Lymphocytes, cytokines and adhesion molecules in chronic graft versus host disease

Sélim Aractingi, Eliane Gluckman, Caroline Le Goué, Louis Dubertret, Edgardo D Carosella

Abstract

**Aims**—To determine which inflammatory and immune pathways are implicated in the development of chronic graft versus host disease (GvHD) and whether differences between these pathways are responsible for the different presentations of chronic GvHD.

**Methods**—Biopsy specimens of diseased and normal skin were obtained from patients presenting with lichen planus-like and sclerodermatous type chronic GvHD. Expression of epidermal cytokines, adhesion molecules and lymphoid surface markers was analysed by means of immunohistochemistry. Apoptosis was detected using the in situ nick end-labelling method.

**Results**—In both GvHD lesion types, CD8+ cells predominated in the epidermis, whereas CD4+ cells were the most prevalent in the dermis. Apoptotic keratinocytes were found in diseased skin only and Fas antibodies labelled a considerable number of keratinocytes. The epidermis in both types of lesions expressed interleukin (IL) 1α, tumour necrosis factor (TNF) α and intercellular adhesion molecule (ICAM)-1, but dermal vascular cell adhesion molecule (VCAM)-1 expression was restricted to specimens of lichen planus-like GvHD. IL1α and E-selectin were expressed in normal looking skin of 55% and 80%, respectively, of patients with lichen planus-like GvHD.

**Conclusion**—The similarity between expression of epidermal cytokines and adhesion molecules (with the exception of VCAM-1) and lymphocyte phenotype in lichen planus-like and sclerodermatous GvHD strongly suggests that the latter occurs as a consequence of the healing process. VCAM-1 distinguishes between lichen planus-like and sclerodermatous lesions. IL1α and E-selectin are potential early markers of chronic GvHD.

Keywords: graft versus host disease, skin, lymphocytes, cytokines, adhesion molecules.

Chronic graft versus host disease (GvHD) is one of the most disabling complications of bone marrow transplantation (BMT). About 30–50% of transplant recipients still living three months after transplantation will present with features of this disease. In contrast to acute GvHD, where a cytotoxic reaction driven by donor T lymphocytes against host antigens has been demonstrated, the pathogenesis of chronic GvHD is poorly understood. Evidence for an interaction between anti-host and autoimmune phenomena is described frequently, but the precise pathways leading to the development of the lesions are still unclear.

**Table 1** Antibodies used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reactivity</th>
<th>Source</th>
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<tbody>
<tr>
<td>CD3</td>
<td>T lymphocytes (CD3 complex associated with ζζ or ζζTCR)</td>
<td>Dako</td>
</tr>
<tr>
<td>CD4</td>
<td>Helper/inducer T lymphocytes</td>
<td>Dako</td>
</tr>
<tr>
<td>CD8</td>
<td>Suppressor/cytotoxic lymphocytes</td>
<td>Dako</td>
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<tr>
<td>CD16</td>
<td>Natural killer cells</td>
<td>Dako</td>
</tr>
<tr>
<td>CD25</td>
<td>IL-2R (β; IL-2Rβ chain)</td>
<td>Dako</td>
</tr>
<tr>
<td>CD30</td>
<td>Activated lymphoid cells</td>
<td>Dako</td>
</tr>
<tr>
<td>CD45R0</td>
<td>Memory T cells</td>
<td>Dako</td>
</tr>
<tr>
<td>CD56</td>
<td>Natural killer cells</td>
<td>Dako</td>
</tr>
<tr>
<td>STCR</td>
<td>ζζ Τ cells receptor</td>
<td>Dako</td>
</tr>
<tr>
<td>ζζTCR</td>
<td>ζζ Τ cells receptor</td>
<td>Immunotech</td>
</tr>
<tr>
<td>CD20</td>
<td>B cells</td>
<td>Dako</td>
</tr>
<tr>
<td>Perform</td>
<td>Perforin expression</td>
<td>Pharmacell</td>
</tr>
<tr>
<td>CD 95 (Fas)</td>
<td>Apo-Fas protein on target cells</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Cytokines</td>
<td>IL1α</td>
<td>Dako</td>
</tr>
<tr>
<td>IL8</td>
<td>IL1 protein</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>TNFa</td>
<td>IL8 protein</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>CD106</td>
<td>TNF protein</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td>VCAM-1: ligand for VLA-4 cells</td>
<td>Novocastra</td>
</tr>
<tr>
<td>E-selectin</td>
<td>Endothelial adhesion molecule</td>
<td>Genzyme</td>
</tr>
<tr>
<td>CD54</td>
<td>ICAM-1: ligand for LFA-1 and Mac-1</td>
<td>Novocastra</td>
</tr>
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</table>

TNF = tumour necrosis factor.
The skin is the major target organ in chronic GvHD and patients may present with lichen planus-like or sclerodermatous lesions, or both. The importance of the interactions between keratinocytes, endothelium and lymphocytes in triggering or amplifying cutaneous inflammation has been underlined in several skin diseases.13-16 As chronic GvHD is characterised by a cutaneous lymphocytic infiltration, these interactions may also play a role in its development. In order to determine which pathways are implicated in chronic GvHD and whether the differences in chronic cutaneous GvHD could be secondary to differences in these pathways, the expression of skin adhesion molecules and cytokines was analysed. The phenotype of the lymphoid infiltrating cells and the mechanisms of epidermal damage were also investigated.

Methods
Transplant recipients who presented between October 1993 and October 1994 with cutaneous lichen planus-like or de novo sclerodermatous lesions were examined by one of us (SA). Patients were considered to have lichen planus-like GvHD if they presented with the typical physical findings and if examination of tissue sections showed epidermal hyperplasia, eosinophilic body formation and a lymphocytic infiltrate in the epidermis and dermis. Patients were considered to have sclerodermatous GvHD if they presented with typical symptoms, if there was no history of lichen planus lesions at the sclerotic sites and if histopathology demonstrated epidermal atrophy, basement membrane flattening and fibrosis of the papillary dermis.

Controls
Biopsy specimens were taken of the diseased and healthy skin. Each specimen was divided in two: one half was embedded in OCT and snap frozen in liquid nitrogen; the other was fixed in formalin, embedded in paraffin wax and stained with haematoxylin and eosin.

Biopsy specimens were obtained from patients who received grafts more than three months previously, who had other skin diseases (one case of late porphyria, two cases of adverse reaction to drugs, one case of solar erythema) or healed lichen planus-like GvHD (one case), and from patients who had undergone total body irradiation and autologous BMT but who had no skin abnormalities.
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<table>
<thead>
<tr>
<th>Table 2 Lymphocyte surface antigens in chronic GvHD</th>
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<tr>
<td>Histological diagnosis</td>
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<td>-------------------------</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Lichen planus-like</td>
</tr>
<tr>
<td>Sclerodermatous</td>
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<tr>
<td>Normal skin</td>
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</table>

(four cases). Normal skin removed during breast plastic surgery was also analysed (four cases).

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Cryostat sections (4-6 μm) were examined immunohistochemically using an indirect biotin antibody conjugated technique. Sections were fixed in cold acetone and incubated with normal serum for 30 minutes at room temperature. Sections were then incubated with each of the specific antibodies directed against the different antigens listed in table 1 for 30 minutes. The sections were washed in phosphate buffered saline (PBS) and then incubated with a biotin conjugated antibody directed against the primary antibody. AEC was used as the chromogen. Lymphocytic infiltration was scored semiquantitatively as follows: -, no labelled cells present; +, scattered labelled cells; ++, moderate infiltration; and +++, dense infiltration.

Negative controls included substitution of the primary antibody by an antibody of an irrelevant specificity but of the same IgG isotype. Positive controls included samples of psoriasis, tuberculoid leprosy, T and B cell lymphoma, and adverse drug reactions.

LABELLING OF APOPTOTIC CELLS

Apoptosis was detected in diseased and normal skin by end labelling fragmented DNA using the ApopTag kit (Oncon, Maryland, USA). Briefly, paraffin wax sections were first deparaffinised and digested with proteinase K. Cryostat sections were fixed in neutral buffered formalin and then post-fixed in ethanol:acetic acid (2:1) for five minutes at room temperature. Slides were incubated in a humidified chamber at 37°C for one hour in a solution containing terminal deoxynucleotidyl transferase (TdTase), digoxigenin labelled dUTP and dATP. The sections were then washed and incubated with the peroxidase labelled antidigoxigenin antibody for 30 minutes. The reaction products were developed with AEC, washed again and the slides counterstained with haematoxylin and eosin. The sections were then examined under a Leitz orthoplan microscope.

Results

HISTOLOGY

On histological examination 13 patients had typical features of lichen planus-like GvHD and five of sclerodermatous GvHD. Lymphocytic infiltration was present in both forms. Epidermal abnormalities were not present and lymphocytes were observed infrequently in the superficial dermis in normal skin of the same patients. The diagnoses of skin diseases in control patients were also confirmed histologically.

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Scattered CD3+ cells were present in the dermis of normal skin in all patients with chronic GvHD. A CD3+ infiltrate was observed in sections of diseased skin in both patient groups. CD4+ and CD8+ subsets were found. However, on semiquantitative analysis, the CD4+ CD8+ infiltrate was more dense in patients with lichen planus-like GvHD (table 2). In both types of GvHD, CD8+ lymphocytes predominated in the epidermis whereas CD4+ cells were more prevalent in the dermis (table 2; figs 1A and 1B). CD8+ cells were also found also in follicular epithelium in those with lichen planus-like GvHD (fig 2). T cells

Figure 3 Diffuse labelling of keratinocytes with anti-Fas antigen in chronic lichen planus-like GvHD (original magnification ×250).

Figure 4 Expression of IL1α in epidermal keratinocytes (original magnification ×100)
Table 3  Cytokines and adhesion molecules expressed in chronic GvHD

<table>
<thead>
<tr>
<th></th>
<th>Lichen planus-like GvHD</th>
<th>Sclerodermatous GvHD</th>
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<tbody>
<tr>
<td></td>
<td>Diseased</td>
<td>Normal</td>
</tr>
<tr>
<td>TNFα</td>
<td>13/13</td>
<td>3/9</td>
</tr>
<tr>
<td>IL1α</td>
<td>13/13</td>
<td>7/9*</td>
</tr>
<tr>
<td>IL8</td>
<td>13/13</td>
<td>9/9</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>12/13**</td>
<td>1/10</td>
</tr>
<tr>
<td>E-selectin</td>
<td>12/13</td>
<td>6/9*</td>
</tr>
<tr>
<td>CD54</td>
<td>13/13</td>
<td>0/9</td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01; t p = 0.062.

had the following phenotype: T cell receptor (TCR) αβ+, TCRγδ−, CD25+, CD3+, CD45RO+. B lymphocytes and natural killer cells were not detected. Perforin expression was not seen. Patients who experienced an adverse reaction to drugs (CD16+, CD56+), those with B cell lymphoma (CD20+) or leprosy (TCRγδ+, perforin+) served as controls. In lichen planus-like GvHD diffuse staining of keratinocyte membranes was noted with Fas antibody (fig 3). This expression was restricted to basal layer in sclerodermatous GvHD. Fas expression was not observed in normal skin.

Table 3 and figs 4–7 summarise cytokine and adhesion molecule expression. Interleukin (IL)

1α and tumour necrosis factor (TNF) α were nearly always expressed in diseased epidermis of both types of GvHD. IL8 was expressed in the cytoplasm of all keratinocytes in diseased and healthy skin of patients in a pattern identical with that in normal controls; the intercellular spaces were unstained (data not shown). Expression of vascular cell adhesion molecule (VCAM)-1 in lichen planus-like and sclerodermatous GvHD differed significantly (p < 0.01; Fisher’s exact test). IL1α was expressed in seven of nine biopsy specimens of normal skin from patients with lichen planus-like GvHD, but not in normal skin of patients with sclerodermatous GvHD (p < 0.05; Fisher’s exact test). Expression of E-selectin was observed in six of nine biopsy specimens of normal skin from patients with lichen planus-like GvHD but not in normal skin of patients with sclerodermatous GvHD (p = 0.062; Fisher’s exact test).

CONTROLS
VCAM-1 expression was not observed in any of the allogeneic BMT recipients with other skin conditions. IL1α and E-selectin were expressed in two of five of these patients. A lymphocytic infiltrate was not observed in patients who underwent total body irradiation followed by autologous BMT. IL1α and E-selectin were expressed in two of four patients who had received radiotherapy for less than one year.

APOTOSIS
Numerous keratinocytic nuclei were labelled in both GvHD types (fig 8A). Labelled keratinocytes were present mainly in the basal layer and in the suprabasal to a lesser degree, especially in patients with lichen planus-like GvHD. Follicular keratinocytes were also labelled (data not shown). Staining was not observed in controls and in normal skin from patients with GvHD (fig 8B).

Discussion
Chronic GvHD is a multisystemic disease which mainly involves the skin, mouth and liver. Its pathogenesis seems to be the consequence of a complex interaction between anti-host and auto-immune phenomena.3-10 Anti-host reactions are demonstrated by the frequent isolation, from peripheral blood of patients with chronic GvHD, of CD4+ clones which proliferate when cultured with cryopreserved host lymphocytes.1 Furthermore, cytotoxic clones directed against host lymphocytes have been isolated from specimens of skin from patients with chronic GvHD.1 However, CD4+ clones recognising the donor Ia group have been isolated from mice with chronic GvHD, suggesting the presence of an autoimmune effect.1 Patients with chronic GvHD may develop lymphoid atrophy and thrombocytopenia, indicating that donor cells are also targeted.1 CD8+ cells predominate in involved tissues1 and electron microscopy studies show that some mononuclear cells are in direct contact with keratinocytes,1 implying direct cytotoxicity. Our results demonstrate that infiltrat-
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Figure 7  E-selectin expression in dermal vessels (original magnification ×250)

Figure 8  (A) Fragmentation of nuclear DNA is evident in keratinocytes from a patient with lichen planus-like GvHD. (B) No DNA fragmentation was seen in uninvolved skin from the same patient.

GvHD. Identical results in chronic GvHD were reported recently by Takata.23 Perforin, however, is expressed by lymphocytes in acute GvHD.23

Keratinocytes from patients with chronic GvHD expressed Fas antigen. This molecule is the receptor for Fas ligand, which may be secreted by some cytotoxic T lymphocytes.24 Keratinocytes do not secrete Fas constitutively, but have the ability to do so in certain circumstances, such as in classic lichen planus.25 Epidermal cell apoptosis, therefore, could be secondary to the secretion of Fas ligand by T lymphocytes acting on keratinocytes. Interestingly, natural killer cells and T\(\gamma\)\(\delta\) cells, which play a role in acute GvHD,26 27 do not seem to be implicated in the chronic form.

IL1\(\alpha\), TNF\(\alpha\) and CD54 were expressed in diseased epidermis and E-selectin in dermal vessels. These molecules can be chemotactic for T cells and permit strong adhesion of lymphocytes to endothelium or keratinocytes and therefore may contribute to the development of cutaneous GvHD. TNF\(\alpha\), E-selectin and VCAM-1 are expressed in skin specimens of patients with systemic sclerosis, an autoimmune disease which is clinically very similar to sclerodermatous GvHD.28-29 The lack of VCAM-1 expression in the specimens of sclerodermatous GvHD in the present study suggests that different pathogenetic mechanisms are involved in these two diseases. Our results show that VCAM-1 expression was restricted to lichen planus-like lesions. In classic lichen planus, as well in acute GvHD, VCAM-1 expression is highly upregulated on dermal endothelial cells.30-32 VCAM-1 is a member of the immunoglobulin superfamily which mediates adhesion of memory T lymphocytes in lichen planus-like and sclerodermatous GvHD have a similar phenotype and distribution pattern. Indeed, CD8+ cells predominated in the epidermis and CD4+ cells in the dermis. The presence of CD8+ cells at the site of cellular damage suggests strongly that these are the effector cells. Invasion of follicle walls by CD8+ cells in lichen planus-like lesions, very similar to the changes described in acute GvHD,20 is in accordance with this hypothesis. The possibility that these observations are the consequence of reactions directed against epidermal stem cells, which are more numerous in lower follicles,31 could not, however, be demonstrated in this study.

Perforin, part of the major cytotoxicity pathway,32 does not seem to play a role in chronic
phocytes expressing VLA-4 to endothelium. 33 TNFα induces the expression of both E-selectin and VCAM-1 molecules 34 on skin endothelial cells, whereas IL4 triggers selective expression of VCAM-1. 35 36 It has recently been shown that administration of antibodies directed against IL4 inhibits features of GVHD in mice. 37 As VCAM-1 was expressed in lichen planus-like GVHD only, where infiltrating T lymphocytes were present, and never in healthy skin (although IL1α and TNFα were sometimes expressed by keratinocytes), VCAM-1 expression could therefore occur secondary to the lymphocytic infiltration and possibly to IL4 expression. Interestingly, Norton and Sloane reported that VCAM-1 was expressed only in patients who developed acute GVHD at a later date. 32

Sclerodermatous GVHD, therefore, is characterized by the production of adhesion molecules and cytokines by keratinocytes and by epidermal apoptosis and lymphocytic infiltration. These features are similar, although milder, than those found in lichen planus-like GVHD. The nature of the T cell infiltrate is also very similar in both GVHD types. These results suggest strongly that de novo sclerodermatous GVHD may represent healing of discrete epidermal events. This hypothesis is in accordance with previous studies showing that the development of sclerotic lesions in a late event in chronic GVHD 84 85 86 and with clinical reports of sclerodermatous GVHD developing at sites of lichen planus lesions. 41 The localization of fibrosis in superficial dermis 32 and the sequential deposition of collagen III and IV in sclerodermatous GVHD, very similar to the processes occurring during tissue repair, 43 also support this hypothesis.

E-selectin and IL1α were expressed in uninvolved skin of 55% and 80% of patients, respectively, with lichen planus-like lesions on biopsy. This was not the case in patients with sclerodermatous GVHD. The significance of the expression of these molecules in the normal skin of patients with lichen planus-like GVHD is difficult to explain. It is possible that an epithelial injury was responsible for the production of IL1α, which in turn induced expression of E-selectin. 34 In patients with acute GVHD TNFα mRNA expression supersedes the appearance of a lymphocytic infiltrate. 44 45 A better understanding of the role of E-selectin and IL1α in the pathogenesis of chronic GVHD may be forthcoming on analysis of these molecules at day 100 after BMT. In conclusion, chronic cutaneous GVHD seems to occur secondary to the infiltration by T cells which in turn induce apoptosis of keratinocytes, possibly via the production of Fas ligand. T cell infiltration may be triggered by the epidermal expression of IL1α and may in turn amplify inflammation by inducing VCAM-1 expression on dermal vessels. In some patients, this results in the formation of sclerotic lesions. Further studies are required to elucidate the pathogenesis of this debilitating chronic disease.

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