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Ribosomal protein S6 is hyper-activated and differentially phosphorylated in epidermal lesions of patients with psoriasis and atopic dermatitis

Running title: S6 phosphorylation and hyper-proliferative epidermis

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Conflict of interest:

Authors have no conflict of interest.

What is already known about this topic?

- Phosphorylation of ribosomal protein S6 (S6) is important for translation initiation.
- S6 becomes phosphorylated via the mammalian target of rapamycin (mTOR) pathway, which is activated in psoriatic skin.

What does this study add?

- In psoriasis and atopic dermatitis S6 phosphorylation is associated with inflammation and epidermal hyper-proliferation.
- S6 is differentially phosphorylated in distinct layers of the diseased epidermis, which may impact keratinocyte proliferation/differentiation

Abstract:

Background: The ribosomal protein S6 (S6) is part of the translation machinery and activated by phosphorylation via the mammalian target of rapamycin (mTOR) pathway, which is activated in psoriatic skin.

Objectives: To investigate which S6 sites are phosphorylated in psoriasis and atopic dermatitis (AD), and to study whether S6 phosphorylation is associated with inflammation and/or keratinocyte hyper-proliferation.

Methods: Healthy skin and skin lesions of patients with psoriasis and AD were investigated by immunostaining using antibodies which stain proliferating cells, leukocytes, and distinct phosphorylated sites of S6.

Results: All psoriasis and AD lesions revealed abnormal S6 phosphorylation in the epidermis. Extent of S6 phosphorylation was diverse, generally stronger in psoriasis, and correlated in both diseases with inflammation. S6 showed differential phosphorylation in distinct epidermal layers, which was most pronounced in hyper-proliferative regions.

Conclusions: Differential S6 phosphorylation may have a role in abnormal keratinocyte proliferation/differentiation.

Introduction:

Ribosomal protein S6 (S6) is part of the 40S ribosomal subunit of the translation machinery¹. Phosphorylation of S6 is required for translation initiation and occurs on a cluster of five serine residues at the carboxyl terminus of the protein^{2,3}. The mTOR pathway seems to be the predominant regulator of S6 activity. mTOR is a serin/threonine kinase that is part of a protein complex involved in the regulation of cell growth, proliferation, protein synthesis and autophagy, and has emerged as an important target for the treatment of certain cancers and inflammatory diseases. mTOR signalling activates S6 kinase which phosphorylates Ser235, Ser236, Ser240 and Ser244 of S6, and inhibition of mTOR causes a drastic reduction in S6 activation⁴. However, S6Ser235 and S6Ser236 can also be phosphorylated via the RAS/ERK pathway⁵. Ser247 of S6 seems to be a target of the casein kinase 1 (CK1) family of protein kinases⁶.

Psoriasis and atopic dermatitis (AD) are inflammatory skin diseases with different aetiology, but often similar clinical appearance due to keratinocyte hyper-proliferation and incomplete differentiation of the epidermis. Clinical data suggest that mTOR inhibitors provide therapeutic benefit for psoriasis^{7,8}. Moreover, mTOR signalling and S6(Ser235/236) phosphorylation was found to be increased in psoriatic skin.⁹

We have investigated S6 activation in depth by using antibodies, which detect specifically S6(Ser235/236), S6(Ser240/244) or S6(Ser240) phosphorylation sites. To evaluate if S6 activation is psoriasis specific or a more general characteristic of epidermal inflammation, we also analysed AD biopsies. In addition, we investigated, whether S6 phosphorylation is associated with inflammation and/or hyper-proliferation.

Material and Methods:

The study was approved by the ethical committee of the University Basel (EK.242/13). All analysed biopsies were from patients that have not undergone therapy known to affect the phosphorylation status of S6.

5µm thin paraffin sections from lesional (psoriasis n=10; AD n=5) and healthy skin were de-waxed, heat pre-treated with 0.1m citrate buffer (pH 6.0) and stained with the following antibodies: Rabbit anti-phospho-S6(Ser235/236) (Cell Signaling #2211; 1:400); rabbit anti-phospho-S6(Ser240/244) (Cell Signaling #2215; 1:400); mouse anti-phospho-S6(Ser240) (DAKO #M7300; 1:100); rabbit anti-Ki67 (DAKO #A0067; 1:200); mouse anti-CD45 (DAKO M0701; 1:100). Immunofluorescence staining was performed by using secondary antibodies coupled to Alexa-fluor488 or Alexa-fluor594 (Invitrogen; 1:500) and mounted with Prolong Gold/DAPI (Invitrogen).

Control immunostaining experiments utilizing tumour xenografts treated with mTOR inhibitor (RAD001) or placebo confirmed that the used phospho-S6 antibodies are highly specific for mTOR regulated phosphorylation sites (data not shown)

Results:

While healthy human skin revealed detectable S6 activation only in the stratum granulosum (**Fig.1, a1-3**), all investigated psoriatic lesions showed abnormal phosphorylation of S6 in supra-basal and differentiating cell layers of the epidermis (**Fig.1, a4-6 and a7-9**). The staining pattern of S6 phosphorylation was diverse amongst psoriasis patients. While some lesions revealed phosphorylation in all epidermal cell layers (**e.g. psoriasis I; Fig.1, a4-6**), others showed phospho-S6 staining in individual cells or cell clusters (**e.g. psoriasis II; Fig.1, a7-9**). Psoriasis I and II illustrate extreme examples of different patients. Such differences were generally less pronounced within lesions.

Abnormal S6 phosphorylation was also observed in all investigated AD samples. The staining pattern showed also variation (**Fig.2, a1-3 and a4-6**), but S6 activation was generally less prominent.

Abnormal S6 phosphorylation was associated with inflammation. Double immunofluorescence staining of phosphorylated S6 and the pan-lymphocyte marker CD45 revealed that S6 phosphorylation was generally much more extensive in severely inflamed areas than in those containing low numbers of inflammatory cells, both in psoriasis (**Fig.1, b1-2 and supporting information**) and in AD (**Fig.2, b1-b2**). The shown images illustrate extreme examples of strongly and weakly inflamed lesions from different patients. Supra-basal cell layers of strongly inflamed lesions contained many phospho-S6/Ki67 double positive keratinocytes (**Fig.1, c2**), whereas proliferating keratinocytes in basal layers were mostly devoid of activated S6. Phosphorylated S6 was not detected in proliferating keratinocytes of healthy human epidermis (**Fig.1, c1**).

Most notably, antibodies against different phosphorylation sites of S6 exhibited distinct staining patterns. This was most evident in strongly inflamed, highly active areas where all supra-basal epidermal layers showed S6 activation (**Fig.1, a4-6**). Such lesions revealed S6(Ser240) phosphorylation predominantly in lower epidermal layers (**Fig.1, a5-6**) containing many proliferating cells (**Fig.1, c2**), whereas S6(Ser235/236) phosphorylation was found both in supra-basal and differentiating compartments (**Fig.1, a4 and a6**). S6 phosphorylation in basal epidermal cells was generally rare and restricted to small areas. In contrast, weakly inflamed areas revealed supra-basal co-phosphorylation of S6(Ser240) and S6(Ser235/236) in only a subset of cells, but pronounced S6(Ser240) phosphorylation in differentiating layers of the epidermis (**Fig.1,a7-9 and supporting information Fig.2d**).

An antibody recognizing both S6(Ser240) and S6(Ser244) phosphorylation also revealed a staining pattern which differed from that obtained with the S6(Ser240) selective antibody, and indicated stronger S6(Ser244) phosphorylation in differentiating compartments of the hyper-proliferative epidermis (**Fig.1, d1-2**).

Discussion:

Our findings confirm that the mTOR pathway is activated in psoriatic skin, but do not support the conclusion that S6 is predominantly activated in upper epidermal layers⁹. Buerger *et al.* used S6(Ser235/236) phosphorylation as downstream marker for mTOR activity, but phosphorylation of this site may also be stimulated by RAS/ERK signalling⁵, known to be active in psoriasis¹⁰. Notably, Wang *et al.*¹¹ reported increased activated ERK1 immunostaining in differentiating compartments of the

psoriatic epidermis, which suggests that RAS/ERK signalling actually contributes to S6Ser(235/236) phosphorylation in psoriasis.

We found that S6 is differentially phosphorylated in the psoriatic epidermis. The S6(Ser240) site is peculiar with respect to its predominant phosphorylation in proliferating compartments of highly active lesions. In contrast, weakly inflamed lesions, which showed scattered S6 phosphorylation in proliferating compartments, revealed S6(Ser240) phosphorylation also in differentiating compartments. This may indicate normalization of epidermal differentiation in regressing lesions, because S6(Ser240) is phosphorylated in the stratum granulosum of healthy skin. Therefore it is tempting to speculate that activation of S6 has a role in terminal differentiation of keratinocytes.

We found increased S6 phosphorylation also in AD, suggesting that mTOR is generally activated in inflammatory skin diseases with epidermal hyper-proliferation. Inflammatory cells secrete growth factors, such as insulin like growth factor-1 (IGF-1), known to activate the mTOR pathway¹², and to inhibit keratinocyte differentiation¹³. Beside this, clinical improvement of psoriasis was found to be associated with decreased IGF-1 expression¹⁴.

S6 activation seems to be dispensable for normal keratinocyte proliferation, because S6 phosphorylation is not evident in dividing keratinocytes of healthy skin. Thus, mTOR inhibitors may selectively target pathologically activated keratinocytes, a hypothesis supported by the observation that mTOR inhibition suppresses UVB-induced keratinocyte hyper-proliferation¹⁵, but does not affect normal epidermal turnover.

mTOR inhibition might help to treat hyper-proliferative skin diseases. Analysis of site specific S6 phosphorylation in skin biopsies could be useful to assess pharmacodynamics of mTOR inhibitors in clinical trials. Finally, differential phosphorylation of S6 may have implications for tumour growth, which should be taken into account when analysing tumour samples.

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Figure legends:

Figure 1: Immunofluorescence images showing differential S6 phosphorylation, CD45 expression and Ki67 positive cells in psoriatic lesions.

In **(a)** staining of phospho-S6(Ser235/236) is shown in red (**a1, a4 and a7**) and staining of phospho-S6(Ser240) in green (**a2, a5 and a8**); overlays of both stainings are shown in **a3, a6 and a9**. In normal skin (**a1-3**) S6 phosphorylation was restricted to the stratum granulosum. In contrast, psoriatic lesions revealed S6 phosphorylation also in supra-basal cell layers (**a4-6 and a7-9**). The staining patterns were highly variable between patients as shown by two examples: Psoriasis lesion I (**a4-6**): staining of phospho-S6(Ser235/236) (**a4**) was visible in all supra-basal epidermal layers and more prominent in differentiating compartments, whereas staining of phospho-S6(Ser240) (**a5**) was mostly restricted to proliferating compartments. Psoriasis lesion II (**a7-9**): Ser235/236 (**a7**) and Ser240 (**a8**) were co-phosphorylated in a small subset of supra-basal cells, but showed distinct phosphorylation patterns in differentiating layers. Double staining of CD45 (**green in b1-2**) and phospho-S6(Ser235/236) (**red in b1-b2**) showed that the extend of S6 phosphorylation is related to inflammation. Double staining of Ki67 (**red in c1-2**) and phospho-S6(Ser240) (**green in c1-2**) revealed that S6 is phosphorylated in many

proliferating keratinocytes of the psoriatic epidermis (**c2**), but not in dividing cells of normal skin (**c1**). A comparison of Ser240/244 and Ser240 phosphorylation in a highly active psoriatic lesion is shown in (**d**), and indicates extended phosphorylation of Ser244 into differentiating compartments of the lesional epidermis. Scale bars: 50µm

Figure 2: Immunofluorescence images showing differential S6 phosphorylation and inflammation in AD lesions.

S6 phosphorylation patterns in AD lesions (**a1-3** and **a4-6**). AD lesion I (**a1-3**): staining of phospho-S6(Ser235/236) (**red in a1**) was most prominent in differentiating compartments, whereas staining of phospho-S6(Ser240) (**green in a2**) was mostly restricted to proliferating compartments. AD lesion II (**a4-6**): staining of phospho-S6(Ser235/236) (**red in a4**) was observed in a subset of epidermal cells scattered throughout the epidermis, and in the stratum granulosum. Most phospho-S6(Ser235/236) positive cells also revealed phospho-S6(Ser240) staining (**green in a5**). Overlays are shown in **a3** and **a6**. Double staining of CD45 (**green in b1 and b2**) and phospho-S6(Ser235/236) (**red in b1 and b2**) showed S6 phosphorylation in all epidermal layers of strongly inflamed AD lesions (**b1**), and restricted S6 phosphorylation in weakly inflamed lesions (**b2**). Scale bars: 50µm.



