Selective Expression of Calcium-Binding Proteins S100A8 and S100A9 at Distinct Sites of Hair Follicles

To the Editor:

Members of the S100 family of calcium-binding proteins have been implicated with the control of proliferation and differentiation events in a variety of cells. This includes regulation of calcium-dependent phosphorylation, maintenance of intracellular calcium homeostasis, and organization of membranes and cytoskeletal filament systems. The genomic localization of human S100 genes in the so-called epidermal differentiation complex 1q21 (Schäfer *et al*, 1995) also suggests a role in the process of keratinocyte differentiation. Consistent with that, some S100 molecules such as S100A10 (calpactin light chain) and S100A11 (calgizzarin) are expressed in terminally differentiated cells (Robinson *et al*, 1997), whereas others such as S100A7 (psoriasin) and S100A12 (calgranulin C) are only upregulated during pathologic keratinocyte differentiation, e.g., in psoriasis (Madsen *et al*, 1992; Mirmohammadsadegh *et al*, 2000).

Two of the S100 molecules, S100A8 (MRP8) and S100A9 (MRP14), show an intriguing tissue distribution: they are exclusively expressed in distinct differentiation stages of myelomonocytic cells as well as by epithelial cells including keratinocytes. S100A8 and S100A9 form noncovalently associated heteromeric complexes in a calcium-dependent manner (Teigelkamp *et al*, 1991), whereas their closest homolog, S100A12, acts as a homodimer (Vogl *et al*, 1999). Epidermal expression of S100A8 and S100A9 is found in a variety of hyperproliferative dermatoses including psoriasis (Gabrielsen *et al*, 1986); however, *in situ* detection of S100A8 and S100A9 has been hampered by the fact that only immunohistochemical methods have been applied using mostly rabbit antisera, which may generate nonspecific staining in epidermis and skin appendages. Moreover, keratinocytes may secrete S100A8 and S100A9 (Katz and Taichman, 1999); thus, it appears inappropriate to solely rely on immunohistochemistry when studying tissue distribution of these proteins. As a thorough analysis of the expression pattern in normal skin and hair follicles is currently not available, we employed radioactive *in situ* hybridization to study the spatial expression profile of S100A8 and S100A9 mRNA and its correlation with established markers of keratinocyte differentiation.

Normal human skin from donors undergoing unrelated skin surgery was paraffin-embedded to guarantee superior morphologic quality. Samples were processed for *in situ* hybridization as described earlier (Goebeler *et al*, 2001) using ^{35}S-labeled antisense probes for S100A8 and S1000A9 (Odink *et al*, 1987). Additionally, serial paraffin sections were immunohistochemically stained either with monospecific rabbit antisera recognizing S100A8, S100A9 (Odink *et al*, 1987), keratin K14 (AF64, Babco, Freiburg, Germany) or with mouse IgG_1 monoclonal antibodies against keratins K6 (Ks6/KA12, Progen, Heidelberg, Germany), K10 (DE-K10, Dako, Hamburg, Germany), and involucrin (Sy5, Sigma, Deisenhofen, Germany), respectively.

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In most cases, S100A8 and S100A9 mRNA or protein were not detectable in normal interfollicular skin. Occasionally, however, a faint patchy expression was found in suprabasal epidermal layers (data not shown). Interestingly, strong signals for both S100A8 and S100A9 mRNA were observed in the anagen hair follicle. Expression of mRNA transcripts and protein was restricted to the hair shaft medulla extending from the suprapapillary region (keratogenous zone) where the expression was rather faint following the course of the medulla well into the lower third of the epithelium of anagen VI hair follicles (**Fig 1*a*–*g***). Additionally, weak expression of S100A9 mRNA but not of detectable protein amounts was observed in the Huxley's layer of the inner root sheath (IRS; data not shown). Hybridizations with sense probes or immunostaining with isotype-matched control antibodies confirmed specificity of the signals (data not shown). Because S100A8 and S100A9 distribution resembles expression patterns of K6 and involucrin in hyperproliferative interfollicular epidermis (Schmidt *et al*, unpublished observation) we also performed immunohistochemistry for these markers on serial sections of hair follicles. K6 labeling was restricted to the inner layers of the outer root sheath (ORS) (**Fig 1*i***) whereas involucrin showed an overlapping staining pattern with S100A8/S100A9 (**Fig 1*h***). While it colocalizes with S100A8/S100A9 in the medulla, additional signals for involucrin were detected in the IRS and, weaker, the cortex of the hair shaft. In contrast, K14, a marker of undifferentiated keratinocytes, showed a completely different distribution (**Fig 1*j***) and no hair follicle-specific signals were observed in the case of K10 (**Fig 1*k***).

Our results demonstrate that both S100A8 and S100A9 genes are abundantly expressed in the medulla of the hair shaft. The colocalization with involucrin in the medulla and lack of congruency with the expression pattern of K14 suggest that only terminally differentiated cells express S100A8 and S100A9. Normal interfollicular epidermis did not express mRNA; thus, a role during regular differentiation of interfollicular keratinocytes is unlikely. Occasionally, however, weak signals were detected suprabasally in a patchy distribution, which might indicate sites of microtrauma.

Of the increasing number of S100 proteins described so far, five, including those investigated here, are expressed at distinct sites of the hair follicle. S100A2 is found in the IRS and, weaker, in the ORS, whereas S100A3 is localized in cuticle and cortex (Kizawa *et al*, 1998; Shresta *et al*, 1998). S100A6 (calcyclin) is detectable in the medulla resembling S100A9 distribution (Wood *et al*, 1991; Kizawa *et al*, 1998). In contrast to S100A8 and S100A9, however, S100A6 is also strongly expressed in the IRS. CLED, a bovine S100 protein homolog, is expressed in the developing Henle's layer and in the ORS (Sun *et al*, 2000). The distinct expression patterns of S100 molecules in different areas of the hair follicle predict a tight transcriptional control of these proteins. It is intriguing that strongest mRNA signals for S100A8 and S100A9 as well as for S100A3 and S100A6 are observed at complementary sites that may reflect a coordinate function during hair shaft formation. S100A8/S100A9 may not only modulate intracellular calcium homeostasis during follicular differentiation as proposed for S100A3 (Kizawa *et al*, 1998) but also fulfill various other regulatory functions such as the control of keratin network reorganization (Goebeler *et al*, 1995). In this respect it is interesting that expression in the medulla resembles distribution of trichohyalin (Tarcsa *et al*, 1997), a

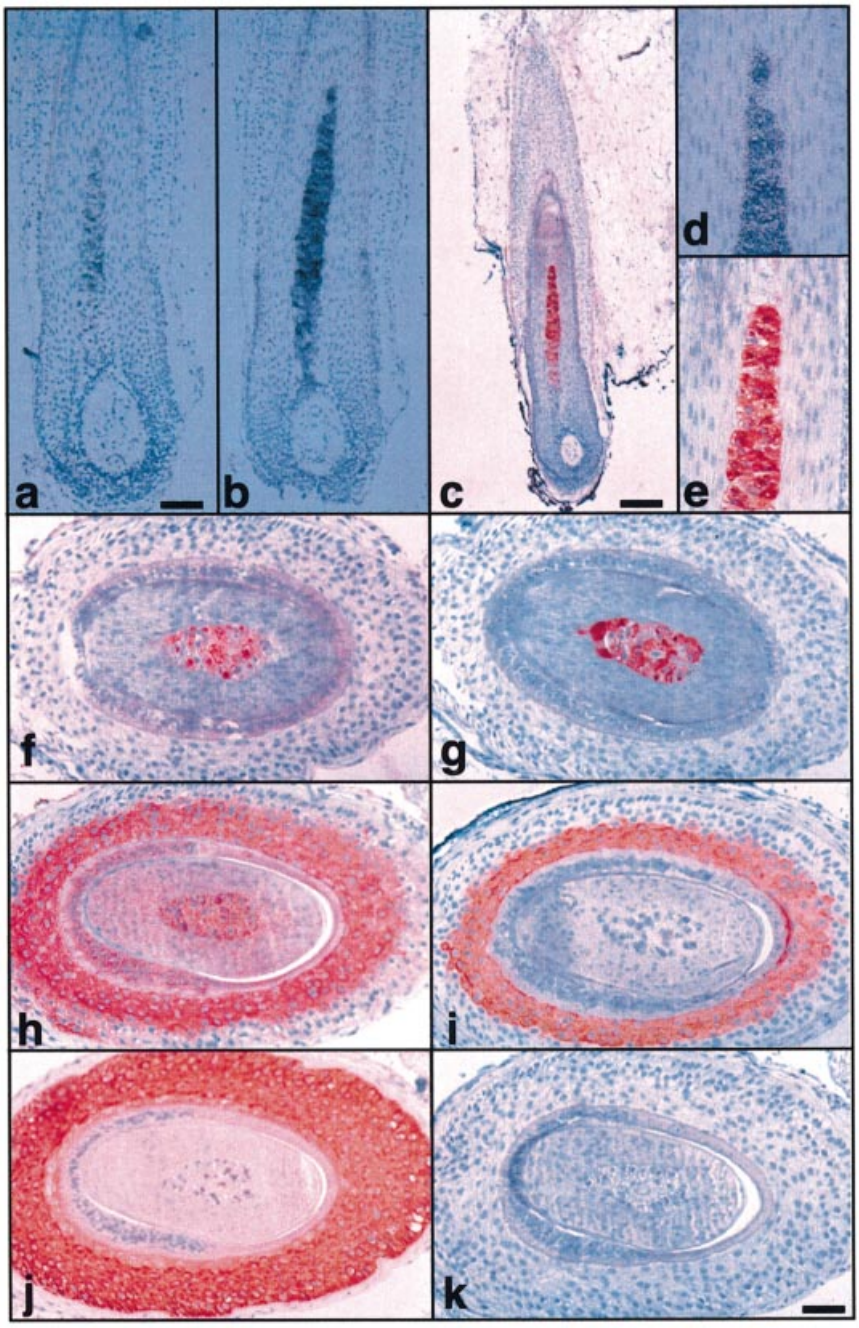

Figure 1. Spatial distribution of calcium-binding proteins S100A8 and S100A9, involucrin and keratins in the human hair follicle. (*a*, *b*, *d*) *In situ* hybridization of longitudinal sections with ^{35}S-labeled antisense probes for S100A8 (*a*) and S100A9 (*b*, *d*). Signals are prominent in the medulla of the hair shaft. (*c*, *e*, *f*, *g*) Immunohistochemical staining of longitudinal (*c*, *e*) and transversal serial sections (*f*, *g*) for S100A8 (*f*) and S100A9 (*c*, *e*, *g*). Immunoreactivity is restricted to the medulla and parallels expression of mRNA transcripts. (*h*, *i*, *j*, *k*) Immunolabeling of serial transversal sections for involucrin (*h*), keratin 6 (*i*), keratin 14 (*j*), and keratin 10 (*k*). Note that involucrin distribution (*h*) partly overlaps with S100A8 and S100A9 expression patterns (*f*, *g*), whereas the latter do not colocalize with keratins K6 and K14. *Scale bars:* (*a*, *b*) 100 μm; (*c*) 200 μm; (*d–k*) 50 μm.

structural protein that has been implicated with regulation of keratin filament lateral association. Trichohyalin contains EF-hand calcium-binding domains with high sequence similarity to S100 proteins (Lee *et al*, 1993). It is tempting to speculate that the partial coexpression of S100A8/S100A9 with trichohyalin might reflect a cooperative action during formation of the medulla.

Further studies are needed to fully clarify the functional relevance of hair follicle-associated expression of S100A8 and S100A9. Because mice with a targeted disruption of S100A8 die around d 7.5 during embryogenesis (Passey *et al*, 1999), analysis of animals with a null mutation of S100A9 should facilitate this task.

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HLA-G Expression in Atopic Dermatitis

To the Editor:

Atopic dermatitis (AD) is a chronic disease usually beginning in childhood. Immunologic features of AD consist in a cytokine balance in the peripheral blood mononuclear cells (PBMC) directed toward Th2 cytokines (Akdis *et al*, 1997; Jirapongsananuruk *et al*, 1998). Skin biopsies from atopy patch test (Thepen *et al*, 1996) as well as early phase of spontaneous AD lesions (Hamid *et al*, 1996) have been shown to bear a mainly Th2 cell infiltrate in contrast to chronic phase spontaneous AD lesions (Hamid *et al*, 1996), infiltrated by IFN-γ and IL12 expressing mononuclear cells (Grewe *et al*, 1994; Hamid *et al*, 1996; Thepen *et al*, 1996). In addition, IL10 is a major cytokine in AD as it is produced in large amounts by PBMC (Ohmen *et al*, 1995; Kallmann *et al*, 1996) and infiltrating lesional cells of AD patients (Ohmen *et al*, 1995).

Recently, the presence of natural killer cells receptors (NKR) on effector T cells has been demonstrated (Lopez-Botet and Bellon, 1999; Huard and Karlsson, 2000). The ligands of these receptors are various MHC class I molecules. HLA-G is a nonclassical MHC class I molecule (Carosella *et al*, 1999) that has a tissue-restricted expression on extravillous cytotrophoblasts (Mcmaster *et al*, 1995) and thymic epithelial cells (Crisa *et al*, 1997). Binding studies have shown that one of the ligands of several inhibitory NKR, such as ILT2, ILT4, KIR2DL4, and p49, was indeed HLA-G (Lanier, 1999). Functionnal assays demonstrated that HLA-G was able to downregulate allogeneic proliferation and cytotoxicity of T cells (Riteau *et al*, 1999; Kapasi *et al*, 2000), as well as antigen specific T cell cytotoxicity (Le Gal *et al*, 1999) and natural killer cytotoxicity (Rouas-Freiss *et al*, 1997). HLA-G expression is induced by IL10 (Moreau *et al*, 1999) or IFN-γ (Yang *et al*, 1996). Because HLA-G may inhibit T cell functions and is induced by a cytokine constantly overexpressed in AD, namely IL10, we studied whether this molecule could be expressed in atopic dermatitis. After local ethical committee approval, adult untreated patients with typical features of AD who gave informed consent were selected. The mean age of the nine included cases was 38.2 y (18–69). Seven of the nine patients had a familial history of atopy and all nine had a long course of AD as the disease began at a mean age of 6 y. The average severity score (SCORAD) at inclusion was 39 (29–63). All biopsies originated from chronic phase AD lesions. As a control, healthy skin was obtained from breast plastic surgery resection.

By immunohistochemistry using 87G, a specific antibody for HLA-G (kindly provided by Dan Geraghty, Fred Hutchinson Cancer Reasearch Center, Seattle, WA), HLA-G expressing cells were demonstrated in all nine patients (**Fig 1**). In contrast, 87G never labeled six specimens of healthy skin (data not shown). The density of positive cells was variable from one patient to another. In AD biopsies, HLA-G positive cells were always found in the papillary and less frequently in the reticular dermis; however, in one case there was in addition few epidermal positive cells distributed in all epidermal layers (**Fig 1**, lower panel). This patient had a very severe disease.

In a second step toward determining which cell types expressed HLA-G in AD, we realized double immunofluorescence studies. Using this technique, 87G positive cells appeared to be labeled by anti-CD3 antibodies (**Fig 2*b***). Controls consisting of incubation with irrelevant mouse IgG2a, followed by antimouse antibodies conjugated with Texas Red and finally FITC conjugated anti-CD3 antibodies, did not show any double staining (**Fig 2*a***), demonstrating that the 87G staining was not due to unspecific binding of the primary or secondary antibody. Using CD14, we were also able to label the HLA-G expressing cells in two out of three patients tested (**Fig 2*c***). In the unique patient who had epidermal 87G positive cells, double immunofluorescence also showed few CD1a/HLA-G positive cells (**Fig 2*d***). These results show that HLA-G was expressed mainly by T infiltrating cells but also to a lesser extent and less frequently by monocyte-macrophagic or even Langerhans cells.

In several situations, HLA-G has been shown to inhibit immune cytotoxicity. Indeed, HLA-G inhibited *in vitro* T cell proliferation, antigen specific and alloreactive cytotoxicity by interacting with different NKR such as ILT2 (Navarro *et al*, 1999). In addition, the inhibition of cytotoxicity of maternal decidual natural killer cells

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