Epidermal Elafin Expression Is an Indicator of Poor Prognosis in Cutaneous Graft-versus-Host Disease

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Graft-versus-host disease (GVHD) remains a common and potentially life-threatening complication of allogeneic hematopoietic stem cell transplantation. In the skin, GVHD can present in an acute (aGVHD), chronic lichenoid (clGVHD), or chronic sclerotic form (csGVHD). Measuring peripheral blood levels of the keratinocyte-derived protease inhibitor elafin has recently emerged as a promising tool for the diagnosis of cutaneous aGVHD. We evaluated whether the analysis of elafin expression in skin would allow distinguishing aGVHD from drug hypersensitivity rashes (DHR) and whether cutaneous elafin expression would correlate with disease severity or altered prognosis of aGVHD and clGVHD/csGVHD. Skin biopsies from aGVHD ($n=22$), clGVHD ($n=15$), csGVHD ($n=7$), and DHR ($n=10$) patients were collected and epidermal elafin expression and its association with diverse clinical/histological parameters were analyzed. Acute GVHD and DHR displayed varying degrees of elafin expression. No elafin was detectable in csGVHD, whereas the molecule was increased in clGVHD as compared with aGVHD. Elafin-high aGVHD/clGVHD lesions presented with epidermal thickening and were associated with poor prognosis—i.e., decreased overall survival in aGVHD and corticosteroid resistance in clGVHD. Although cutaneous elafin does not seem to discriminate aGVHD from DHR lesions, our study strongly suggests an association between cutaneous elafin expression and poor prognosis for patients with cutaneous GVHD.

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HCT) is a potentially curative therapy for numerous hematological disorders. The broader application of HCT is limited by its most frequent and potentially life-threatening complication, namely graft-versus-host disease (GVHD; Ferrara *et al.*, 2009). GVHD can occur in an acute or a chronic form (aGVHD and cGVHD), in both of which the skin is the most frequent and usually the first affected organ (Higman and Vogelsang, 2004; Ferrara *et al.*, 2009). Despite major progresses in HCT practice, available treatment options for GVHD patients are limited and corticosteroids remain the first-line therapy for both aGVHD and cGVHD (Wolff *et al.*, 2010, 2013). Early diagnosis of skin involvement in GVHD is crucial for an appropriate therapy in an attempt to prevent disease progression.

Skin aGVHD usually presents as a maculopapular or morbilliform rash (Wagner and Murphy, 2005; Hausermann *et al.*, 2008). Histopathological features of aGVHD include satellite cell necrosis (dyskeratotic keratinocytes surrounded by lymphocytes) and the vacuolization of basal epidermal layers. Chronic cutaneous GVHD shares major characteristics with lichen planus during the early stages of disease progression (chronic lichenoid GVHD, clGVHD). Clinically, this is evidenced by the presence of lichenoid papules. Histopathologically, clGVHD shows a wedge-shaped hypergranulosis and basal cell liquefaction of the epidermis, as well as a prominent band-like lymphocytic infiltrate in the dermis. In a more advanced stage, cGVHD tends to exhibit signs of sclerosis (chronic sclerotic GVHD, csGVHD).

It is challenging both clinically and histopathologically to differentiate between aGVHD and its main differential diagnosis, namely drug hypersensitivity rashes (DHR). This distinction has critical therapeutic consequences—i.e., either the

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Abbreviations: aGVHD, acute graft-versus-host disease; clGVHD, chronic lichenoid graft-versus-host disease; csGVHD, chronic sclerotic graft-versus-host disease; DHR, drug hypersensitivity rashes; HCT, allogeneic hematopoietic stem cell transplantation; HE, hematoxylin and eosin; IH, immunohistochemistry; TNFα, tumor necrosis factor alpha; TGFβ, transforming growth factor beta

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initiation of a systemic corticosteroid regimen to control GVHD or the discontinuation of the drug(s) causing DHR. Numerous approaches have aimed at identifying parameters to diagnose aGVHD more accurately and to distinguish it from DHR (Byun *et al.*, 2011). Elafin has (in a retrospective proteomic approach) been identified as an accurate plasmatic biomarker of cutaneous aGVHD that correlates with disease severity and even has a prognostic value (Paczesny *et al.*, 2010; Levine *et al.*, 2012). The investigators also showed that elafin was overexpressed in seven out of ten aGVHD skin specimens but was absent in a similar number of DHR biopsies (Paczesny *et al.*, 2010).

Elafin is a serine protease inhibitor that is mostly produced by epithelial cells. In the skin, keratinocytes are the main source of this molecule. Although elafin is not detectable in normal skin, it is secreted abundantly in psoriasis and other inflammatory (Tanaka *et al.*, 2000; Kamsteeg *et al.*, 2010), as well as neoplastic (Alkemade *et al.*, 1993), skin disorders. Elafin acts in various ways on the cutaneous immune homeostasis by not only exerting antiprotease effects but also immunomodulatory and antiproliferative ones (Williams *et al.*, 2006; Verrier *et al.*, 2012). Hence, keratinocyte-derived elafin seems to favor the development of a T helper type1 response (Roghanian *et al.*, 2006), but it can also enhance the resolution of inflammation by facilitating phagocytosis of apoptotic leukocytes (Williams *et al.*, 2006).

In this study, we sought to analyze cutaneous elafin expression in the acute and chronic forms of GVHD and to explore whether cutaneous elafin was associated with distinct histopathological or clinical features related to the severity or outcome of GVHD. In addition, we wanted to evaluate the potential of cutaneous elafin as a marker to distinguish aGVHD from DHR.

RESULTS

Varying degrees of elafin expression in aGVHD and DHR

To explore the potential of cutaneous elafin as a marker to distinguish aGVHD from DHR, we quantified cutaneous elafin in these two diseases at both the mRNA and protein levels. We performed a quantitative real-time polymerase chain reaction (reverse transcriptase-PCR) with RNA extracted from whole-skin biopsies of 22 aGVHD patients, as well as 10 DHR patients. This analysis of elafin mRNA levels (normalized to healthy control skin) did not reveal any difference between aGVHD ($n=22$) and DHR ($n=10$; Figure 1a), and we could confirm our data in an independent cohort of aGVHD patients (Supplementary Figure S1 online).

In accordance with this finding, immunohistochemistry (IH) staining for elafin on paraffin sections of the same patients revealed various elafin expression patterns (Figure 1b and e). In some sections, elafin was restricted to the stratum granulosum, whereas in others it extended to the stratum spinosum (Figure 1e). In contrast, healthy control skin samples ($n=7$) were completely negative, and lesional psoriasis skin (stained as a positive control, $n=5$) was highly positive for elafin (Figure 1c). The percentage of elafin-positive keratinocytes as quantified using the HistoFAXS software (TissueGnostics GmbH, Vienna, Austria) was not significantly higher in aGVHD as compared with DHR (Figure 1d).

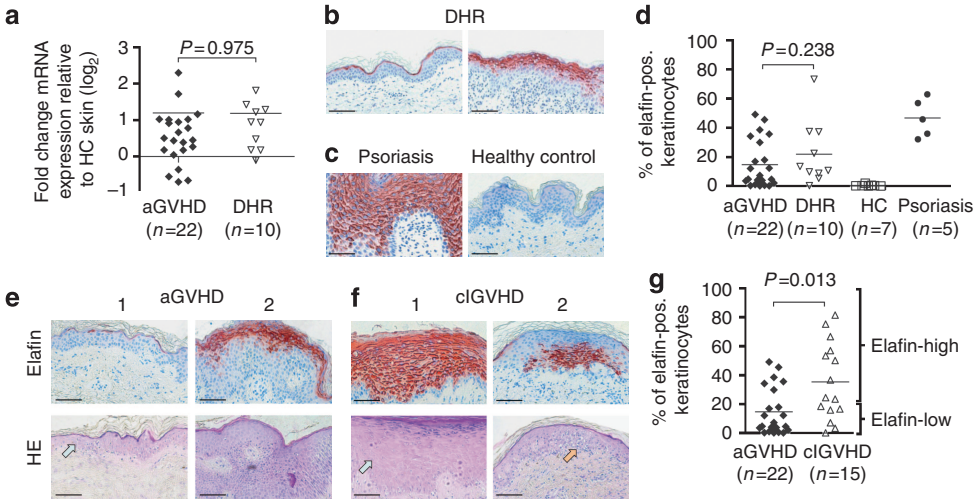

Figure 1. Various elafin expression patterns in cutaneous graft-versus-host disease (GVHD). (**a**) mRNA expression of elafin in acute graft-versus-host disease (aGVHD) and drug hypersensitivity reaction (DHR), normalized to healthy control (HC) skin. Quantitative real-time reverse transcriptase-PCR (RT-PCR) was performed after TRizol lysis of skin biopsies. (**b**, **c**) Representative pictures (scale bar = 100 μm in all pictures) of immunohistochemistry (IH) elafin staining in DHR, HC, and psoriasis. Images were taken using a PixeLINK PL-B623CF color digital camera (Zeiss, Oberkochen, Germany). (**d**) The percentage of elafin-positive keratinocytes in aGVHD as compared with DHR measured using HistoQuest imaging analysis software. Expression in HC and lesional psoriatic skin is shown as a negative control and a positive control, respectively. (**e**, **f**) Pictures of elafin staining in aGVHD and chronic lichenoid graft-versus-host disease (clGVHD) skin (two different patients each) with corresponding hematoxylin and eosin (HE) sections shown below (scale bar = 100 μm in all pictures). Blue arrows indicate correlation of epidermal thickness with increased elafin expression. The orange arrow points to the prominent inflammatory infiltrate in an elafin-high skin area. (**g**) Percentage of elafin-positive keratinocytes in aGVHD vs. clGVHD. Cutoff (at 20%) between the elafin-high and the elafin-low groups.

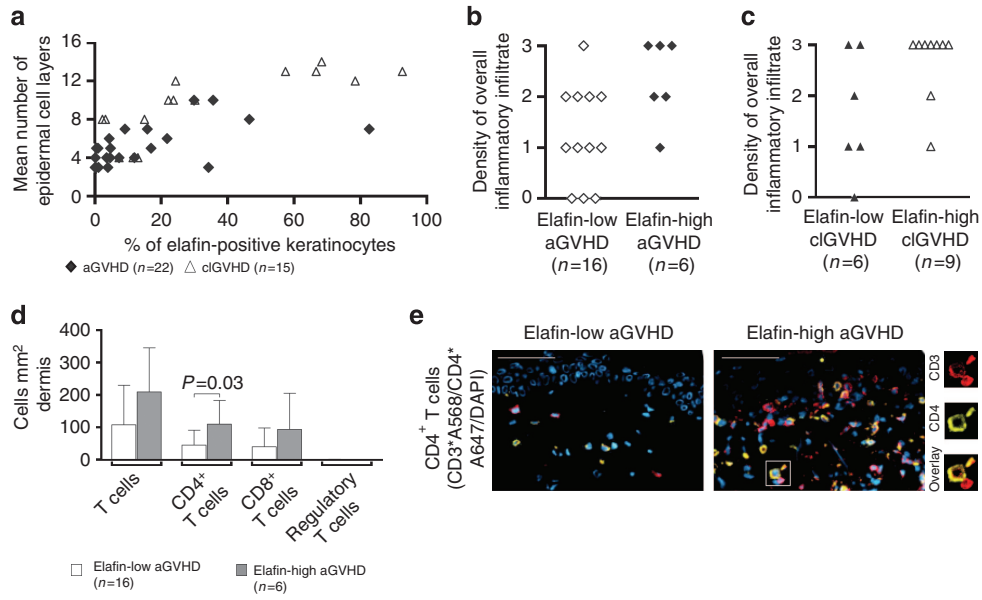

Figure 2. Epidermal thickening and a prominent CD4$^+$ T-cell infiltrate in elafin-high graft-versus-host disease (GVHD) lesions. (**a**) Mean number of epidermal cell layers and the percentage of elafin-positive keratinocytes in acute graft-versus-host disease (aGVHD)/chronic lichenoid graft-versus-host disease (clGVHD) patients. The mean number of cell layers was calculated as (minimal number of cell layers epidermis + maximal number of layers epidermis)/2. (**b**, **c**) Quantification of the overall inflammatory infiltrate in elafin-low versus elafin-high groups of the aGVHD and clGVHD cohorts. (**d**) Quantitative analysis of the dermal T-cell infiltrate (T cells: CD3$^+$, CD4$^+$ T cells: CD3$^+$CD4$^+$, CD8$^+$ T cells: CD3$^+$CD8$^+$; regulatory T cells (Treg) cells: CD4$^+$Foxp3$^+$) in elafin-high as compared with elafin-low aGVHD skin lesions. (**e**) Representative images of immunofluorescence (IF) stainings of CD4$^+$ T cells merged with DAPI in an elafin-high and an elafin-low aGVHD skin lesion. Scale bar = 50 μm in both pictures. The epidermis is located on the upper side in both images. Images were taken using a PCO PixelFly camera (Zeiss).

Different patterns of epidermal elafin expression in aGVHD and clGVHD

As elafin expression has only been analyzed in cutaneous aGVHD, we asked whether the chronic forms of GVHD would exhibit similar elafin expression patterns. To explore keratinocytic elafin expression in these patient groups, we performed an IH staining for elafin on skin sections of 15 clGVHD and seven csGVHD patients. Although no, or only very low, elafin expression was seen in csGVHD (data not shown), clGVHD lesions exhibited various expression patterns. Only in a few sections elafin staining was restricted to the stratum granulosum, whereas in the other skin specimens it was detectable in the stratum spinosum with an either patchy or continuous pattern (Figure 1f). The percentage of elafin-positive keratinocytes was significantly higher ($P = 0.013$) in clGVHD as compared with aGVHD (Figure 1g).

Elafin expression is associated with epidermal thickening

The expression of elafin in aGVHD and clGVHD skin biopsies was heterogeneous. To evaluate a possible association of upregulated elafin expression with histopathological or clinical features, aGVHD and clGVHD patient cohorts were divided into an elafin-low subgroup and an elafin-high subgroup (cutoff shown in Figure 1g). Patients were considered elafin-low if the elafin staining was restricted to the stratum granulosum, which corresponded to less than 20% elafin-positive keratinocytes. According to this classification, 27% of the aGVHD (6/22) and 60% of the clGVHD patients (9/15) belonged to the elafin-high groups.

To address the potential association of elafin expression with distinct histopathological features, parameters known to be relevant (Lerner *et al.*, 1974) for GVHD diagnosis were assessed on hematoxylin and eosin (HE)–stained skin sections. The parameters analyzed were vacuolization, dyskeratosis, overall inflammatory infiltrate, hyperkeratosis, and hypergranulosis, as well as epidermal thickness. These features were evaluated by a blinded histopathologist (PP) and compared between elafin-high versus elafin-low patients of our aGVHD and clGVHD cohorts.

Dyskeratosis and vacuolization were not associated with higher elafin expression in aGVHD or clGVHD (Supplementary Figure S2a–b online). Epidermal thickening, assessed as the mean number of epidermal cell layers found in a section, correlated with increased elafin expression (Figure 1e and f, blue arrows on HE images; Figure 2a). A statistically significant difference was found between elafin-high and elafin-low groups in aGVHD ($P = 0.003$) and clGVHD ($P = 0.008$). Additional signs of epidermal thickening that could be observed in clGVHD, i.e., acanthosis and hypergranulosis, were more often present in elafin-high specimens (Supplementary Figure S2c–d online).

We found the inflammatory infiltrate to be more prominent in elafin-high skin areas (Figure 1f, HE image, Figure 2b and c). To substantiate this finding, we performed immunofluorescence stainings of CD4$^+$ and CD8$^+$ T cells, as well as regulatory T cells. This revealed a trend toward higher elafin expression in the presence of a prominent CD4$^+$ and CD8$^+$ T-cell infiltrate (Supplementary Figure S3a–c online)

with an increase of $CD4^+$ T cells in the dermis of elafin-high aGVHD samples as compared with elafin-low ones (Figure 2d and e). Regulatory T cells were almost absent in all of the skin lesions.

TNFα and TGFβ are dysregulated in elafin-high GVHD skin lesions

On the basis of the observation that increased elafin expression was associated with altered histopathological features, i.e., epidermal thickening and a more prominent inflammatory infiltrate, we were interested in the mechanisms driving elafin expression in GVHD.

We hypothesized that the expression of cytokines known to modulate keratinocytic elafin release could be altered in skin displaying high elafin expression. Therefore, we quantified mRNA levels of IL-1β and tumor necrosis factor alpha (TNFα), both potent inducers, and transforming growth factor beta (TGFβ), an inhibitor of elafin secretion by keratinocytes. We found a significant ($P=0.03$) increase of TNFα (Figure 3a) in elafin-high as compared with elafin-low aGVHD skin. In contrast, TGFβ was downregulated ($P=0.03$) in elafin-high lesions as compared with elafin-low clGVHD lesions (Figure 3b). Hence, cytokines appear to have a role in the distinct expression patterns of elafin in GVHD.

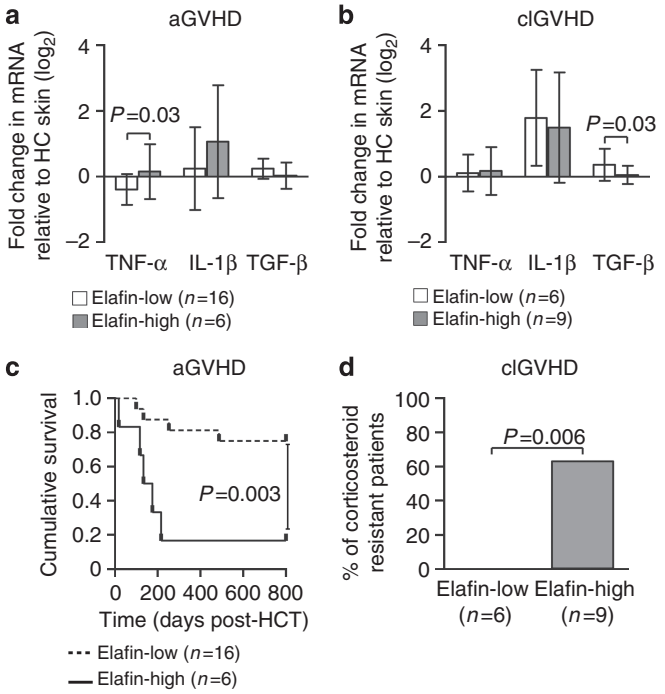

Figure 3. High elafin expression in graft-versus-host disease (GVHD) is associated with corticosteroid unresponsiveness and decreased overall survival (OS). (**a**,**b**) Comparison of mRNA expression of tumor necrosis factor alpha (TNFα), IL-1β, and transforming growth factor beta (TGFβ) in elafin-high as compared with elafin-low groups of (**a**) acute graft-versus-host disease (aGVHD) and (**b**) chronic lichenoid graft-versus-host disease (clGVHD) patients. Quantitative real-time reverse transcriptase-PCR (RT-PCR) was performed after TRizol lysis of skin biopsies. Data are normalized to β_2m of each specimen and represent the mean ± SD of ($\log_2$) fold change in mRNA expression relative to healthy control (HC) skin ($n=10$). (**c**) Cumulative survival after 2 years of elafin-high versus elafin-low aGVHD patients shown as a Kaplan–Meier curve. (**d**) Percentage of corticosteroid-resistant clGVHD patients in the elafin-high versus elafin-low groups.

Elafin is associated with poor prognosis of GVHD

Finally, we sought to determine whether high elafin expression in cutaneous GVHD had a clinical correlate and was associated with a more severe form or poor prognosis of the disease. Clinical characteristics related to the severity or the outcome of GVHD were analyzed and compared between elafin-high and elafin-low groups of aGVHD and clGVHD patients. The analysis of these parameters revealed that neither the histological or clinical staging (Supplementary Table S1 online) of GVHD skin involvement nor the GVHD overall grading differed between elafin-high and elafin-low groups of the aGVHD or clGVHD cohorts. However, the prognosis of patients was worse in aGVHD and clGVHD patients with elafin-high skin lesions. Elafin-high aGVHD patients had a significantly decreased 2-year overall survival ($P=0.003$; Figure 3c) and elafin-high clGVHD patients were more frequently refractory to corticosteroids ($P=0.006$; Figure 3d).

DISCUSSION

In the present study, we could show that both aGVHD and clGVHD skin lesions exhibit diverse elafin expression patterns associated with distinct molecular, histopathological, and clinical features. In addition, the analysis of cutaneous elafin did not allow distinguishing between aGVHD and DHR skin eruptions. We report comparable levels of elafin expression at the mRNA and protein levels in both DHR and aGVHD rashes. Remarkably, elafin expression was higher in clGVHD as compared with aGVHD but not detectable in csGVHD. In both aGVHD and clGVHD, high elafin expression was associated with epidermal thickening and a trend toward a more prominent T-cell infiltrate. Although none of the clinical/histological grading criteria were associated with high elafin expression, elafin-high aGVHD patients had a decreased overall survival and clGVHD patients were more frequently refractory to corticosteroids. TNFα, a potent inducer of elafin expression in keratinocytes, was increased in elafin-high as compared with elafin-low aGVHD skin, whereas the inhibitor TGFβ was downregulated in elafin-high clGVHD skin specimens.

The distinction between acute cutaneous GVHD and DHR is challenging, yet therapeutically important. Our findings from two independent cohorts strongly indicate that the analysis of elafin in the skin is not a useful marker to support the histopathological diagnosis of cutaneous aGVHD and to discriminate between this disease and DHR. Our observations partially differ from those made by others (Paczesny *et al.*, 2010) who stained for elafin in the skin of aGVHD ($n=10$), as well as of DHR ($n=10$) patients, and found expression in the majority of aGVHD (70%) but in none of the DHR samples. Conceivably, these divergent results are because of differences in either clinical, procedural, or data collection/analysis-related factors. Potentially critical clinical parameters in

Table 1. Patient characteristics

	aGVHD ($n=22$)	clGVHD ($n=15$)		DHR ($n=10$)
Patient age[1]	47.6 ± 13.2	46.8 ± 13.2		53.7 ± 13.9
Patient gender				
F	11	9		4
M	11	6		6
Skin clinical staging				
Stage 1	4	3	Generalized MPR[2]	7
Stage 2	7	7	Erythrodermia[2]	1
Stage 3	11	5	Erythrodermia, few blisters[3]	1
			Suberythrodermia, mucosal enanthema[4]	1
Diagnosis before HCT				
ALL	2	6		
AML	9	5		
sAML	3	2		
CLL	1			
CML	1			
HL		1		
NHL	4	1		
MDS	2			
Stem cell source				
PBSC	20	15		
BM	2			
Donor type				
MUD	13	6		
MMUD	6	5		
SIB	3	4		
Gender mismatch (donor/recipient)				
F/F	10	2		
M/F	1	7		
F/M		2		
M/M	11	4		
Conditioning				
RIC	13	8		
MAC	9	7		
GVHD prophylaxis				
CSA MMF	12	5		
CSA MTX	9	9		
CSA	1	1		

Abbreviations: aGVHD, acute graft-versus-host disease; ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; BM, bone marrow; clGVHD, chronic lichenoid graft-versus-host disease; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia; CSA, cyclosporine A; DHR, drug hypersensitivity rashes; F, female; HCT, allogeneic hematopoietic stem cell transplantation; HL, Hodgkin's lymphoma; M, male; MAC, myeloablative conditioning; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MMUD, mismatched unrelated donor; MPR, maculopapular rash; MTX, methotrexate; NHL, non-Hodgkin's lymphoma; PBSC, peripheral blood stem cells; RIC, reduced-intensity conditioning; sAML, secondary acute myeloid leukemia; SIB, sibling; TEN, toxic epidermal necrolysis.

[1]Mean (years) ± SD.

[2]No blisters, no mucosal involvement.

[3]No mucosal involvement (no TEN).

[4]No blisters (no TEN).

DHR patients are the diversity of clinical manifestations (in our patients: 7/10 generalized maculopapular rash, 3/10 (sub)erythroderma; details see Table 1) and the HCT status of patients (HCT recipients vs. non-HCT recipients). DHR manifestations in the study of Paczesny *et al.* (2010) were denominated as maculopapular rash but not further detailed. A limitation of our work is that DHR patients of the study by Paczesny *et al.* (2010), but not of our study, were HCT recipients. Unpublished data from our lab indicate, however, that elafin expression is not altered in HCT patients ($n=4$) as compared with non-HCT patients with DHR. In aGVHD patients, the underlying diagnosis before HCT, as well as the course and severity of the disease (see Table 1), could be important. Our data suggest that disease prognosis rather than severity is associated with an altered elafin expression. Meanwhile, it is difficult to evaluate the potential impact of the other parameters because Paczesny *et al.* (2010) provided information of their entire aGVHD plasma elafin validation cohort ($n=58$), but not specifically of the ten aGVHD patients (part of this cohort), in which elafin expression was evaluated in the skin. Among procedural aspects, biopsy timing, as well as the type of conditioning and GVHD prophylaxis, could be important but are not specified for the Paczesny *et al.* (2010) cohort. With regard to data analysis/collection, IH elafin staining in the Paczesny study was evaluated by two blinded independent pathologists considering sections to be positive if more than 50% of the epidermis was stained. We used the HistoFAXS software system to more precisely and objectively measure the percentage of elafin-positive keratinocytes and concurrently quantified elafin expression at an mRNA level. These methodological differences cannot sufficiently explain the divergent results, as a re-analysis of our data using the method of Paczesny *et al.* (2010) (data not shown) confirmed our findings.

The various mechanisms through which elafin acts on the cutaneous immune homeostasis make it a candidate effector molecule of interest in the pathogenesis of GVHD. Besides its major role as an inhibitor of neutrophil-derived elastases, elafin has antimicrobial, immunomodulatory, and tissue-remodeling properties (Williams *et al.*, 2006; Verrier *et al.*, 2012).

Particularly interesting with regard to our results is the observation that elafin can inhibit elastase-induced keratinocyte proliferation (Meyer-Hoffert *et al.*, 2004). In contrast to normal skin where it is not detectable (Pfundt *et al.*, 1996; Kamsteeg *et al.*, 2010), elafin is abundantly secreted in diverse skin conditions exhibiting hyperproliferative features. These include psoriasis (Kamsteeg *et al.*, 2010), wound healing (van Bergen *et al.*, 1996), diverse skin neoplasms (Alkemade *et al.*, 1993), and lichen planus (unpublished data from our lab). Our study not only revealed increased elafin expression in clGVHD, another disease characterized by keratinocytic hyperproliferation (Shulman *et al.*, 2006), but also demonstrated a strong association between high elafin expression and epidermal thickening. On the basis of these observations, it would be conceivable that elafin production may represent a compensatory mechanism to regulate keratinocytic hyperproliferation.

This hypothesis is further supported by our observation that TNFα mRNA expression is slightly increased in elafin-high aGVHD specimens. TNFα potently induces elafin production in keratinocytes and is a key mediator in aGVHD, where it is abundantly secreted after conditioning therapy for HCT. The TNFα upregulation and TGFβ (an inhibitor of elafin release) downregulation are in line with the previously described concept that elafin production by keratinocytes is triggered during inflammatory reactions characterized by high levels of TNFα and IL-1β. Nevertheless, this finding needs to be confirmed in a larger cohort.

In addition, elafin has been shown to favor the development of T helper type1 responses by enhancing dendritic cell accumulation and activation. As we could recently demonstrate that cutaneous clGVHD is characterized by a mixed T helper type1/ T helper type17 response (Brüggen *et al.*, 2014), higher elafin expression in clGVHD supports this idea. It remains to be elucidated whether our finding of an increased $CD4^+$ T-cell infiltrate in elafin-high aGVHD is, at least partially, attributable to elafin production. Alternatively, it might be due to molecules that are concurrently increased with elafin (such as TNFα).

Although in our hands cutaneous elafin expression is not a useful diagnostic marker for aGVHD and does not correlate with disease severity, our results show that high elafin expression in GVHD skin lesions heralds a worsened disease prognosis—i.e., decreased overall survival in aGVHD patients and corticosteroid unresponsiveness in clGVHD patients. Epidermal elafin could therefore serve as a predictive marker, provided that the diagnosis of GVHD has unequivocally been established. Studies by others (Paczesny *et al.*, 2010) indicate that plasmatic elafin levels could be helpful diagnostic and predictive biomarkers in cutaneous aGVHD. Preliminary data from our lab support this notion (data not shown).

In conclusion, our study shows that elafin expression in the skin is associated with poor prognosis in both the acute and chronic forms of cutaneous GVHD. Staining for cutaneous elafin in addition to the standard HE histopathological evaluation can be easily done, and it may facilitate the early identification of patients with an unfavorable prognosis, provided that the diagnosis of GVHD has previously been established. This would allow the introduction of alternative treatment options and ultimately improve patient management.

MATERIALS AND METHODS

Study design and patient characteristics

A total of 44 HCT recipients treated in the Bone Marrow Transplantation Unit of Vienna's Medical University between 2007 and 2012 were enrolled in the study. In all, 22 patients suffered from aGVHD, 15 had clGVHD, and seven had csGVHD. Additional eight aGVHD cases from an independent cohort from Hematology/Transplantation, Hospital Saint Louis, Paris (collected in 2012), as well as ten cases of non-HCT recipients with DHR collected between 2009 and 2012 in the Dermatology Department of the University Hospital Zurich, were included.

In all GVHD patients, the diagnosis of GVHD was based on the National Institutes of Health (NIH) consensus criteria and confirmed

by histopathology. At the time of biopsy, none of the aGVHD patients were under corticosteroid treatment. Clinical aGVHD grading was based on modified Glucksberg criteria (Przepiorka *et al.*, 1995), and histopathological aGVHD grading was based on Lerner criteria (Lerner *et al.*, 1974). DHR patients clinically presented generalized maculopapular exanthema ($n=7$) or (sub)erythrodermia ($n=3$; for details, see Table 1). Patients with toxic epidermal necrolysis or grade 4 cutaneous aGVHD were not included. For cGVHD, the clinical and histological evaluations were performed according to the NIH consensus criteria (Filipovich *et al.*, 2005). Corticosteroid responsiveness was defined as the reduction in GVHD activity in one or more organs without progression in any organ or site and the ability to taper corticosteroids. Patient characteristics of GVHD and DHR patients are listed in Table 1. Psoriasis sections for IH stainings were taken from routine biopsies for diagnostic analysis.

The study was approved by the Ethics Committee of the Medical University of Vienna (EK 607/07) and the Ethics Committee of the University of Zurich (EK 647) and conducted according to the Declaration of Helsinki principles. All study subjects gave written informed consent and participated voluntarily.

Skin samples

6-mm skin punch biopsies were taken under local anesthesia. One part was embedded in formalin and used for conventional HE histopathological analysis, as well as for the elafin staining. The residual tissue material was divided in half. One half was used for TRIzol (Invitrogen Life Technologies, Vienna, Austria) fixation (reverse transcriptase-PCR), and the other half was embedded in optimal cutting temperature (OCT; TissueTek, Sakura Finetek Europe B.V., Alphen aan der Rijn, Netherlands) compound (immunohistology) and subsequently deep-frozen in liquid nitrogen. Tissue specimens were stored at $-20\,^{\circ}C$ (OCT) and $-80\,^{\circ}C$ (TRIzol) until further processing.

Evaluation of histopathological parameters and IH staining for elafin

Details on the histopathological evaluation, as well as on the IH staining for elafin, can be found in Supplementary Material and Methods.

Quantification of epidermal elafin expression

To quantify epidermal elafin expression, slides were scanned using the digital HistoFAXS imaging system (TissueGnostics GmbH). The percentage of elafin-positive keratinocytes was measured using the HistoQuest imaging analysis software (TissueGnostics GmbH). The detection baseline was adapted to the isotype control, and all images were acquired and analyzed using identical hardware and software settings.

Immunofluorescence stainings

Frozen tissue was cut into 5-μm-thick sections and mounted on capillary gap microscope slides (Dako, Glostrup, Denmark). Cryosections were air-dried for 20 minutes, fixed in ice-cold acetone (Sigma-Aldrich, Vienna, Austria) for 10 minutes, and stored at $-20\,^{\circ}C$.

Triple immunofluorescence stainings on sections of GVHD patients and healthy control ($n=7$) were performed and evaluated as previously described (Brüggen *et al.*, 2014). Negative controls were obtained by substituting IgG for the primary antibody. Labeled cells were expressed as the number of cells ± SD per either millimeter (mm) basement membrane (epidermis) or mm^2 (dermis).

Real-time reverse transcriptase-PCR

After TRIzol lysis, the samples were thawed on ice and homogenized with a FastPrep-24 homogenizer (MP Biomedicals; Solon, OH) according to the manufacturer's instructions. RNA concentration was determined on a NanoDrop ND-100 spectrophotometer (Peqlab Biotechnology; Erlangen, Germany). Reverse transcription, preamplification, quantification, and data analysis were performed as previously described (Brüggen *et al.*, 2014). Details on the primers used are listed in Supplementary Material and Methods.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 21 for Windows (IBM, Armonk, NY). For all comparisons of means between groups (elafin expression, epidermal thickness, infiltrate, PCR-data), a Mann–Whitney test was used and a P-value of <0.05 was considered statistically significant. Spearman's test was used to analyze the correlation between clinical/histological grading and elafin expression. Corticosteroid responsiveness and nonrelapse mortality in elafin-high and elafin-low groups was evaluated with a X^2 test. Log-rank test was used to compare the survival times. Bonferroni correction for multiple comparisons was conducted and a Bonferroni-adapted P-value of <0.01 was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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