR

was performed to assay collagen types 1 and 3 (COL1A1, COL1A2, COL3A1; **A**), fibronectin-1 (**B**), laminin- β_1 (**C**), α -smooth-muscle actin (**D**) and TGF- β types 1–3 (**E**) using gene-specific primers. **F** Proliferation of cultured fibroblasts was analysed by BrdU incorporation after treatment with IFN- γ , psoriasin and koebnerisin.

psoriasin (S100A7) and koebnerisin (S100A15) on fibroblast proliferation. Cultured fibroblasts were exposed to psoriasin and koebnerisin peptides at concentrations that were effective to suppress the expression of ECM components. Each recombinant psoriasin and koebnerisin peptide significantly inhibited the proliferation of fibroblasts

as measured by reduced BrdU uptake (data not shown). The antiproliferative effect was further enhanced when both S100 peptides were used in combination (fig. 3F). In contrast, incubation of fibroblasts with IFN- γ increased BrdU uptake, which is indicative of a stimulation of fibroblast proliferation.

Discussion

Transformation of a wound clot into granulation tissue requires a delicate balance between ECM protein deposition and degradation, and when disrupted, abnormalities in scarring appear [1]. Despite a plethora of *in vivo* and *in vitro* studies investigating the complex mechanisms underlying hypertrophic scar formation, its pathophysiology remains poorly understood. Diverse proteins have been suggested to be involved in excessive scar formation; however, to date, little attention has been paid to the S100 proteins.

Genes of the S100 family encode small (9–13 kDa), calcium-binding proteins of the EF hand type that regulate tissue maturation, inflammation and antimicrobial defence [12]. Dysregulated levels of certain S100 peptides have previously been detected during wound repair of epithelia and other tissues [14–16, 23]. Upregulated in the hyperproliferative epidermis, S100A8 and S100A9 promote the growth of epithelial keratinocytes [24–26], whereas S100A2 drives TGF- β -dependent mesenchymal cell migration [20]. S100B mediates myofibroblast proliferation and might contribute to the scar formation observed in the infarcted myocardium [27]. Increased fibroblast activation and chronic fibrotic kidney disease have been associated with S100A4, which has been established as a diagnostic marker for systemic fibrotic disorders [28].

In this context, we aimed to elucidate the significance of two S100 proteins, psoriasin (S100A7) and koebnerisin (S100A15), for the pathogenesis of keloid formation.

Psoriasin and koebnerisin form a unique subgroup of the S100 protein family and were discovered to be upregulated in psoriatic skin [17, 29]. Although being highly homologous peptides, psoriasin and koebnerisin have distinct functions as epidermal antimicrobial peptides, leucocyte attractants and pro-inflammatory ‘alarmins’ [18]. Compared with psoriasin, koebnerisin is also expressed by additional cell types in the skin, such as endothelial cells and smooth muscle cells. Here, we noticed a predominant interface pattern (fig. 1A), which may also be due to the fact that staining intensity varies among the different cell types and depends on the imaging conditions. We also discovered that skin fibroblasts serve as another source of koebnerisin (fig. 1B). Interestingly, when compared to normal skin, staining of keloid tissue sections for both S100 proteins was significantly reduced within the epidermis and underlying dermis.

Both koebnerisin mRNA isoforms lead to the same protein and were expressed in normal fibroblasts, but were downregulated in keloid-derived fibroblasts. Psoriasin transcript, however, could not be detected in either type of fibroblasts. Whole tissue immunoblot analysis further demonstrated that psoriasin and koebnerisin staining intensity was overall weaker in keloid tissue when compared with normal skin (fig. 1).

Recent evidence suggests that the type of immune response predisposes to excessive scarring [5]. While the development of a Th2 response has been strongly linked to fibrogenesis, a predominance of Th1 CD41 cells has been shown to almost completely attenuate the formation of tissue fibrosis via production of IFN- γ [6, 7]. Both psoriasin and koebnerisin are inducible by Th1-derived cytokines in epidermal keratinocytes [11, 30], and the lack of inducibility of both S100 by Th2-derived cytokines may account for the low expression levels of psoriasin and koebnerisin in keloid tissue [31].

Importantly, low S100 protein expression corresponded to high levels of profibrotic markers, such as collagens, in keloid tissue (fig. 2A, B). This data suggests that subepidermal fibroblasts in keloid tissue might be exposed to decreased levels of secreted psoriasin and koebnerisin that, in turn, could affect the production of collagens and other extracellular matrix compounds.

In fact, when fibroblasts were exposed to either S100 peptide, the expression of both collagen types 1 and 3 was attenuated (fig. 2C, D; fig. 3A). Also, both psoriasin and koebnerisin peptides individually decreased the expression of other ECM compounds, such as fibronectin-1, laminin- β_2 and α -smooth-muscle actin, without affecting fibroblast viability. The suppressing patterns of psoriasin and koebnerisin on the ECM components were different and are in line with distinct functional mechanisms of these S100 peptides. Despite their high homology, extracellular psoriasin and koebnerisin activate different classes of receptors [21]. Psoriasin activates the pattern recognition receptor ‘receptor of advanced glycosylated end products’, whereas koebnerisin functions through a pertussis toxin-sensitive Gi protein-coupled receptor. When coregulated under pathophysiological conditions, both S100 peptides can exert synergistic effects. Here, we could show that co-exposure of fibroblasts to psoriasin and koebnerisin leads to a synergistic inhibition of the ECM components (fig. 3). In some instances, their combined effect was comparable to that of IFN- γ , which has previously been established as one of the most potent inhibitors of ECM-related genes, such as type I collagen, fibronectin and laminin [32–34].

TGF- β signalling has been implicated as key regulator in early stages of ECM production and in keloid formation. While TGF- β_1 and - β_2 represent the most important stimulators of collagen and proteoglycan synthesis [35, 36], TGF- β_3 reduces connective tissue deposition [37]. We could demonstrate that the combination of psoriasis and koebnerisin significantly attenuated the expression of TGF- β subtypes 1–3; however, the suppressive effects of IFN- γ on TGF- β_2 and - β_3 were much more pronounced. Nevertheless, incubation of cultured fibroblasts with psoriasis and koebnerisin did significantly attenuate fibroblast proliferation, which could account for the suppressive effects of psoriasis (S100A7) and koebnerisin (S100A15) on

ECM production. Thus, although the exact molecular mechanism underlying the S100 antifibrotic effects remains currently unclear, a better understanding of these two proteins may give additional insights into the complex pathophysiology of fibroproliferative diseases of the skin.

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