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Differential expression of interferon-induced genes and other tissue-based biomarkers in acute graft-versus-host disease versus lupus erythematosus in skin

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Summary

Background.—In both acute graft-versus-host disease (GVHD) and lupus erythematosus (LE), the patient's own tissues are subjected to immunological assault via complex mechanisms influenced by interferon (IFN) and other cytokines. Though not typically confused clinically, these entities have overlapping histopathological findings in the skin.

Aim.—To assess whether GVHD can be differentiated from LE using molecular methods on skin specimens.

Methods.—We developed a quantitative reverse transcription PCR assay based on previously identified tissue-based biomarkers of cutaneous GVHD, and compared gene expression in GVHD with that in LE.

Results.—Both entities showed robust expression of IFN-induced genes and of genes encoding proteins involved in antigen presentation, cell signalling and tissue repair. Levels of gene expression differed significantly in GVHD compared with LE, particularly those of interferon-induced genes such as *MXI*, *OAS3*, *TAPI* and *STAT3* ($P < 0.01$). Three logistic regression models could differentiate the two entities with a high degree of certainty (receiver operating characteristic area under the curve was 1.0).

Conclusion.—The study demonstrates the feasibility of distinguishing between microscopically similar inflammatory dermatoses using tissue-based molecular techniques.

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Introduction

Acute graft-versus-host disease (GVHD), a serious complication of hematopoietic cell transplantation (HCT), occurs when the transplanted immune cells attack host tissues.¹ In a conceptually analogous fashion, lupus erythematosus (LE) results when a patient's own immune system breaks tolerance and mounts an immune reaction against autoantigens.² Although acute cutaneous GVHD and cutaneous involvement by LE are not typically confused clinically, owing to the different clinical settings and dermatological manifestations,³ these two entities may have overlapping histopathological changes. Specifically, both entities demonstrate varying degrees of vacuolar interface changes at the dermoepidermal junction, which may extend down the follicular epithelium.⁴ Additionally, both diseases characteristically lack prominent dermal eosinophils or epidermal spongiosis.⁵ Although certain histopathological features, such as dermal mucin or perivascular lymphocytic inflammation, may favour a diagnosis of LE over acute GVHD, the skin biopsy changes seen in acute GVHD can be indistinguishable from those seen in LE (Fig. 1). Superficial dermal clusters of interferon (IFN)-producing plasmacytoid dendritic cells may also be present in both entities.^{5,6}

The molecular underpinnings of GVHD and LE do not appear to have been directly compared previously. However, the pathogenesis of both entities is known to be influenced by type I IFNs (IFN- α and IFN- β), which trigger an immunological cascade via various pathways, including the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway.^{2,4,7-9}

Given the observation that GVHD and LE have conceptually and immunologically similar pathophysiological mechanisms, we aimed to quantitatively compare the molecular profiles of acute cutaneous GVHD and cutaneous LE, and to determine whether tissue-based molecular signatures could reliably differentiate between these two entities

Methods

This research was approved by the institutional review board of Mayo Clinic prior to its initiation.

Case selection

We selected formalin-fixed, paraffin-embedded (FFPE) skin biopsies from the clinical archives that were interpreted as GVHD or LE, and we recorded the anatomical site from which the biopsy had been taken. We also recorded whether or not patients were receiving immunosuppressive therapies at the time of biopsy and, if so, which medications. Diagnoses were confirmed by examination of the haematoxylin and eosin (H&E)-stained slides and by patient clinical history.

Samples from patients with LE were subclassified as acute cutaneous LE, subacute cutaneous LE, chronic cutaneous LE, or cutaneous LE not otherwise specified, as assessed by a dermatologist at the time of clinical care. Whether or not the patient had concomitant systemic LE (SLE) was also documented. For patients to be classified as having SLE, they

had to meet established American College of Rheumatology criteria, as assessed by a dermatologist or rheumatologist at the time of clinical care. Cases with vacuolar interface inflammation on skin biopsy were included, while those with clinical or microscopic features suggestive of alternative diagnoses were excluded.

Histopathological grade of vacuolar interface changes, based on established acute GVHD histopathological criteria for the skin,¹⁰ was determined for each case in a blinded fashion. We also semi-quantitatively assessed degree of inflammation (mild, moderate, abundant) for each case in a blinded fashion.

Quantitative reverse transcriptase PCR

Our group recently reported tissue-based biomarkers of acute GVHD of the skin using unbiased proteomics methods.⁷ In our previous study, each of these biomarkers was found to be overexpressed at least 20-log fold in acute cutaneous GVHD compared with normal post-HCT skin by laser-capture microdissection with liquid chromatography–tandem mass spectrometry.⁷ For the present study, we developed a quantitative RT-PCR assay to test a selection of these acute GVHD biomarkers. Specifically, we selected genes to test on the basis of their biological function, with an attempt to represent various immunological functions, as well as on commercial availability of relevant primers. We also tested control genes (*IL-8*, *CXCL1* and *Melan-A*).

RNA was purified from 6–10 sections 10 µm thick cut from diagnostic biopsy FFPE tissue, as previously described.¹¹ Quantitative RT-PCR was performed for each gene (BioMark HD System and dynamic array integrated fluid circuits; Fluidigm, South San Francisco, CA, USA). In total, 43 specific targets in 32 genes (26 experimental and 6 housekeeping genes) were amplified for each set of cDNA (standards and experimental samples). The following cDNAs were run per array: standards in triplicate, and control (internal standard) and experimental cDNAs in duplicate. All cDNA was pre-amplified (TaqMan Preamp Master Mix, Applied Biosystems, Foster City, CA, USA) and array-based quantitative PCR was performed with TaqMan Gene Expression Master Mix (Applied Biosystems). Primer sequences are given in Supplementary Table 1. After thermal cycling, raw count (Ct) data for standards was checked for linear amplification and transformed to copy number values. Copy numbers were normalized to the housekeeping genes. Averaged, normalized gene copy numbers were compared with an internal standard for inter-experiment variation. Data that did not pass both linear amplification and reproducibility checks were discarded.

Statistical analysis

For the primary analysis, we compared all cases of GVHD with all cases of LE. We performed the following comparisons as secondary analyses: (i) GVHD cases from anatomical sites other than the torso and lower extremities (as these anatomical sites were not represented among the LE specimens) vs. all LE cases; (ii) GVHD cases from anatomical sites other than the torso and lower extremities vs. cases of systemic LE only; and (iii) skin involved by LE from both patients with and patients without SLE. For each comparison, two different statistical analyses were performed. First, the quantified expression of each gene was compared between GVHD and LE using Wilcoxon test, with

statistical significance set as $P < 0.05$. For the development of a model to differentiate acute GVHD from LE, all cases were included in analysis. All genes were subjected to a variable selection process to identify candidate genes that can potentially differentiate between GVHD and LE. This process created 1000 randomly seeded, logistic regression models, each configured to use a three-fold crossvalidation. The variables with nonzero coefficients in all 1000 models were considered for final model building. During this phase, logistic regression models were generated for all possible combinations of the final variables. All models with no interdependent variables and the best area under the curve (AUC) for differentiating between GVHD and LE were considered for final reporting. This recursive and layered model building allowed us to identify equally well-performing gene expression-based classifiers, while reducing the likelihood of overfitting the data.

Results

For the primary analysis, we included 14 cases of LE and all cases of acute GVHD available in a study set of this disease ($n = 49$). Clinical features are outlined in Table 1.

Mean histopathological grade of vacuolar interface inflammation between acute GVHD cases and LE cases did not differ significantly ($P = 0.7$). Although LE cases tended to show more inflammation than did GVHD cases, particularly in the perivascular compartment, this difference was not statistically significant ($P = 0.1$). Genes showing significant quantitative differences in mRNA expression in GVHD compared with LE are summarized in Table 2. Of all the IFN-inducible genes tested, the only one that showed equivalent expression between GVHD and LE was *GBP2* (GVHD/LE ratio 1.00; $P = 0.91$), with all others showing significantly greater expression in LE (Table 2). The only gene expressed at significantly higher levels in GVHD compared with LE was *IVL* (involucrin) (GVHD/LE ratio 1.2; $P < 0.01$). Expression of control genes (*IL8*, *CXCL1* and *Melan-A*) showed no significant differences between the two groups (data not shown).

For the secondary analyses, we discovered that these trends persisted when biopsy specimens of acute GVHD derived from sites other than the torso and lower extremities were excluded (Supplementary Table 2) or when cases of cutaneous-only LE were excluded (Supplementary Table 3). When skin involved by LE from patients with SLE ($n = 7$) was compared with skin involved by LE from patients without SLE ($n = 7$), no differences in gene expression were detected (Supplementary Table 4).

Using data from the primary analysis, we were able to generate three distinct models, each using expression values of 2 of the 5 markers that offered excellent diagnostic accuracy (AUC = 1.0) in the samples tested: *ITGA5*, *LOXL1*, *PTK2*, *TAP1* and *OAS3* (Table 3).

Discussion

Previously, we found that several type I IFN-inducible proteins, including MX1, OAS3, TAP1 and STAT3, are overexpressed in acute cutaneous GVHD compared with normal post-HCT skin.⁷ In the current study, we demonstrated that expression of type I IFN-inducible genes in skin specimens was significantly more robust in LE, regardless of clinical subtype, compared with acute GVHD. This finding confirms the results of prior work on blood and

mesenchymal stem cells implicating type I IFNs in these conditions,^{12,13} and additionally validates that the influence of IFN is also observed in affected skin tissue. Although expression of most IFN-inducible genes tested was significantly higher in LE than GVHD, *GBP2* (guanylate binding protein 2, the only IFN- γ -inducible gene tested, was expressed at levels that were equivalent between the groups. In addition, thrombospondin, a protein that suppresses IFN- γ and is also involved in early wound healing,¹⁴ was expressed to a higher degree in LE than GVHD. *GBP2* has been observed to be elevated in early liver GVHD in oligonucleotide microarray studies¹⁵ and in Langerhans cells regulating cutaneous GVHD.¹⁶ Based on data from the current study, it appears that the ratio between IFN- γ effect (with *GBP2* as a tissue-based proxy gene) and type I IFNs (with *MX1*, *OAS3*, *TAP1* and *STAT3* as tissue-based proxy genes) may be higher in GVHD than in LE. The role of IFN- γ is complex but is generally thought to have a regulatory effect on GVHD. Therefore, the role of IFN- γ in tissues may be an interesting avenue for further study.

Emerging evidence indicates that serum levels of IFN- α , IFN- γ and related cytokines may correlate with various clinical subtypes and/or severity in LE.^{17,18,25-27} The present study was not designed to test whether the relationship between IFN-inducible gene expression and clinical subtypes or disease severity carries over to tissue; future studies may be enlightening in this regard.

Genes encoding proteins integral to cellular adhesion, signalling and migration, such as the integrins, the tyrosine kinase PTK2, STAT3 and the laminin subunit LAMB1, were also expressed in both conditions, to a greater degree in LE. It has been shown that the lupus band partially colocalizes to laminin-1¹⁹ and that circulating anti-laminin-1 autoantibodies can be detected in the sera of most patients with cutaneous LE.²⁰ It is unknown in LE if perhaps overexpression of laminin leads to the development of antilaminin antibodies or perhaps keratinocyte apoptosis unmasks laminin subunit epitopes, leading to the development of antilaminin antibodies as an epiphenomenon.

The gene *PSMA3* encodes a subunit of proteasomes, the cellular machinery required for antigen processing and presentation central to both processes. This gene was overexpressed in both LE and GVHD, with the former having greater expression. The use of proteasome inhibitors for the treatment of both appears promising.^{21,22}

Several genes induced by cellular stress (*SQRDL*, *LOXL1* and *HYOU1*) were expressed in both conditions, to a greater degree in LE than GVHD. Other hypoxia-related factors, particularly HIF-1 α (hypoxia-inducible factor-1 α), are also thought to be involved with the development of autoimmune diseases.²³ Therapies targeting heat shock proteins are being trialled in both LE and GVHD.^{24,25}

One of the novel aspects of this study is that histopathologically similar inflammatory dermatoses were compared and differentiated using molecular methods. In other areas of dermatopathology, such as interpretation of atypical melanocytic proliferations, it is well understood that prediction of tumour biological behaviour is not always possible based on histopathology alone, and that molecular methods may improve accuracy.¹¹ This study demonstrates the feasibility of designing molecular tissue-based assays to reliably

distinguish two related inflammatory skin dermatoses with disparate clinical features. This proof of concept provides promise for other potential, clinically useful assays, such as one to distinguish acute cutaneous GVHD from post-HCT drug or viral rash with vacuolar interface changes, a true clinicopathological conundrum with practical implications for patients. Another strength of our study is that, by using cases with similar histopathological grades of vacuolar interface dermatitis, we controlled for degree of epidermal apoptosis and basilar degeneration as potential confounding variables.

A limitation of the study is that gene expression differences may, at least in part, reflect other confounding patient variables, such as the effect of conditioning or of immunosuppression in patients with GVHD or an autoimmune tendency in patients with LE. The higher rates of immunosuppression with patients with GVHD may correlate with reduced gene expression. However, we do not believe this factor accounts for the observed differences in gene expression entirely, as control genes were expressed in similar levels between the two groups. Owing to the nature of these clinical conditions, it was not possible to control for these patient variables. Discrepancies in anatomical distribution of skin biopsy collection sites between the groups represents another study limitation, although comparisons of LE with GVHD (excluding cases from the torso and lower extremities) and to more closely mirror the anatomical sites affected by LE yielded similar findings. Disease severity could not be determined accurately owing to variations in documentation inherent to the retrospective study design. In addition, this study did not emphasize the influence of tumour necrosis factor (TNF)- α , a cytokine known to be involved in the development of LE and GVHD, because TNF- α -associated proteins happened not to be featured prominently in the previous proteomics studies on which the current assay was based.⁷ However, issue-based expression of TNF- α induced genes could be the subject of future study. It was not feasible to determine whether skin that was biopsied and included in the study had been treated with topical immunomodulatory agents prior to harvesting. Finally, this study evaluated mRNA expression only. Future studies evaluating expression of corresponding proteins may be valuable in validating these findings.

Conclusion

LE and GVHD share molecular similarities in the skin, particularly with regard to expression of genes induced by type I IFN, those instrumental in cellular signalling and adhesion, and those responsive to tissue injury. However, these genes are expressed to a differential degree, as demonstrated by the development of several molecular models that can distinguish GVHD from LE with a high level of accuracy in tested samples. Although preliminary, our observations generate several hypotheses regarding therapeutic targeting of the genes overexpressed in affected skin. This study also demonstrates the novel principle of characterizing gene expression in skin biopsy specimens in inflammatory dermatoses that share similarities in pathogenic mechanisms and histopathological features.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What's already known about this topic?

- Although clinically distinct, LE and acute GVHD have overlapping histopathological features and pathophysiological mechanisms.

What does this study add?

- A tissue-based quantitative RT-PCR assay allowed for reliable differentiation between acute GVHD and LE using molecular data alone.

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Figure 1.
(a) Lupus erythematosus; (b) acute graft-versus-host disease. (haematoxylin and eosin, original magnification (a,b) $\times 100$. Both entities may show vacuolar interface inflammation along the dermal–epidermal junction, extending down the hair follicles.

Table 1

Clinical features of patients with either acute graft-versus-host disease of the skin or cutaneous involvement by lupus erythematosus.

Parameter	Acute GVHD (<i>n</i> = 49)	LE (<i>n</i> = 14)
Sex, M./F (%)	28/11 (57/43)	2/12 (14/86)
Age at skin biopsy, years; mean (range)	54.9 (20.9–74.4)	60.6 (36.3–80.1)
Hematopoietic cell transplantation graft type (GVHD; <i>n</i> = 48), <i>n</i> (%) [*]		
Peripheral stem cells	39 (81)	NA
Bone marrow	5 (10)	
Unrelated cord blood	4 (8)	
Haematological diagnosis requiring HCT (GVHD), <i>n</i> (%)		
AML	17 (35)	NA
MDS	15 (31)	
CML	6 (12)	
NHL	2 (4)	
ALL	3 (6)	
Multiple myeloma	1 (2)	
Other leukaemia	1 (2)	
Conditioning regimen (GVHD; <i>n</i> = 47 [*]), <i>n</i> (%)		
Fludarabine/TBI	6 (13)	NA
Fludarabine/TBI/cyclophosphamide	6 (13)	
Busulfan/cyclophosphamide	6 (13)	
Busulfan/fludarabine	8 (17)	
Melphalan/fludarabine	12 (26)	
Cyclophosphamide/TBI	7 (15)	
BEAM	1 (2)	
Cladribine/thiotepa/thymoglobulin	1 (2)	
Relationship of donor to recipient for HCT (GVHD; <i>n</i> = 47 [*]), <i>n</i> (%)		
Full sibling	22 (47)	NA
Haplotype-matched	1 (2)	
Other relative	1 (2)	
Unrelated	23 (49)	
Clinical subtype of LE skin lesions and SLE status, <i>n</i> (%)		
Acute cutaneous LE, with SLE	NA	6 (43)
SCLE, without SLE		4 (29)
SCLE, with SLE		2 (14)
Cutaneous LE, NOS; without SLE		2 (14)
Systemic immunosuppressive medications at time of skin biopsy, <i>n</i> [†]		
Yes	44/47** (94)	3 (21)
Tacrolimus	19	0
Ciclosporin	20	0

Parameter	Acute GVHD (<i>n</i> = 49)	LE (<i>n</i> = 14)
Mycophenolate mofetil	8	0
Systemic corticosteroids	11	2
Methotrexate	0	2
Hydroxychloroquine	0	2
Anatomical site of skin biopsy, <i>n</i> (%)		
Head/neck	3 (6.1)	3 (21.4)
Upper extremity (including shoulder)	13 (26.5)	6 (42.8)
Lower extremity	12 (24.5)	0
Back	7 (14.3)	2 (14.3)
Torso (abdomen/flank)	11 (22.4)	0
Chest	3 (6.1)	3 (21.4)

ALL, acute lymphocytic leukemia; AML, acute myeloid leukaemia; BEAM, BCNU (carmustine), etoposide, Ara-C (cytarabine) and melphalan; CML, chronic myeloid leukaemia; GVHD, graft-versus-host disease; HCT, haematopoietic cell transplantation; LE, systemic lupus erythematosus; MDS, myelodysplastic syndrome; NA, not applicable; NOS, not otherwise specified; SCLE, subacute cutaneous lupus erythematosus; TBI, total body irradiation.

* Data not available for all patients

[†] totals may exceed total number of patients on systemic immunosuppressant medications, as patients could be taking > 1 systemic immunosuppressive medication.

Table 2

Genes, their biological function and the expression ratio of genes in acute graft-host-disease to those in lupus erythematosus

Gene	Full name	Biological function	Ratio (GVHD/LE)	P*
<i>MXI1</i> [†]	MX dynamin-like GTPase 1	Metabolism of GTP; antiviral	0.53	< 0.001 *
<i>OAS3</i> [†]	2'-5'-oligoadenylate synthetase 3	Cellular protein synthesis inhibition; antiviral	0.58	< 0.001 *
<i>TAP1</i> [†]	Transporter 1, ATP binding cassette subfamily B member	Transport of degraded cytosolic peptides within the endoplasmic reticulum to allow assembly of class I molecules	0.28	< 0.001 *
<i>STAT3</i> [†]	Signal transducer and activator of transcription 3	Transcription activation	0.67	0.001 *
<i>GBP2</i>	Guanylate-binding protein 2	IFN- γ -induced GTPase	1.00	0.91
<i>THBS2</i>	Thrombospondin	Cellular adhesion and migration; suppresses IFN- γ and TNF- α	0.74	< 0.001 *
<i>SQRDL</i>	Sulfide quinone reductase	Decreasing toxic levels of sulphide	0.73	< 0.001 *
<i>PTK2</i>	Protein tyrosine kinase 2	Cell surface adhesion, signalling	0.42	< 0.001 *
<i>PSMA3</i>	Proteasome subunit alpha 3	Processing of class I MHC peptides	0.71	< 0.001 *
<i>LOXL1</i>	Lysyl oxidase-like 1	Synthesis of connective tissue, including collagen and elastin	0.57	< 0.001 *
<i>ITGA5</i>	Integrin subunit alpha 5	Cell surface adhesion, signalling	0.85	< 0.001 *
<i>HYOU1</i>	Hypoxia upregulated protein 1	Protects against hypoxia-induced apoptosis	0.53	< 0.001 *
<i>LAMB1</i>	Laminin subunit beta 1	Cellular attachment, chemotaxis	0.24	< 0.01 *
<i>IVL</i>	Involucrin	Formation of cell envelope	1.20	< 0.01 *

GVHD, graft-versus-host disease; IFN, interferon; LE, lupus erythematosus; MHC, major histocompatibility complex. TNF, tumour necrosis factor. Genes were assessed by quantitative reverse transcription PCR from formalin-fixed, paraffin-embedded skin biopsy specimens.

* Statistical significance was set at $P < 0.05$

[†] known to be induced by type I interferons.

Table 3

Top-performing logistical regression models developed to differentiate acute graft-versus-host disease and lupus erythematosus of the skin. Models are based on quantitative expression of the genes listed, as determined by quantitative RT-PCR from formalin-fixed, paraffin-embedded skin biopsy specimens. AUC, area under the curve.

Gene	Estimate	<i>P</i> *	AUC
Model 1			
TAP1	−0.4	< 0.01 *	1
LOXL1	−3.85	< 0.001 *	
ITAG5	−1.43	0.25	
Intercept	44.075		
Model 2			
TAP1	−2.049	0.04	1
LOXL1	−9.703	< 0.001 *	
OAS2	−1.435	0.14	
Intercept	107.318		
Model 3			
TAP1	−1.902	< 0.001 *	1
LOXL1	−9.926	< 0.001 *	
Intercept	95.153		

* Statistical significance was set at $P < 0.05$.