Significance of the S100A4 Protein in Psoriasis

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The S100A4 protein is reported as a pivotal player in the tumor microenvironment with a metastasis-promoting function. Moreover, the upregulation of S100A4 is found in other non-malignant human disorders as cardiac and pulmonary systems and rheumatoid arthritis. In this study, we investigated the expression and significance of S100A4 in psoriasis. We found significant upregulation of S100A4 in the dermis of psoriatic skin compared with normal skin. This pattern of S100A4 expression differs considerably from that of other S100 proteins, S100A7 and S100A8/9, with predominant expression in the epidermis of psoriasis. Furthermore, we revealed a massive release of the biologically active forms of S100A4 from psoriatic skin. Interestingly, we found stabilization (increase) of p53 in the basal layer of epidermis in close proximity to cells expressing S100A4. To examine the possible implication of S100A4 in the pathogenesis of psoriasis, we analyzed the effect of S100A4 blocking antibodies in a human psoriasis xenograft SCID mouse model and observed a significant reduction of the epidermal thickness and impairment in cell proliferation and dermal vascularization. In conclusion, we showed strong upregulation and release of S100A4 in the upper dermis of psoriatic skin and found evidence indicating that S100A4 might actively contribute to the pathogenesis of psoriasis.

Journal of Investigative Dermatology (2010) **130,** 150–160; doi:10.1038/jid.2009.206; published online 30 July 2009

INTRODUCTION

Psoriasis is a complex chronic skin disease with a histopathological appearance of compromised inflammation, epidermal hyperplasia, and vascular alterations. Several observations point to T cells and inflammatory cytokines as key players in psoriasis pathogenesis (Griffiths and Barker, 2007; Lowes *et al.*, 2007). Subsets of dendritic cells were shown to be fundamentally important in the orchestration of psoriasis as well (Cumberbatch *et al.*, 2006). A prominent change in dermal microvascularity suggests that angiogenesis may also be an essential component in the progression and pathogenesis of the disease (Folkman, 1972; Barker, 1991; Kuroda *et al.*, 2001).

The S100 proteins comprise a family of calcium-binding proteins, localized within the epidermal differentiation complex on human chromosome 1 (1q21) (Engelkamp *et al.*, 1993). Several S100 proteins, S100A7, S100A8/9, and S100A12, were found to be upregulated in psoriatic skin (Mirmohammadsadegh *et al.*, 2000; Broome *et al.*, 2003). A weak and sparse expression of S100A4 was shown earlier in normal skin (Boni *et al.*, 1997; Shrestha *et al.*, 1998). In addition, in mouse the expression of S100A4 has been reported in regions of the epithelial sac of hair follicles on hair damage and regeneration (Ito and Kizawa, 2001). However, S100A4 has been extensively investigated in cancer development. Metastasis-inducing ability of S100A4 was shown by several approaches in rodents and humans. The tight association of S100A4 with metastases suggests it as a reliable cancer prognostic marker (Helfman *et al.*, 2005). Recent findings have shown a remarkable increase of S100A4 in the stroma compartment of different malignant tumors (Schmidt-Hansen *et al.*, 2004a, b; Grum-Schwensen *et al.*, 2005; Cabezon *et al.*, 2007) and in non-malignant diseases (reviewed in (Grigorian *et al.*, 2008)), such as rheumatoid arthritis (RA) (Klingelhofer *et al.*, 2007), myocardium infarction (Schneider *et al.*, 2007), and pulmonary artheropathy (Greenway *et al.*, 2004). A common feature of these diseases underlying their pathogenesis is the activation of ''host'' cells (fibroblasts and immune cells) and factors (cytokines, growth factors, matrix metalloproteinases, reactive oxygen species, and others). Interestingly, we showed earlier the basic expression of S100A4 under normal conditions in monocytes, T-lymphocytes, and granulocytes and its upregulation on their ''inflammatory'' challenge (Grigorian *et al.*, 1994).

S100A4 is categorized as an active extracellular factor with powerful capacity to influence gene expression by modulation of mitogen-activated protein kinases and transcription factors p53 and NF-κB. S100A4 stimulates expression and proteolytic activation of MMPs, angiogenesis, and cell invasion that could be abrogated by using S100A4-neutralizing antibodies in mice (Schmidt-Hansen *et al.*, 2004a, b; Yammani *et al.*, 2006; Schneider *et al.*, 2007).

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Abbreviations: NIF, normal tissue interstitial fluids; NN, normal non-involved skin; PIF, psoriatic tissue interstitial fluids; PN, psoriasis non-involved skin; PP, psoriasis skin plaque

Received 26 November 2008; revised 24 April 2009; accepted 6 June 2009; published online 30 July 2009

S100A4 is externalized from macrophages, fibroblasts, and activated lymphocytes into the tumor microenvironment rather than from the tumor cells (Cabezon *et al.*, 2007), where the protein is known to stimulate motility and alter the metastatic potential of cancer cells (Schmidt-Hansen *et al.*, 2004a; Grum-Schwensen *et al.*, 2005), probably by the modulation of key transcription factors, tumor suppressor p53, and NF-κB (Grigorian *et al.*, 2001).

An association of S100A4 and inflammation was first observed by detecting S100A4 messenger RNA in the synovial tissue of RA patients (Masuda *et al.*, 2002). Later, we showed in RA patients massive S100A4 protein expression in various cell types of the synovial tissue and its release as a biologically active form into synovial fluid and plasma (Klingelhofer *et al.*, 2007).

Here, we present data on the involvement of S100A4 in another chronic inflammatory disease, psoriasis, which shares cellular and molecular similarities with other autoimmune inflammations, including RA. We observed a significant expression of S100A4 in various cell types in the upper dermal compartment of psoriatic skin compared with non-involved skin and especially with healthy skin. Our results showed a significant release of S100A4 in psoriatic skin apparently in its oligomeric (active) conformation. Moreover, blocking of S100A4 by means of specific antibodies results in diminishing the thickness of psoriatic skin, vascularization, and keratinocyte proliferation. We suggest that cumulatively our data implicate S100A4 in the pathogenesis of psoriasis and allow us to consider it as to our knowledge previously unreported potential therapeutic target.

RESULTS

Expression of S100A4 in psoriatic skin

To assess the expression of S100A4 in psoriasis, we analyzed skin biopsies from lesional psoriatic skin (PP), non-lesional psoriatic skin (PN), and healthy skin (normal non-involved skin (NN)) by immunohistochemical techniques using human monoclonal anti-S100A4. We observed a significant increase of S100A4 expression in PP compared with PN ($P<0.05$) and NN ($P<0.005$) (Figure 1a–c). The S100A4 expression in PP was intense in the upper layers of the dermis (stratum papillae) compared with PN (1.8 fold) and NN (4.2 fold) (Figure 1d). In PN and NN, the expression was in a few cells in minor clusters (Figure 1a and b).

To compare the expression pattern of S100A4 with other S100 proteins previously reported as being upregulated in psoriasis (Mirmohammadsadegh *et al.*, 2000; Broome *et al.*, 2003), we examined the matched biopsy samples for the expression of S100A7, S100A8/9. We observed a striking difference in the topography of S100A4 compared with other S100 proteins. S100A7 was expressed exclusively in the epidermis, S100A8 and S100A9 were expressed predominantly in the epidermis but also to a low degree in the dermis (Figure 2). Expression of S100A4 is undetectable in the epidermis by this approach. The level of S100A4 is much higher in the dermis of PP compared with PN, whereas expression of S100A7 is detected in the epidermis of PP but not in PN (Figure 2). The expression level of S100A8 and S100A9 (Figure 2) was not strikingly different in PP and PN.

To identify cells expressing S100A4 in psoriatic skin, we performed double immunofluorescence stainings with antibodies against S100A4 and cell-specific markers (Figure 3). The data obtained clearly colocalized S100A4 with markers specific for T-lymphocytes (CD3), macrophages (CD68), dendritic cells (CD1a), non-epithelial cells of mesenchymal origin, mostly fibroblasts (Vimentin), and finally alpha-smooth muscle actin-positive cells (ASMA) located mostly in myofibroblasts and vascular pericytes. Single S100A4-positive cells in the epidermis were revealed to be T-lymphocytes and Langerhans cells in accordance with cell-specific markers. We did not find coexpression of S100A4 with B-cells (CD20) or hematopoietic stem cells (CD34). These observations are in good agreement with our previous data on S100A4 distribution in tumor stroma and synovial tissue from patients with rheumatoid arthritis (Cabezon *et al.*, 2007; Klingelhofer *et al.*, 2007).

The S100A4 release in psoriatic skin

In previous studies on various models (cancer, cardiac, and RA), we observed a notable release of S100A4 into extracellular space and postulated its functional activity in the corresponding microenvironments. Therefore, next we studied the S100A4 release in PP and compared it with NN. The S100A4 concentrations in psoriatic tissue interstitial fluids (PIF) and normal tissue interstitial fluids (NIF) were estimated by means of sandwich ELISA. The average concentration for PP was $1.38 \pm 0.82\,\mu g\,ml^{-1}$ with the range $0.37–3.3\,\mu g\,ml^{-1}$ ($n=9$) compared with NN $0.51 \pm 0.10\,\mu g\,ml^{-1}$, with the range $0.36–0.58\,\mu g\,ml^{-1}$ ($n=4$) being statistically significant ($P<0.005$) (Figure 4). However, we did not find any significant difference in plasma levels of S100A4 from psoriasis patients and healthy subjects (data not shown). These data suggest that S100A4 released in psoriatic skin is either confined locally or is not enough to be detectable in the plasma.

Status of the S100A4 and p53 proteins in psoriasis plaques

Various conformational forms of S100A4, dimer, and a mounting level of oligomers were shown earlier both *in vitro* and *in vivo* (Ambartsumian *et al.*, 2001; Schmidt-Hansen *et al.*, 2004a; Klingelhofer *et al.*, 2007). Moreover, the biological activity of the protein was attributed mainly to high-molecular multimers. To assess a conformational structure of S100A4 in the psoriatic milieu, we examined S100A4 both in PP and PN biopsies (Figure 5a) and in psoriatic and normal tissue interstitial fluids (Figure 5d) by western blot analysis. Samples of tissue lysates (Figure 5a) and tissue interstitial fluids (Figure 5d) were subjected to PAAG electrophoresis in reduced condition (protein was boiled in 1% β-mercaptoethanol and SDS). After blotting onto a membrane, S100A4-specific protein bands were developed using highly specific S100A4 mAb. The data obtained revealed that the level of the S100A4 protein at 11 kDa is evidently much higher in PP than in PN (Figure 5a) and especially in the case

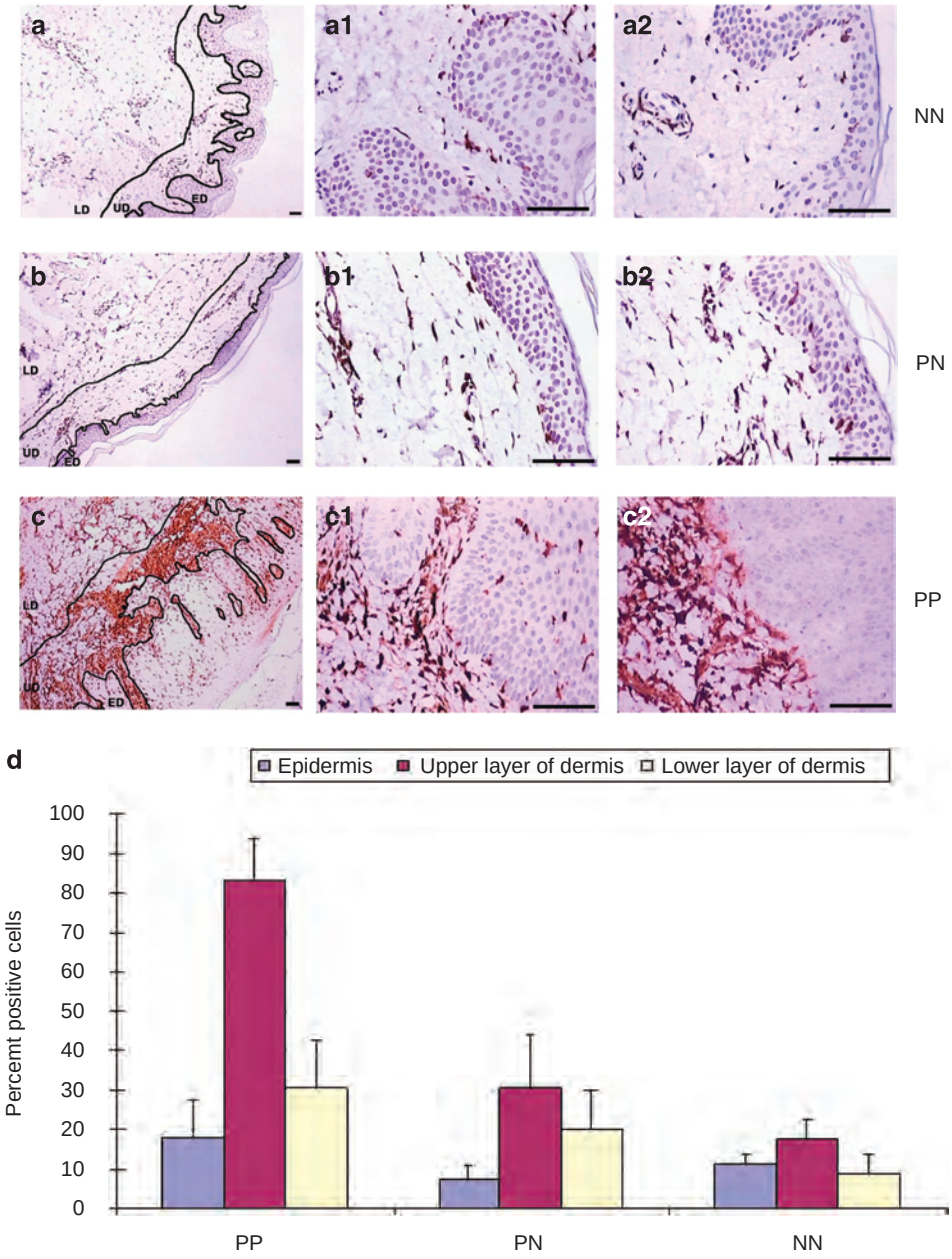

Figure 1. S100A4 is upregulated in the upper layers of dermis in psoriatic skin. The protein expression of S100A4 was studied by immunohistochemical staining in biopsies from nine PP (**a**), nine PN (**b**), and four NN (**c**). The biopsies were scored with percentage positive cells in the epidermis (ED), upper layers of the dermis including the superficial vascular plexus and papillary dermis (UD), and lower layers of the dermis including the deep vascular plexus and reticular dermis (LD) (**a**–**c**). (**d**) Illustrated are the mean scored for each layer with SEM. Scale bars = 100 μm.

of PP1 with abundant high-molecular S100A4. To confirm whether the detected high-molecular S100A4-positive signals were specific, we loaded the PN1 and PP1 samples along with the recombinant S100A4 protein in its oligomeric form isolated after size-excluded chromatography in non-reducing buffer (without β-mercaptoethanol and boiling) (Figure 5c-1) and high-reducing buffer (with 5% β-mercaptoethanol, 2% SDS, and boiling) (Figure 5c-2). At non-reducing conditions (Figure 5c-1) and at low-reducing conditions (Figure 5a), we observed an intense signal in the high-molecular zone with a significant difference between PN1 and PP1. Oligomeric S100A4 was detected only as a high-molecular weight protein (Figure 5c-1). In contrast, in high-reducing conditions the high-molecular signals were not present and strong bands corresponding to the monomeric S100A4 emerged (Figure 5c-2) with the estimated ratio between the monomeric S100A4 signals in low/high-reducing conditions 1:2.7 in PP1 and 1:2 in PN1. This may indicate that the proportion of high-molecular S100A4 in psoriatic skin is higher than in non-involved psoriatic skin. In both cases, the pre-incubation of

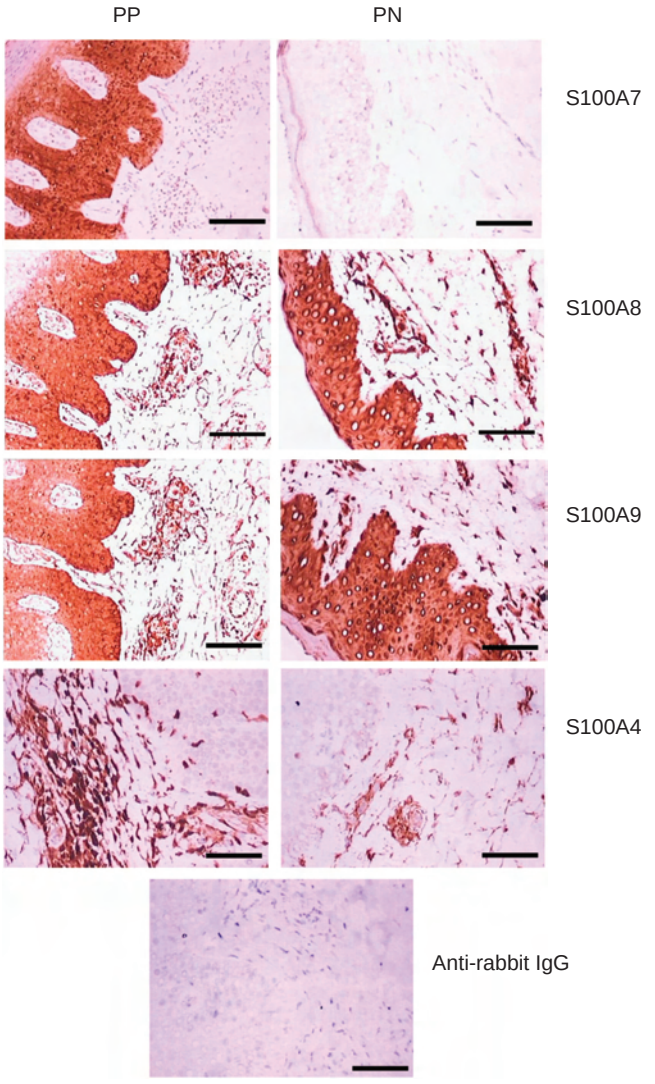

Figure 2. S100 expression is upregulated in psoriatic skin. Immunohistochemical stainings of patient-matched PP and PN biopsies for S100A7 mAb, S100A8 mAb, S100A9 mAb, and S100A4 mAb. Furthermore, control of the secondary antibody by replacing the primary antibody with rabbit IgG is displayed. Scale bars = 100 μm.

antibodies before the immunoprobing completely abolished the appearance of S100A4-positivity in non-reducing and high-reducing conditions (Figure 5c-3 and -4), confirming the high specificity of the antibodies used. More striking was the evidence of strong enrichment of high-molecular forms of S100A4 in PIFs compared with NIFs in low-reduced conditions. In NIFs, mainly an 11 kDa S100A4-specific band corresponding to the S100A4 monomer was revealed, whereas in PIFs a heterogonous pattern of the S100A4 protein, ranging up to more than 250 kDa, was easily detected.

To investigate whether the high level of S100A4 in PP and low level in PN reflected its transcriptional activation and/or various aspects of the protein turnover (for example, stabilization owing to modifications or enhanced secretion),

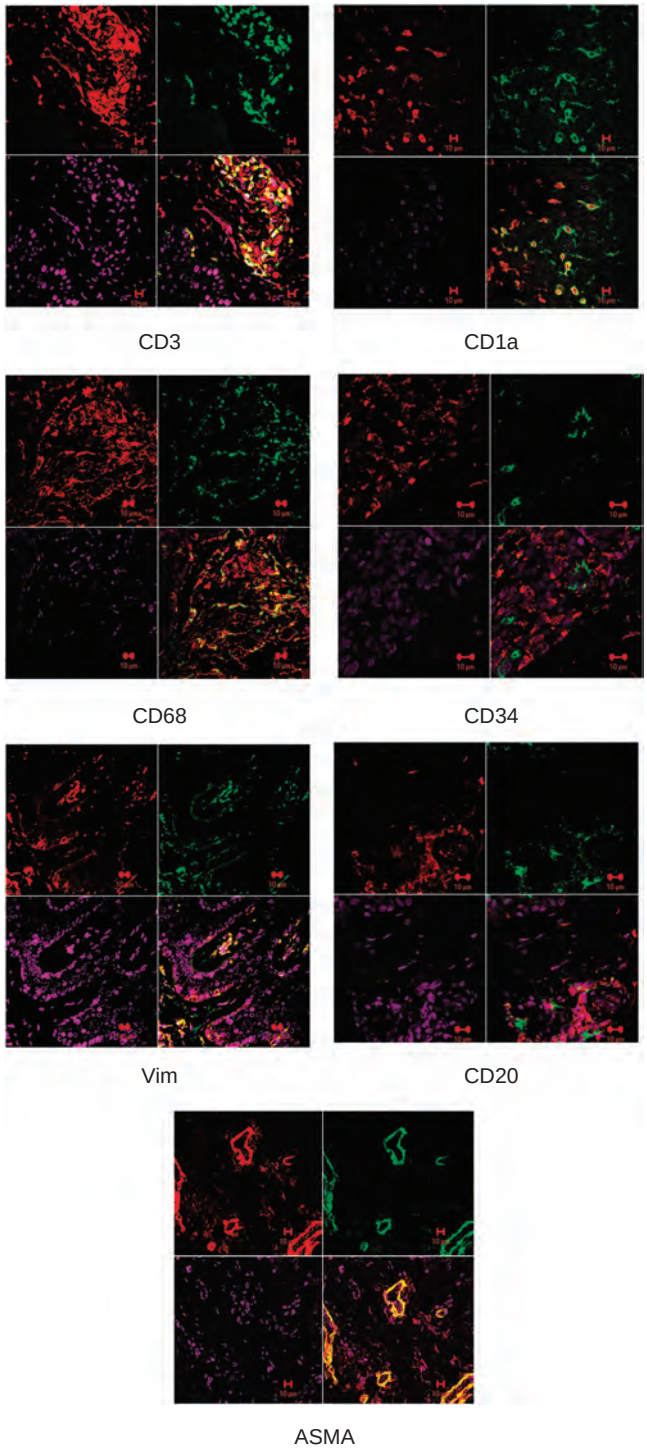

Figure 3. Cell-specific localization of S100A4 in psoriatic skin. Confocal microscopy images of S100A4 (red) colocalization with cell-specific markers (green) in psoriatic skin biopsies assessed by double immunofluorescence staining with antibodies against: anti-CD3 (T-lymphocytes), anti-CD1a (dendritic cells), anti-CD68 (macrophages), anti-CD34 (stem cells), Vim (cells of mesenchymal origin), anti-CD20 (B-lymphocytes), ASMA (mostly pericytes). Cell nuclei were visualized with DNA-specific dye To-Pro (blue). Scale bars = 10 μm.

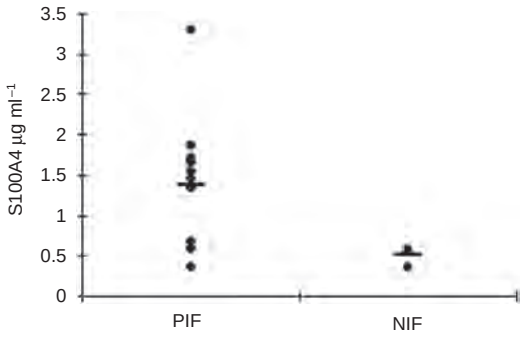

Figure 4. S100A4 is released from psoriatic skin to the interstitial fluid. Biopsies from involved psoriatic skin ($n=11$) and healthy skin ($n=4$) were transferred to DMEM and cut into small pieces and incubated for 24 hours. S100A4 levels in the interstitial fluid from PP biopsies (PIF) and NN biopsies (NIF) were determined by sandwich ELISA assay. Dots are individual measurements and bars are median. The data were calibrated by the most abundant protein band common from all samples found by SDS-PAGE.

we studied the same biopsy samples for S100A4-specific q-RT-PCR (Figure 5b). The data obtained showed a significant transcriptional upregulation of S100A4 in PP compared with PN ($P=0.03$).

Previous studies on the models of cancer progression and RA reveal that S100A4 has a potential in modulating the activity of the tumor suppressor p53 protein (Grigorian *et al.*, 2001). Here, we also tested the expression of p53 in PP and PN using p53 mAb in immunohistochemical stainings. A clear, strong p53-specific positive pattern was observed in the basal layer of the epidermis (Figure 6a and c) adjoining to the dermal compartment enriched with active S100A4 in PP biopsies (Figure 1c-1 and c-2), whereas in PN, with a lower S100A4 expression (Figure 1b-1 and b-2), scattered p53-positive cells were observed all through the epidermal layer (Figure 6b and d). These data may associate upregulation and/or stabilization of p53 with upregulation of S100A4 in PP.

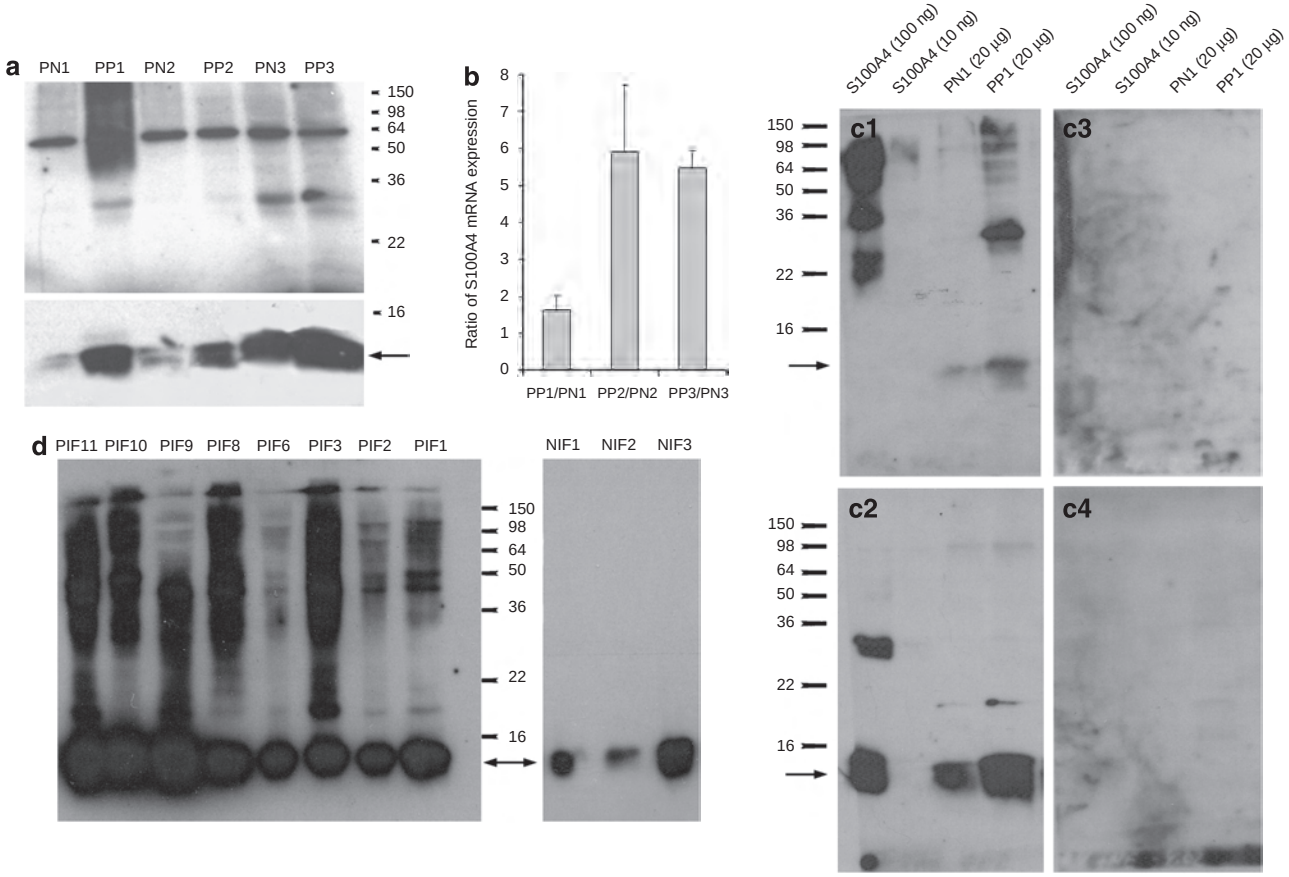

Figure 5. Oligomeric S100A4 was detected in biopsies and in supernatant from psoriatic skin. (**a**) Proteins (20 µg) were subjected to SDS–PAGE and analyzed by western blotting using S100A4 mAb. Protein lysates loaded in a low-reducing buffer (1% β-mercaptoethanol and boiling) from PP ($n=3$) and corresponding PN biopsies ($n=3$) were analyzed. (**b**) Transcriptional expression of the same samples was analyzed using q-RT-PCR. Displayed are the $2^{-\Delta\Delta ct}$ and SEM, representing the ratio in involved psoriatic skin S100A4 gene expression normalized to the GAPDH endogenous reference gene and relative to the patient-matched non-involved psoriatic skin ($n=3$). A paired *t*-test was carried out on each duplicate S100A4 C_t normalized to GAPDH C_t between involved and non-involved psoriatic skin ($P=0.03$). (**c-1**–**c-4**) Oligomeric recombinant S100A4 in two concentrations (100 and 10 ng) and protein lysates from PN1 and PP1 (20 µg) were subjected to PAAG electrophoresis in non-reducing buffer (without β-mercaptoethanol and boiling) (**c-1** and **c-3**) and high-reducing buffer (5% β-mercaptoethanol and boiling) (**c-2** and **c-4**). After blotting, the membranes were probed with S100A4 mAb *per se* (**c-1** and **c-2**) and S100A4 mAb pre-incubated with recombinant S100A4 protein in the molar ratio of mAb/protein 1:5 (**c-3** and **c-4**) for specific neutralization of S100A4. (**d**) Biopsies from involved psoriatic skin ($n=8$) and healthy skin ($n=3$) were transferred to DMEM and cut into small pieces and incubated for 24 hours. S100A4 levels in the released interstitial fluid from PP biopsies (PIF) and NN biopsies (NIF) were analyzed in a low-reducing buffer. Arrows designate S100A4 at 11 kDa.

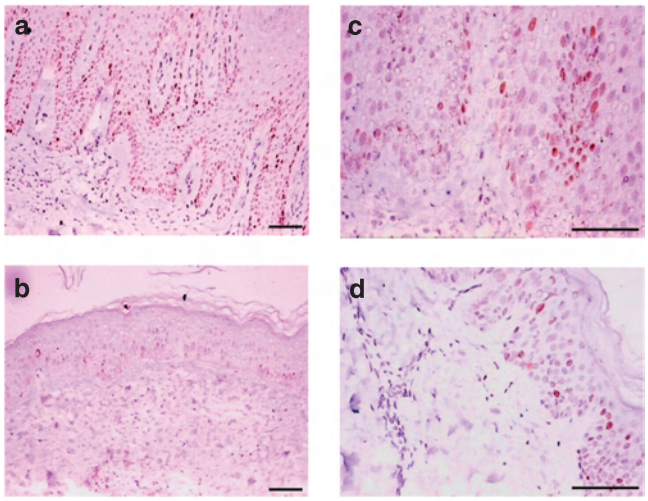

Figure 6. p53 expression in psoriatic skin. The p53 expression was assessed by immunohistochemical staining using p53 antibodies on PP biopsies ($n=2$) (**a** and **c**) and compared with corresponding PN biopsies ($n=2$) (**b** and **d**). Scale bars = 100 μm.

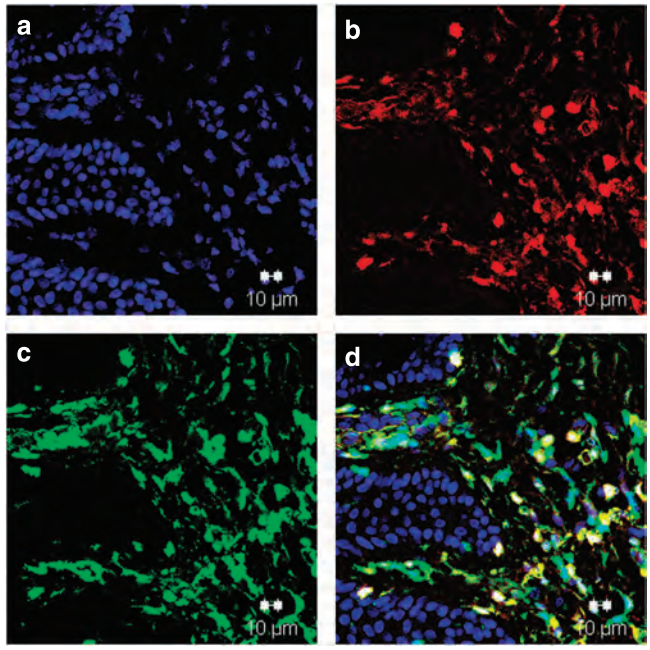

Figure 7. Both human and murine S100A4 are expressed in the human psoriasis xenograft model. One SCID mouse was xenografted with psoriatic skin and a biopsy was obtained 14 days after transplantation. Double immunofluorescence staining of the biopsy was carried out. A section was labeled with: (**a**) 4′, 6-diamidino-2-phenylindole (DAPI) to identify cell nuclei, (**b**) S100A4 mAb (mouse anti-human monoclonal S100A4) and (**c**) S100A4 pAb (rabbit anti-human and mouse polyclonal S100A4). In (**d**), images were merged. Scale bars = 10 μm.

Effect of S100A4 pAb on psoriasis features in a human psoriasis xenograft SCID mouse model

To investigate whether the expression of S100A4 in psoriatic skin is significant for the pathogenesis of the disease, we tested the effect of S100A4 pAb in a human psoriasis xenograft SCID mouse model. First, we determined human or murine S100A4 protein expression in the human psoriasis skin xenograft in biopsies obtained 14 days after transplantation. Staining the biopsies with human monoclonal anti-S100A4 recognizing only human S100A4, and S100A4 rabbit pAb recognizing both human and mouse S100A4 revealed among an ample quantity of pAb-positive cells a pool of cells also positive for human monoclonal anti-S100A4 (Figure 7). These data show that cells and S100A4 from both species, respectively, contribute to the shaping of the psoriatic milieu in this model. Therefore, to target the entire pool of the S100A4 protein in the human psoriasis xenograft mouse model, we used affinity-purified polyclonal antibodies. Antibodies were injected intraperitoneally in two different dose regimes in two mice in each group. Three control mice were injected with rabbit IgG (see the protocol in Materials and Methods). Two weeks after the last treatment, the mice were killed and various parameters of the psoriatic lesions were examined. We observed an apparent dose-dependent effect of S100A4 pAb on the hallmark features of psoriasis in mice. Thus, the epidermal thickness was significantly reduced (1.5-fold) when comparing the low dose with controls ($P<0.05$) and highly significant (3-fold) when comparing the high dose with controls ($P<0.005$) (Figures 8a and 9). Moreover, the amount of proliferating cells (Ki-67$^+$) were significantly reduced at both S100A4 pAb dose regimes compared with controls ($P<0.05$) (Figures 8b and 9). Staining for T cells (CD3$^+$) was not altered in the S100A4 pAb-treated mice compared with control mice (data not shown).

Importantly, we found signs of impaired vascularization in psoriatic skin after administration of antibodies. Thus, staining of skin biopsies with antibodies to alpha-smooth muscle actin revealed both a reduced number of blood vessels and defective capillary walls (thinner layer of alpha-smooth muscle actin-positive pericytes), which can possibly lead to vascular leakage (Figure 9).

DISCUSSION

Psoriasis is a disorder of the immune system characterized by the uncontrolled proliferation of keratinocytes. Existing evidence supports the concept of psoriasis as a chronic skin inflammation mediated by T cells, DCs, and inflammatory cytokines. A massive upregulation of several small calcium-binding S100 proteins has been reported as well. Many members of the S100 family are encoded in the epidermal differentiation complex located on chromosome 1q21 (Engelkamp *et al.*, 1993). This region is of particular interest, as it encodes several other genes expressed in epidermal keratinocytes (Mischke *et al.*, 1996; South *et al.*, 1999). Thus, the expression of S100A7 and S100A8/9, studied by means of real-time PCR, was shown to be strongly (around 100 folds) elevated in psoriatic plaques (Wolk *et al.*, 2006). In addition, hyperproliferation and abnormal differentiation of psoriatic keratinocytes are associated with a massive upregulation and secretion of S100A8/9 (Benoit *et al.*, 2006). However, the role of the proteins and pathways implicated remains unclear so far.

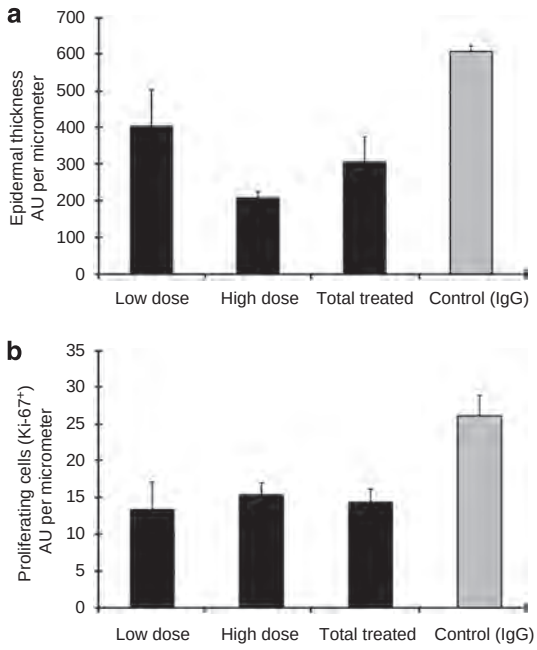

Figure 8. Treatment with S100A4 pAb blocked the S100A4 expression in the human psoriasis xenograft model. Polyclonal antibodies S100A4 were given three times in a low dose 5 mg kg^{-1} × 3 (n = 2) or high dose 10 mg kg^{-1} × 3 (n = 2), and in control mice 10 mg kg^{-1} rabbit IgG × 3 (n = 3) in week 1 followed by three times half dose administration in week 2. One week after the last treatment, the mice were killed and biopsies were obtained. The effect of the treatment was assessed by the epidermal thickness (**a**), and number of proliferating cells, Ki-67$^+$ nuclear antigen (**b**). Data were analyzed by imaging digital pixel-quantifying software ImageJ. Illustrated are the mean and SEM of analyzed arbitrary units (AU) per μm for low and high dose, total treated (mean of low and high), and controls (IgG).

Our interest in studying the expression and functional significance of S100A4 in psoriasis was based on a large body of evidence on its implication in the inflammatory constituent in various pathological milieu, such as tumor stroma and RA (Schmidt-Hansen *et al.*, 2004a, b; Grum-Schwensen *et al.*, 2005; Cabezon *et al.*, 2007; Klingelhofer *et al.*, 2007).

First, we studied the expression of S100A4 in psoriatic skin and compared it with an adjacent, non-involved skin from the same patients and skin biopsies from healthy subjects, and found massive upregulation of S100A4 in the upper dermal part in psoriatic skin compared with its much lower levels in non-involved psoriatic skin and especially in healthy skin. Furthermore, q-RT-PCR analysis data indicated that the upregulation of S100A4 occurs on the transcriptional level. After *ex vivo* incubation of biopsies, we observed a significant release of S100A4 into the extracellular space. More interestingly, S100A4 released from psoriatic skin is folded in the high-molecular oligomeric structure compared with S100A4 released from healthy skin. Earlier, we showed in various models (stimulation of tumor cell, differentiation of primary neurons and cardiomyocytes) that the biologically active function of S100A4 is attributed to its oligomeric, but not dimeric forms as it is postulated for several members of the S100 family (Novitskaya *et al.*, 2000; Ambartsumian *et al.*, 2001; Schmidt-Hansen *et al.*, 2004a; Klingelhofer *et al.*, 2007). Later on, the association of S100 protein oligomerization and biological function was attributed to other members of the S100 family, such as S100A8/A9 (Leukert *et al.*, 2006) and S100B (Ostendorp *et al.*, 2007) as well.

In our experiments, we were curious to compare the expression pattern of S100A4 with that of other psoriasis-associated S100 proteins, S100A7 and S100A8/9. Upregulation of these proteins was shown earlier in the epidermal compartment of the skin. We detected highly specific expression of S100A7 exclusively in the epidermis of psoriasis, but it was undetectable in non-involved psoriatic skin, although a moderate expression of S100A7 was observed by others in the stratum basale and spinosum of normal epidermis (Broome *et al.*, 2003). S100A8/9 revealed a rather high expression pattern in the epidermis, whereas it showed a clustered and scattered pattern in the dermis in both involved and non-involved psoriatic skin. The data obtained definitely show a distinctive pattern of S100A4 expression in inflamed skin compared with other S100A proteins.

The main difference is that unlike other S100 proteins, the expression of S100A4 colocalized with cell-specific markers on various cell types of non-epithelial origin, such as fibroblasts, pericytes, T-lymphocytes, macrophages, and dendritic cells but not B cells and hematopoietic stem cells (CD34$^+$). These data are in concordance with our previous observations on the cell-specific expression of S100A4 in tumor stroma and synovial tissue in patients with rheumatoid arthritis (Schmidt-Hansen *et al.*, 2004a, b; Grum-Schwensen *et al.*, 2005; Cabezon *et al.*, 2007; Klingelhofer *et al.*, 2007). Furthermore, we have shown earlier that the upregulation of S100A4 in tumor stroma has a functional significance in tumor metastases (Grum-Schwensen *et al.*, 2005; Cabezon *et al.*, 2007). These data along with data obtained in other groups, which have an important role of S100A4 in the activation of stem cells at the onset of hair follicle regeneration and proliferation (Ito and Kizawa, 2001), allow us to consider the implication of S100A4 in the pathogenesis of psoriasis.

Earlier, we reported a strong functional and physical association between the tumor suppressor p53 protein and S100A4, namely the binding of S100A4 with the C-terminal domain of p53 (Chen *et al.*, 2001; Grigorian *et al.*, 2001; Schmidt-Hansen *et al.*, 2004a). Moreover, we showed that the extracellular oligomeric S100A4 is able to enhance the level of p53 and modulate a gene expression profile in synovial fibroblasts from RA patients (Klingelhofer *et al.*, 2007). That could be explained by the finding that the interaction between S100A4 and p53 affects the oligomerization and, as a result, functional activity of p53 by binding of S100A4 to the p53 tetramerization domain (Fernandez-Fernandez *et al.*, 2005). In agreement with earlier studies (Tadini *et al.*, 1989), we also found a strong p53-positivity in the basal layer of epidermis in psoriatic lesions in close proximity to the dermal zone enriched with S100A4-expression, suggesting that S100A4 could modulate p53 function resulting in a cell-hyperproliferative condition in

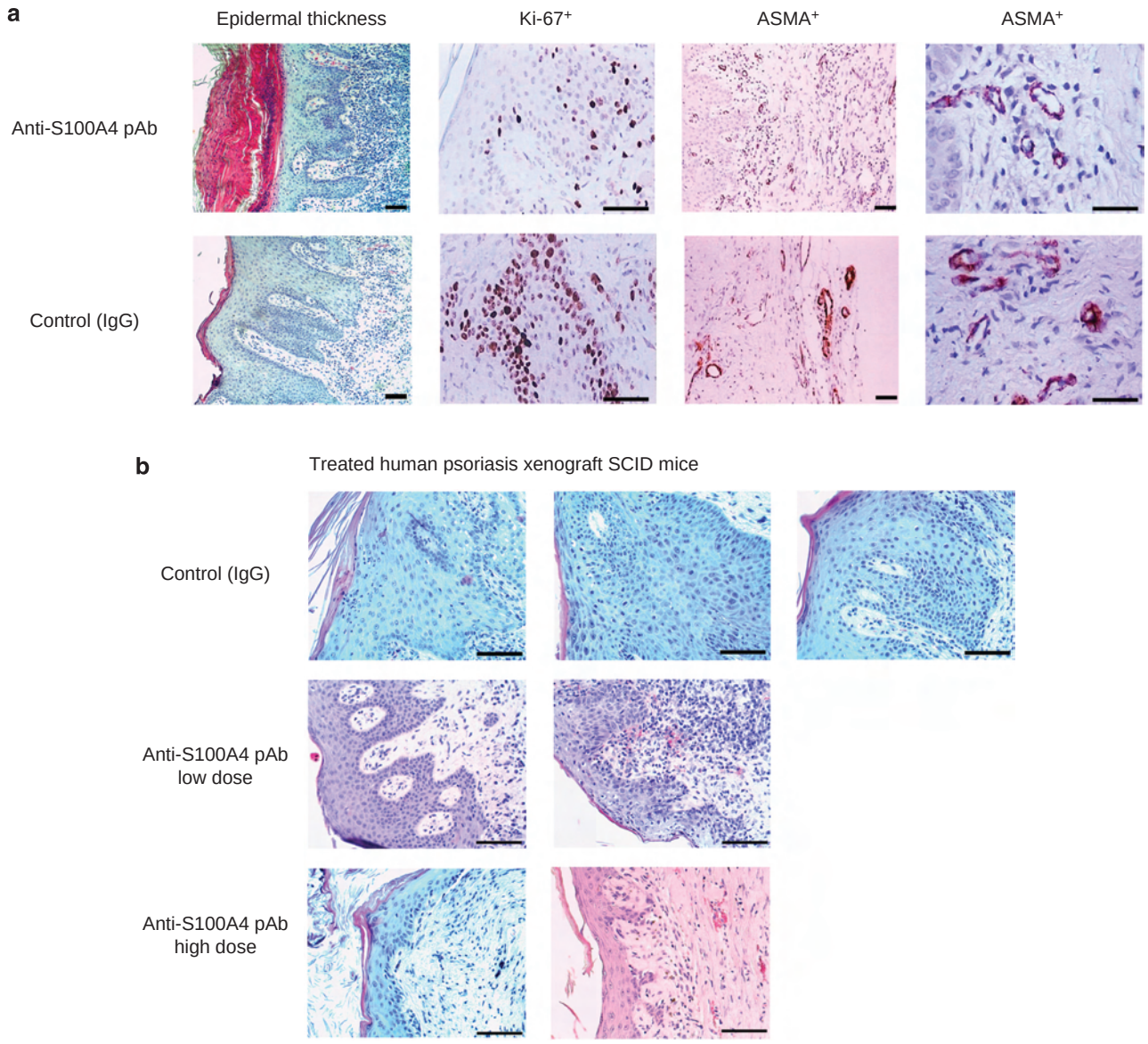

Figure 9. Treatment with S100A4 pAb in psoriasis results in changed epidermal thickness, number of proliferating cells and endothelial cell morphology. Human psoriasis xenograft SCID mice were treated with S100A4 pAb in two-dose regimens. (**a**) Biopsies were obtained from treated mice and subjected to hematoxylin–eosin staining, immunohistochemistry staining for ki-67, and ASMA stainings. Illustrated is the averaged epidermal thickness. (**b**) The epidermal thickness of all treated mice is illustrated. Scale bars = 100 μm.

psoriasis. We propose that the modulation of p53 could be associated with the S100A4-induced alteration of its oligomerization status, which may profoundly affect p53 function (Joerger and Fersht, 2008).

We found solid evidence on the essential role of S100A4 in the pathogenesis of psoriasis by successful blocking of S100A4 in the human psoriasis xenograft SCID mouse model. This humanized model is suitable for studying psoriasis pathogenesis and therapeutic interventions (Boehncke and Schon, 2007). It was shown that in the xenografted human skin, direct anastomoses between murine and human blood vessels are formed with the participation of both murine and human endothelial cells (Boehncke, 1997). Here, we show that the dermal compartment of the xenograft is populated with both mouse and human cells producing S100A4 specific for both species. Therefore, we chose S100A4 pAb for targeting of the protein in this ''mixed'' mouse/human model. We carried out the antibody treatment in two-dose regimens, a high and a low, during 3 weeks. Control mice received high doses of rabbit IgG. In both the tested regimes, we did see a statistically significant and rather strong reduction of epidermal thickness. Furthermore, the blocking of S100A4 protein resulted in decreased proliferative activity of cells in the basal layer assessed by Ki-67 immunopositivity. In addition, we observed evident impairment in the vascular system of the xenografted psoriatic skin that is marked by fewer and smaller vessels with weaker staining for alpha-smooth muscle actin pointing to a less developed vessel wall. We have observed

these phenomena earlier in the microenvironment of tumors raised in S100A4-deficient mice, which are not able to support sufficiently tumor development and metastases (Grum-Schwensen *et al.*, 2005). Moreover, we reported earlier a pro-angiogenic function of S100A4 (Ambartsumian *et al.*, 2001), which could give additional explanations of the damaged vascularization on diminishing S100A4. In conclusion, we suggest that S100A4 is an essential contributor to the pathogenesis of psoriasis. We assume that cells activated (for example, T-cells) at the initial stages of the disease produce factors (cytokines, growth factors, etc) stimulating high-level production and release of the S100A4 protein in the dermis of psoriatic skin. Further, the biologically active S100A4 could trigger and fuel pathways implicated in the modulation of gene expression (for example, tumor suppressor p53) and thereby have an effect on the cell proliferation, angiogenesis, and additional attraction of immune cells. This circuit likely shapes the pathogenesis and progression of psoriasis. We suggest that the inhibition of S100A4 has a potential in quenching the excited molecular events in psoriasis and propose that further exploration of the role of S100A4 and anti-S100A4 tools will be profitable in the therapy of psoriasis.

MATERIALS AND METHODS

Patients

Thirteen Caucasian patients with moderate to severe psoriasis vulgaris were randomly selected from the Department of Dermato-Allergology, Copenhagen University Hospital Gentofte. None of the patients have used any systemically immunosuppressive medications 4 weeks before the study and no local treatment 2 weeks before participating in the study. Eight healthy subjects were recruited in the control group. The study was carried out in agreement with the Declaration of Helsinki Principles and approved by the Danish National Committee on Biomedical Research Ethics. All subjects gave informed consent.

Punch biopsies (4 mm) were taken from involved psoriasis skin lesions (PP) and non-involved psoriasis skin (PN). One skin biopsy was obtained from each of the healthy subjects (NN).

For immunohistochemistry analysis, biopsies were fixed in 4% buffered formalin and embedded in paraffin. For the analysis of S100A4 in PIFs and NIFs, biopsies were transferred to 0.5 ml of DMEM with 50 IU ml^{-1} penicillin, 50 IU ml^{-1} streptomycin (all from Cambrex, Verviers, Belgium), cut into small pieces, and incubated at 37 °C, 5% CO_2, in 24-well incubation plates for 24 hours, followed by centrifugation to remove the cells and debris.

For western blot analysis and q-RT-PCR, the biopsy samples were snap-frozen, ground in a mortar with a pestle in liquid nitrogen, and immediately transferred to either a lysis buffer containing proteinase mix inhibitors (Sigma-Aldrich, Brøndby, Denmark) or a lysis/binding buffer for RNA purification (Applied Biosystems, Warrington, UK). After incubation in lysis buffer, the samples were homogenized with a rotor stator (IKA, Staufen, Germany).

Immunohistochemical and immunofluorescence studies

For immunohistochemical and immunofluorescence analyses, 4 μm paraffin sections were used. To unmask the antigens, sections were boiled in 0.01 M Tris–EDTA buffer (pH 9.0) in a microwave oven for 2.5 minutes at 600 W followed by 6 minutes at 220 W, rinsed in TBS supplemented with 1% FCS, and incubated with primary antibodies overnight in a cold room. We used the following dilutions of the antibodies: polyclonal and monoclonal anti-S100A4; 1:2,000 (produced in the Department of Molecular Cancer Biology, Danish Cancer Society); polyclonal anti-S100A9; 1:4,000, anti-S100A7; 1:2,000 and anti-S100A8; 1:4,000 (all provided by Dr J Celis, Danish Cancer Society, Copenhagen, Denmark); anti-alpha smooth muscle actin (ASMA), 1:4,000; (Sigma-Aldrich, Brøndby, Denmark); anti-p53 DO1, 1:500 (provided by Dr J Bartek, Danish Cancer Society); and monoclonal mouse anti-human anti-Ki-67 (MIB-1); diluted 1:50 (Dako, Glostrup, Denmark).

The detection procedures were conducted according to the manufacturer's protocol for the EnVision detection system/alkaline phosphatase (Dako).

All stained sections were counterstained with hematoxylin (Merck, Darmstadt, Germany).

Negative controls were performed with omission of the primary antibody. For the double-labeling experiments, the following antibodies were used: polyclonal and monoclonal anti-S100A4 (diluted 1:2,000; produced by M. Grigorian); monoclonal anti-CD68 (diluted 1:100; Dako); anti-CD1a (diluted 1:200; Dako); anti-CD3 (diluted 1:300; Abcam, Cambridge, UK); anti-vimentin (diluted 1:500; Dako); anti-CD20cy (diluted 1:100; Dako); anti-CD34 (diluted 1:200, Dako). Secondary antibodies coupled to Alexa 488 or Alexa 568 were used in dilution 1:1,500 (Molecular Probes, Leiden, The Netherlands). Samples were analyzed by confocal microscopy using an LSM 510 microscope (Carl Zeiss MicroImaging, Oberkochen, Germany).

Sandwich ELISA

PIFs and NIFs from biopsies were analyzed for S100A4 concentrations by sandwich ELISA, using the modifications mentioned in Klingelhofer *et al.* (2007). Briefly, the ELISA system was calibrated with the purified recombinant His-tagged S100A4 protein in a concentration range of 0.31–200 ng ml^{-1}. Microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were coated with 100 μl of a 1.25 μg ml^{-1} volume of S100A4 pAb in a 0.025 M sodium carbonate buffer, pH 9.5, and were incubated overnight in a cold room. Plates were then washed with 1 × PBS–0.1% Tween 20 (washing buffer) and 2 × SuperBlock PBS (Pierce, Rockford, IL). PIFs and NIFs from the biopsies, at various dilutions, were added to SuperBlock PBS and incubated for 1 hour at 30 °C, with agitation. Plates were washed and incubated with 1.25 μg ml^{-1} of S100A4 mAb for 1 hour at 30 °C. After washing, HRP-labeled rabbit anti-mouse antibodies (1:2,000 dilution; Dako) in SuperBlock PBS diluted 1:5 with PBS were added and incubated for 1 hour at 30 °C. Plates were washed and developed TMB Plus (3, 3′,5′-tetramethylbenzidine plus hydrogen peroxide; Kem-En-Tec, Taastrup, Denmark). Absorbance at 450 nm was measured using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA).

Western blot analysis

Total protein (20 μg) in low-reducing protein loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue) were boiled and loaded on either 4–20 or 15% gradient SDS-PAGE. For high-reducing conditions, 5% β-mercaptoethanol, and for non-reducing conditions 0% β-mercaptoethanol was used. To test for specificity of antibodies,

polyclonal anti-S100A4 antibodies were pre-incubated with the oligomeric recombinant, S100A4 protein, in the molar ratio 1:5. Protein-containing samples were transferred onto an Immobilon TM transfer membrane (Millipore, Bedford, MA). The membrane was blocked for 20 minutes with 5% non-fat milk in Tris-buffered saline buffer plus 5% FCS and then incubated with S100A4 pAb (1:1,000) for 1 hour, followed by incubation with horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (Dako) at a dilution of 1:2,000. Positive bands were visualized by enhanced chemiluminescence using an ECL SuperSignal reagent in a western blotting detection system (Pierce).

Transcriptional level of S100A4

Quantitative real-time polymerase chain reaction (q-RT-PCR) was performed to investigate the mRNA expression level of S100A4 in psoriatic and non-involved psoriatic skin from the same patient samples as analyzed for western blot analysis. Total RNA was isolated using the *mir*Vana miRNA Isolation Kit (Applied Biosystems) following the manufacturer's instructions. Quality measurements of the RNA were assessed using an Agilent RNA 6000 Nano Assay on an Agilent 2100 Bioanalyzer (Agilent Technologies, Naerum, Denmark) having an RNA integrity number of more than 7.0. For the generation of cDNA, we reverse-transcribed 4.5 ng total RNA using random hexamer primers and Transcriptor Reverse Transcriptase (Exiqon, Vedbaek, Denmark). The S100A4 gene (TTGTGTCCACCT TCCACA and GCTGTCCAAGTTGCTCATCA) (TAG, Copenhagen, Denmark) and the household gene, GAPDH (AAGGGTCTACA TGGC and CGACCACTTTGTCAA) (DNA Technology, Aarhus, Denmark), were amplified using the Brilliant SYBR green QPCR master mix (Stratagene, Aarhus, Denmark) and detected by q-RT-PCR and analyzed by MxPro (Stratagene). The S100A4 mRNA was normalized to GAPDH and the difference in expression by comparing PP with PN was determined by $2^{-\Delta\Delta ct}$.

The human psoriasis xenograft SCID mouse model

For the human psoriasis xenograft model, a patient with severe plaque psoriasis was recruited. A keratome biopsy of psoriatic skin with an area of 8×3 cm, containing epidermis, was obtained after informed consent from the patient. The study was approved by the Danish National Committee on Biomedical Research Ethics and the Danish Experimental Animal Inspectorate. Immunocompromised SCID mice were kept under pathogen-free conditions throughout the study. The keratome biopsy was divided into eight pieces of 1.5×1.5 cm and transplanted using Histoacryl topical skin adhesive (TissueSeal, MI, USA) onto the back of eight 2-month-old C.B-17 SCID mice (Taconic, Ry, Denmark). All transplantations were successfully accepted. Two weeks after transplantation, mice were randomized into three groups for the intraperitoneal injections of (i) control (10 mg kg^{-1} rabbit IgG) ($n = 3$) (vehicle, Sandoz, Basel, Switzerland); (ii) S100A4 pAb at a dose of 10 mg kg^{-1} on days 1, 3, and 5 followed by 5 mg kg^{-1} on days 7, 11, and 14 ($n = 2$); (iii) S100A4 pAb at a dose of 5 mg kg^{-1} on days 1, 3, and 5 and 2.5 mg kg^{-1} on days 7, 11, and 14 ($n = 2$) (pAb was raised in the Department of Molecular Cancer Biology). On day 25, mice were killed, and xenografts were harvested and fixed in formalin for paraffin embedding. Sections were processed for histological analyses by staining with H&E (Merck, Glostrup, Denmark), and immunoprobed for Ki-67 and ASMA.

Histological evaluation

All sections were blinded before evaluation and investigated. To assess the epidermal thickness, H&E-stained sections were evaluated by importing a digital image of each section to a digital image processing program (Adobe Photoshop Elements 3, Alleroed, Denmark) where the epidermis (without the stratum corneum) was cropped out and exported to a digital pixel-quantifying software (ImageJ, http://rsbweb.nih.gov/, Bethesda, MD), where each epidermal layer was converted to an 8-bit black and white format with the highest possible threshold for color intensity. Finally, the pixels were measured and related to the epidermal length in μm, and the data were summarized as the mean ± SEM.

To assess the number of proliferative cells, sections stained for Ki-67 were evaluated by importing the picture to the digital image processing program (Adobe Photoshop elements 3) where all positive ki-67 cells were cropped out and exported to the digital pixel-quantifying software (ImageJ), where the picture was converted to an 8-bit black and white format with the highest possible threshold for color intensity. Finally, the pixels were measured and related to the epidermal length in μm, and the data were summarized as the mean ± SEM.

The sections stained for p53, ASMA, S100A7, S100A8, and S100A9 were evaluated in the epidermis and upper layer of dermis for the number of positively stained cells per micrometer length of the section and for morphology changes.

Statistical analysis of data

All tests were two sided *t*-tests.

CONFLICT OF INTEREST

The authors state no conflicts of interest.

ACKNOWLEDGMENTS

The authors of this article thank: Inge Skibshøj and Eugene Lukanidin Deparment of Molecular Cancer Biology, Danish Cancer Society; Eric Santoni-Rugiu, Margit Bæksted, and Jette Pedersen, Bartholin Institute, Rigshospitalet; Mette Ramm, Department of Dermatology, Copenhagen University Hospital Gentofte; Marianne Fregil, Department of Oncology, Copenhagen University Hospital Herlev, for their enthusiastic participation in this project. Finally, we thank the Danish Psoriasis Foundation, EU grant TuMIC-Health-F2-2008-201662 and Københavns Amts Forskningsfond for financial support.

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