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Decreased Srcasm expression in hyperproliferative cutaneous lesions

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Abstract

Background—Src-family tyrosine kinases (SFKs) are signaling proteins that regulate keratinocyte proliferation and differentiation. Srcasm is a recently identified molecule that downregulates SFK activity and promotes keratinocyte differentiation. To determine if Srcasm expression correlates with keratinocyte differentiation, we characterized the level of Srcasm expression in some cutaneous lesions that exhibit increased keratinocyte proliferation.

Methods—Formalin-fixed sections of randomly selected seborrheic keratoses and basal cell carcinomas were analyzed for Srcasm and Ki-67 immunohistochemical staining. Anti-Srcasm and anti- Ki-67 staining were performed in parallel.

Results—All seborrheic keratoses displayed decreased Srcasm staining in areas comprised of basaloid keratinocytes that exhibited an increased Ki-67 index. Higher Srcasm staining levels were detected near pseudo-horn cysts where keratinocytes exhibited a lower Ki-67 index. All multicentric and nodular basal cell carcinomas displayed a prominent loss of Srcasm staining in association with a marked increase in Ki-67 staining.

Conclusions—Our results support the hypothesis that Srcasm protein levels are decreased in the hyperproliferative keratinocytes found in seborrheic keratoses and basal cell carcinomas. Increased Srcasm protein levels are detected in keratinocytes undergoing differentiation. Decreased Srcasm levels may be part of the pathophysiologic mechanism in cutaneous lesions exhibiting keratinocyte hyperproliferation.

Introduction

Further analysis of molecules that regulate cellular growth and differentiation is important for understanding the pathophysiology of skin disorders associated with keratinocyte hyperproliferation, including cutaneous carcinomas and benign tumors. Src-family tyrosine kinases (SFKs) constitute a group of non-receptor tyrosine kinases known to regulate keratinocyte differentiation.^{1, 2} At the molecular level, SFKs are activated downstream of growth factor receptors and transmit signals to other signaling molecules, ultimately leading to activation of transcription factors and altered gene expression.³

Src-activating and signaling molecule (Srcasm) is a recently described substrate of SFKs, that can downregulate levels of activated SFKs.^{2, 4, 5} Increased Srcasm expression in human primary keratinocytes regulates the activity of MAP kinases, increases transcription from serum-response elements, and promotes differentiation.² Increased Srcasm expression in vivo suppresses epidermal hyperproliferation induced by over-expression of Fyn, an SFK, in transgenic mice.⁴ In addition, Srcasm levels are decreased in human squamous cell

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carcinoma (SCC) as determined by western blotting and immunohistochemistry, suggesting that decreased Srcasm levels are associated with keratinocytic neoplasia.²

Although previous immunohistochemical data implicate Srcasm in promoting keratinocyte differentiation, these studies were limited to a subset of lesions comprised of actinic keratoses, squamous cell carcinoma in-situ (SCIS), and SCCs. Therefore, it is not known whether decreased Srcasm expression is associated with hyperproliferative keratinocytes in other types of cutaneous lesions. To determine if Srcasm expression is decreased in other epidermal tumors and non-carcinomatous hyperproliferative lesions, we characterized the levels of Srcasm in basal cell carcinomas (BCCs) and seborrheic keratoses (SKs).

The data show that Srcasm levels are decreased in basal cell carcinomas and specific regions of seborrheic keratoses. The BCCs and the hyperproliferative regions of SKs demonstrated prominent Ki-67 staining and concordant decreased Srcasm staining. The portions of SKs adjacent to pseudo-horn cysts demonstrated infrequent Ki-67 staining and high levels of Srcasm staining. Ki-67 staining of BCCs and SKs confirmed an inverse relationship between Srcasm levels and keratinocyte proliferation in BCCs and SKs. The immunohistochemical staining pattern of Srcasm suggests that it may represent a general component of the cell differentiation pathway in keratinocytes. Decreased Srcasm staining in keratinocytes appears to be a feature of non-melanoma skin cancers.

Materials and Methods

Immunohistochemistry

Biopsy sections were retrieved from the archives of the Department of Dermatology, division of dermatopathology, at the University of Pennsylvania in Philadelphia, PA. In all cases, recut slides were stained with hematoxylin/eosin and examined to confirm the diagnoses. 16 seborrheic keratoses (SKs), 7 basal cell carcinomas (BCCs), and 5 samples of unremarkable epidermis were stained with affinity-purified anti-Srcasm antibody and anti-Ki-67.²

In brief, samples were heated, de-paraffinized, re-hydrated, and washed using standard solutions and procedures. Antigen retrieval was performed by incubating the slides in 10 mM citrate buffer (pH 6) at 85.0°C for 20 minutes. The tissue sections were blocked at room temperature for 30 minutes with 10% goat serum; then incubated for 105 min at room temperature with a 1:50 dilution of affinity-purified, rabbit anti-Srcasm antibody, or for 15 minutes with anti-Ki-67. Consecutive levels were stained anti-Srcasm and anti-Ki-67. Affinity-purified biotinylated goat anti-rabbit polyclonal immunoglobulin (BD Biosciences Pharmingen, USA) was applied to the tissue sections at a 1:200 dilution for 30 minutes. Pre-diluted Streptavidin-Horseradish Peroxidase (BD Biosciences Pharmingen, USA) was applied to the tissue sections and incubated for 30 minutes and histochemical development was performed using a liquid 3, 3'-Diaminobenzidine tetrahydrochloride (DAB) substrate kit (Zymed Laboratories Inc., San Francisco, Ca, USA), for 100 seconds. The slides were subsequently counterstained in hematoxylin, dipped three times in bluing reagent, dehydrated, and cleared. Adjacent slides were stained at least twice to confirm staining levels.

Analysis of staining intensity

All slides were evaluated independently by two individuals (JTS and MM) for staining intensity and the extent of staining in lesional cells and unremarkable epidermis. Staining intensity was graded as follows: 1-none, 2-weak, 3-moderate, 4-strong. No lesion exhibited a 0 staining intensity. Extent of staining was assessed as follows: 1- 0 to 25%, 2- 25 to 50%, 3-50 to 75%, 4- 75 to 100%. For each specimen, a staining index was determined by

multiplying the intensity factor by the extent factor. The maximum index is 16. The data reported represent the average staining index for each biopsy class with a standard deviation. A Ki-67 staining index was calculated by determining the percent of nuclei with positive staining for each specimen, at least 100 nuclei were assessed in specific regions, sometimes requiring serial sections.

In the seborrheic keratoses, given the dichotomous staining pattern, the Srcasm staining index was determined for the areas comprised of basaloid cells and areas adjacent to pseudo-horn cysts. Staining patterns were studied for all lesions and representative sections were photographed.

Statistical calculations

To determine the p values associated with the differences in staining indices, a two-tailed student's t test was performed to analyze the means and standard deviation of the two indicated groups.

Results

In unremarkable human epidermis, the intensity of Srcasm staining correlates well with the degree of cellular differentiation. In unremarkable epidermis, weak staining for Srcasm was seen in basilar keratinocytes, and more prominent staining was detected in the stratum spinosum and granulosum (Fig. 1A). A low percentage of Ki-67 positivity was detected in sections of unremarkable epidermis (Fig. 1B). In both multicentric and nodular BCCs, Srcasm expression was markedly diminished, whereas the adjacent, non-tumorigenic epidermis exhibited Srcasm staining typical of unremarkable epidermis (Fig. 1C). In contrast to Srcasm staining levels, Ki-67 stained a high percentage of nuclei in all BCCs (Fig. 1D); Ki-67 positive nuclei were less frequent in unremarkable epidermis adjacent to the BCCs.

Seborrheic keratoses displayed decreased Srcasm staining in the regions comprised of basaloid keratinocytes (Figs. 2A and C) and higher levels of Srcasm staining in keratinizing cells located closer to the stratum corneum and the pseudo-horn cysts (Figs. 2B and D). Similar to BCCs, the Ki-67 index in seborrheic keratoses was inversely correlated with Srcasm levels. However, in seborrheic keratoses, the inverse relationship between the level of Srcasm staining and the Ki-67 index could be easily discerned within the same lesion (Fig. 2).

To further characterize the inverse relationship between Srcasm levels and Ki-67 staining in the biopsies examined, we performed a semi-quantitative assessment of these two variables. As expected, the percentage of Ki-67 positive cells in BCCs was markedly increased compared to unremarkable skin, and this difference was statistically significant (Fig. 3A and Table 1). The Srcasm-staining index in BCCs was markedly decreased compared to unremarkable skin (Figure 3B). Interestingly, in seborrheic keratosis lesions, areas of increased Ki-67 staining were noted in portions of the keratoses comprised predominately of basaloid, non-keratinizing cells (SK pro), while areas comprised of keratinizing cells (SK dif) exhibited less frequent Ki-67 positivity (Fig. 3A). Both regions of SKs were evaluated for Srcasm staining levels. Once again, an inverse correlation between the degree of keratinocyte proliferation and Srcasm staining was found (Figs. 2 and 3B). Overall, these data support the hypothesis that proliferating keratinocytes are associated with low levels of Srcasm staining while differentiating keratinocytes are associated with higher levels of Srcasm staining (Fig. 3). The Srcasm and Ki-67 staining differences assessed between unremarkable epidermis or SKdiff and BCCs or SKpro are statistically significant (Table 1).

DISCUSSION

The equilibrium between keratinocyte proliferation, differentiation, and shedding maintains the functional integrity of the skin.⁴ Disruption of the molecular signaling pathways responsible for this orderly progression leads to altered patterns of proliferation, and the development of cutaneous lesions including basal cell carcinomas and seborrheic keratoses. Key cellular signaling events relevant to the pathophysiology of these lesions likely include activation of growth factor receptor tyrosine kinases and Src-family tyrosine kinases. Cellular mechanisms that negatively regulate pro-growth pathways in keratinocytes are important for limiting proliferation and, in essence, function as a suppressor of proliferation. Srcasm, a substrate of Src-family tyrosine kinases, is expressed in the epidermis, most highly in post-mitotic keratinocytes, and it negatively regulates Src-family tyrosine kinase signaling in vitro and in vivo.^{1, 4, 5} These features suggest that Srcasm may play a role in regulating the levels of tyrosine kinase signaling in keratinocytes and may be associated with the transition of proliferating keratinocyte to differentiating keratinocyte.

To better understand the role of Srcasm in human cutaneous disease, we studied the expression pattern of Srcasm protein in basal cell carcinomas and seborrheic keratoses. Compared with unremarkable epidermis, the uniform and markedly decreased Srcasm staining in multicentric and nodular basal cell carcinomas suggests that decreased Srcasm levels are a common feature of these carcinomas. These data coupled with previous work demonstrating decreased Srcasm expression in actinic keratoses, squamous cell carcinoma in situ, and squamous cell carcinomas suggests that Srcasm levels are commonly downregulated in both of the most prevalent cutaneous carcinomas.² Since basal cell carcinomas are associated with increased signaling through the *patched* pathway, it will be important to characterize how abnormal signaling in this pathway relates to decreased Srcasm levels.

Within seborrheic keratoses, decreased Srcasm staining was seen in lesional cells exhibiting Ki-67 positivity, and increased Srcasm staining, approximating levels seen in unremarkable epidermis, was found in lesional cells undergoing keratinization. Our knowledge regarding the pathophysiology of seborrheic keratoses has expanded recently. Activating mutations in the fibroblast growth factor receptor 3 and the catalytic subunit of PI-3 kinase have been detected in seborrheic keratoses.^{6, 7} It is interesting to note that phosphorylated Srcasm can bind the regulatory subunit of PI-3 kinase; however, the significance of this interaction on PI-3 kinase signaling remains under investigation.⁵ This dichotomous staining pattern of Srcasm in seborrheic keratoses supports the hypothesis that Srcasm levels are inversely related to keratinocyte proliferation and are directly associated with keratinocyte differentiation.

The increased Ki-67 staining in basal cell carcinoma and seborrheic keratoses supports prior studies.^{8, 9, 10} The strong inverse correlation between Srcasm and Ki-67 staining suggests that Srcasm may function to antagonize keratinocyte proliferation and that higher levels of Srcasm are associated with keratinocyte differentiation (Fig. 4). Experimentation with transgenic mice over-expressing the Fyn tyrosine kinase demonstrates that increasing Srcasm levels can correct the tyrosine kinase-induced epidermal hyperplasia; in effect, these observations are corroborated by the Srcasm staining pattern in SKs and BCCs.⁴

Interestingly, Srcasm staining in seborrheic keratoses and basal cell carcinomas appears inversely related to the patterns previously reported for the epidermal growth factor-receptor (EGFR).¹¹⁻¹³ These data would suggest that increased Srcasm staining levels are associated with a decrease in EGFR levels. Further study of the association of Srcasm with EGFR and

other signaling molecules may reveal the mechanism by which the Srcasm protein may influence keratinocyte differentiation and proliferation.

These data help generalize the hypothesis that there is an inverse correlation between Srcasm expression and keratinocyte proliferation in a variety human cutaneous lesions exhibiting hyperproliferation (Fig. 4). However, this model appears to apply to only neoplasia, as Srcasm levels in psoriasis are elevated compared to unremarkable epidermis.¹⁴ Further characterization of additional inflammatory disorders such as lichen planus will help determine if elevated Srcasm levels are generally elevated in inflammatory skin conditions. Therefore, one could hypothesize that levels of Srcasm may be elevated in inflammation-induced hyperproliferations and decreased in neoplastic proliferations; this dichotomy may be helpful in distinguishing inflamed lesions from neoplastic lesions. In inflamed benign keratoses, the Srcasm levels should be equal to or greater than the levels in unremarkable epidermis. In neoplastic lesions, the Srcasm levels should be less than that seen in adjacent unremarkable epidermis.

Together, these and previous studies support the notion that Srcasm represents a novel-anti-proliferation gene for keratinocytes, perhaps even a tumor suppressor gene; additional studies will determine if Srcasm plays an important pathophysiologic role in benign and malignant cutaneous lesions.

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Figure 1. Immunohistochemical staining for Srcasm and Ki-67 in unremarkable human skin and basal cell carcinoma

Sections of unremarkable epidermis (A and B) were stained for Srcasm (A) and Ki-67 (B). Note prevalent Srcasm staining and infrequent Ki-67 positivity. Sections of basal cell carcinoma (C and D) were stained for Srcasm (C) and Ki-67 (D). Lesional cells demonstrate a paucity of Srcasm staining coupled with prominent Ki-67 staining.

FIGURE 2. Immunohistochemical staining for Srcasm and Ki-67 in seborrheic keratoses
Sections of two representative seborrheic keratoses were stained for Srcasm (A and C) and Ki-67 (B and D). Note the inverse relationship between Srcasm staining and Ki-67 positivity.

FIGURE 3. Ki-67 (A) and Srcasm (B) staining indices in unremarkable human epidermis, BCCs, basaloid areas of seborrheic keratoses (SK pro), and keratinizing regions of seborrheic keratoses (SK dif)

The percentage of cells exhibiting Ki-67 positivity was determined by examining representative areas of lesions and evaluating at least 100 cells. Results are tabulated in A. Srcasm staining was quantified using a staining index formula, (see methods), and results are tabulated in B. Standard deviations in values are indicated.

Srcasm Modulation Hypothesis:

Figure 4. Schematic Diagram of Srcasm Modulation Hypothesis

Increased levels of Srcasm staining are associated with a low Ki-67 index in unremarkable epidermis and seborrheic keratoses. Low levels of Srcasm staining are associated with high Ki-67 indices in proliferative regions of seborrheic keratoses and basal cell carcinomas. There appears to be an inverse relationship between Srcasm staining levels and the Ki-67 index.

Table 1

Student T-test p values for the Ki-67 indices (A) and the Srcasm indices (B).

A			
Ki-67 Index	BCC	SKpro	SKdiff
Unremarkable	p<0.01	p<0.01	p=0.75
SKdiff	p<0.01	p<0.01	

B			
Srcasm Index	BCC	SKpro	SKdiff
Unremarkable	p<0.01	p<0.01	p=0.19
SKdiff	p<0.01	p<0.01	