

2. Cell

Immunohistology of skin and oral biopsies in graft-versus-host disease after bone marrow transplantation and cytokine therapy

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Background: Early diagnosis of graft-versus-host-disease (GVHD) after bone marrow transplantation is often difficult, particularly when the patients are immunosuppressed by chemotherapy or irradiation.

Objective: To investigate the influence of cytokines on skin lesions after bone marrow transplantation.

Methods: Biopsy specimens of skin and oral mucosa were obtained from bone marrow transplant patients with GVHD and were subjected to histologic and immunohistochemical examination.

Results: Administration of granulocyte-macrophage colony-stimulating factor caused atopic dermatitis-like lesions in two patients, who had infiltration of neutrophils, eosinophils, and lymphocytes around the hair follicles of the skin and no signs of GVHD in other organs. Only patients who were treated with cytokines developed acute GVHD. Immunohistochemical examination of skin biopsies from 18 patients with acute GVHD and 11 patients with chronic GVHD after cyclophosphamide administration or irradiation showed that the maculopapular skin lesions characteristic of acute GVHD contained infiltrates of CD4⁺ and CD8⁺ lymphocytes. There was also an increase in numbers of epidermal keratinocytes expressing intercellular adhesion molecule-I and HLA-DR antigens.

Conclusion: These findings support the involvement of cytokines in GVHD and suggest that immunostaining of skin biopsies may be useful for the early diagnosis of this condition. (*J Allergy Clin Immunol* 1997;100:S73-6.)

Key words: Graft-versus-host disease, skin, intercellular adhesion molecule-1, keratinocyte, atopic dermatitis, granulocyte-macrophage-colony stimulating factor

Graft-versus-host-disease (GVHD) was originally described as a T-cell-mediated disease. However, recent studies have identified autoantigens in GVHD as well as a role for several cytokines.¹ GVHD is treated with intense immunosuppressive therapy targeting T lympho-

Abbreviations used

BMT:	Bone marrow transplantation
G-CSF:	Granulocyte-colony stimulating factor
GM-CSF:	Granulocyte-macrophage colony stimulating factor
GVHD:	Graft-versus-host disease
HLA:	Human leukocyte antigen
ICAM-1:	Intercellular adhesion molecule-1
LFA-1 α :	Lymphocyte function associated antigen-1 α
M-CSF:	Macrophage-colony stimulating factor

cytes, especially cytolytic T lymphocytes. Distinguishing clinically between the dermatologic manifestations of GVHD and exanthems caused by irradiation, chemotherapy, or viral infections is often difficult. Biopsy diagnosis of the early dermatologic manifestations of GVHD can also be difficult, because the initial histologic changes are usually nonspecific.

A variety of skin symptoms can occur after bone marrow transplantation (BMT). Several cytokines, including granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF), and macrophage-colony stimulating factor (M-CSF) are used for salvaging peripheral blood leukocytes before BMT. To investigate the mechanisms causing skin lesions after BMT, we examined the effect of treatment with several cytokines by using immunohistologic methods. The aim of the present study was to characterize the infiltrating mononuclear cells and epidermal keratinocytes in the skin during acute and chronic GVHD in order to establish the value of immunohistochemistry for making an early differential diagnosis.

MATERIALS AND METHODS

Patients

All patients who underwent BMT for hematologic disorders at Kinki University Hospital between 1983 and 1996 were reviewed. All patients were treated with high-dose cyclophosphamide (120 mg/kg) or total body irradiation (10 to 12 Gy). After 1989, patients who developed skin eruptions after BMT were also treated with various cytokines to hasten engraftment.

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TABLE I. Effect on the skin of GM-CSF, G-CSF, and M-CSF

Treatment	Type of skin lesion					Total no. of patients
	Acute GVHD	Chronic GVHD	Acute plus chronic GVHD	Atopic dermatitis	Dry skin	
GM-CSF	0	0	0	2	2	5
G-CSF	15	8	8	0	9	40
M-CSF	3	1	1	0	2	5
No cytokines	0	2	0	0	2	18
Total	18	11	9	2	15	68

These included GM-CSF (300 µg/kg/day intravenously for 21 days), G-CSF (2 to 20 µg/kg/day intravenously for 14 days), or M-CSF (2 to 20 µg/kg/day intravenously for 14 days). These studies were approved by our hospital review board.

Biopsies and histologic examination

Thirty-five 3-mm punch skin biopsy specimens and 21 oral mucosa biopsy specimens were obtained from 75 BMT recipients. The skin biopsy samples were obtained during episodes of acute GVHD with skin manifestations in 22 patients and during episodes of chronic GVHD with skin involvement in 13 patients. Formalin-fixed biopsy specimens were embedded in paraffin, and 14-µm examination sections were processed and stained with hematoxylin and eosin.

Immunohistochemistry

Tissue samples were immersed in Tissue-Tek (Lab Products, Miles Laboratories, Inc., Naperville) and snap-frozen in liquid nitrogen. Frozen sections (4 to 5 µm) were cut and labeled with a streptavidin-biotin LSAB kit (Dako, Carpinteria). After air-drying and fixing in acetone, sections were treated with 3% hydrogen peroxide for 5 minutes followed by washing in Tris-NaCl buffer. The sections were then blocked with normal goat serum for 5 minutes, incubated with a monoclonal mouse anti-human antibody for 30 minutes, and washed twice in Tris-NaCl buffer, (pH 7.4). As the second antibody, a biotinylated rabbit anti-mouse antibody was used. After washing twice with Tris-NaCl buffer, the slides were incubated with horseradish peroxidase-conjugated streptavidin, exposed to a mixture of 3-amino-9-ethyl carbazole and hydrogen peroxide in 0.05 mol/L acetate buffer, (pH 5.0), counterstained for 1 minute in Mayer's hematoxylin, and mounted with Aquamount.

The following monoclonal antibodies were used: CD1a (Leu 6; Becton Dickinson, California), CD3 (Leu 4; Becton Dickinson), CD4 (Leu 3a; Becton Dickinson), CD8 (Leu 2a; Becton Dickinson), CD18 (anti-lymphocyte function associated antigen-1α [LFA-1α]; Dako), CD54 (anti-intercellular adhesion molecule-1 [ICAM-1]; Cosmo), and HLA-DR (DK22; Dako).

Immunostained sections were graded from - to 4+ on the basis of the number of infiltrating cells. Sections with no or occasionally positive cells were graded as -; sections with a few more positive cells were graded 1+; sections with moderate infiltrates were graded 2+; sections with marked infiltrates were graded 3+; and those with very prominent infiltrates were graded 4+. All sections were reviewed by one operator.

TABLE II. Immunostaining of skin cells in 18 patients with acute GVHD

Grade of infiltration	ICAM-1	HLA-DR	LFA-1α	CD1
4+	0	0	0	0
3+	4	0	2	0
2+	8	14	16	12
1+	5	3	0	6
0-	1	1	0	0
Total positive (2+ to 4+)	12	14	18	12

RESULTS

GM-CSF and atopic dermatitis-like eruptions

We first investigated the effect of cytokine therapy on skin lesions by comparing the number of patients developing skin eruptions before 1988 (without cytokine therapy) with those doing so after 1988 (on cytokine therapy). Table I summarizes the types of skin eruptions in the patients receiving cytokine therapy. For this analysis, we selected 68 of the 75 BMT patient seen over 13 years at our hospital. Forty-one patients were treated at the Department of Dermatology for skin problems. Cytokines were used to increase the numbers and activity of granulocytes in 50 of the 68 patients. GM-CSF induced atopic dermatitis-like eruptions in two patients, whereas folliculopapular eruptions were seen in three cases (see Table I). Histologically, infiltration of neutrophils, eosinophils, and lymphocytes was noted around the hair follicles, without any sign of GVHD in other organs. There were no patients with acute GVHD of the skin among those who did not receive cytokine therapy.

Skin abnormalities in acute GVHD

The results of immunohistologic examination of 18 skin biopsy samples are summarized in Table II. Fourteen biopsy samples were obtained from patients who were not receiving corticosteroids for acute GVHD and six biopsy samples were obtained from another group of patients receiving corticosteroids. On histologic examination, the epidermis was normal in 6 out of 18 cases. The extent of cellular infiltration in these biopsies ranged from 1+ to 3+. On immunohistologic examination, ICAM-1 was detected in 12 specimens and HLA-DR-positive epidermal cells were detected in 14 samples. The number of CD1-positive cells was low in six samples. However, CD4⁺ and CD8⁺ infiltrates were observed in all specimens with a variable grade of lymphocytic infiltration ranging from 1+ to 4+. The CD4/CD8 ratio was increased in 10 of the 18 biopsy specimens. LFA-1α-positive cells were present in large numbers in the dermis of all specimens. Lymphoid infiltrates were more intense in the dermis than in the epidermis, but most biopsy samples displayed the same subsets of mononuclear cells in both regions.

Skin abnormalities in chronic GVHD

Eleven biopsy samples revealed normal histology, except for parakeratosis of the hair follicles in 10 samples. However, by immunohistochemical analysis CD4⁺ and CD8⁺ T-cell subtypes were present in both the epidermis and the dermis of all specimens. The cell clusters in the epidermis and dermis consisted mostly of CD4⁺ lymphocytes, but the number of CD8⁺ lymphocytes and LFA-1a⁺ cells was also increased in most specimens, compared with normal skin. The degree of dermal infiltration ranged from 1+ to 2+ (data not shown).

Abnormalities of the oral mucosa in chronic GVHD

To assess the mucosal cellular response during chronic GVHD, oral biopsy samples were obtained from 12 patients with dry skin. Histologic examination showed infiltration of mononuclear cells into the lip glands of all patients. CD4⁺ or CD8⁺ T cells were found in the dermis of all specimens, whereas ICAM-1⁺ and HLA-DR⁺ cells were detected in the basal layer of the epidermis in 7 out of 12 biopsy samples (Table III).

DISCUSSION

Cytokines are considered to mediate many of the clinical manifestations of GVHD.¹ These agents are initially released during the conditioning regimen, and their levels increase after T cell recognition of histocompatibility antigens. The data in Table I indicate that acute GVHD was induced by cytokine therapy.

With GM-CSF therapy, the circulating eosinophil and neutrophil counts increased after BMT, and the skin eruptions that occurred in two patients were follicular papules covering the whole body, similar to the lesions of atopic dermatitis.^{2,3} Histologically, infiltration of neutrophils, eosinophils, and lymphocytes was observed around the hair follicles, without any signs of GVHD in other organs. The serum levels of IgE in these two patients were higher than in the other subjects. There have been only a few reports of high IgE levels in BMT patients.^{4,5} In this context, Leiferman⁶ identified the development of an IgE-mediated late-phase reaction in atopic dermatitis and described a role for eosinophils in this disease. The bone marrow donors for these patients may also have had certain primary genetic traits related to atopic dermatitis. In addition, it is known that treatment with recombinant GM-CSF enhances eosinophil activity and IgE production.⁷ This combination could cause atopic dermatitis-like skin eruptions. In the chronic GVHD group, all patients had dry skin, which is an important feature of atopic dermatitis. In addition, accumulation of CD4⁺ or CD8⁺ cells in the dermis occurs in this disease. Together, these findings indicate that GM-CSF may be involved in the pathogenesis of a subset of atopic dermatitis.

We studied skin biopsy samples obtained from BMT patients during GVHD. Cellular infiltrates in cutaneous structures are seen more clearly after immunohistologic

TABLE III. Immunostaining of oral mucosa cells in 12 patients with chronic GVHD

Grade of infiltration	ICAM-1	HLA-DR	LFA-1α	CD1
4+	0	0	0	0
3+	2	3	4	0
2+	5	4	3	12
1+	2	1	0	0
0–	3	4	5	0
Total positive (2+ to 4+)	7	7	7	12

staining compared with routine histological staining.⁸ We demonstrated that typical infiltrates within the maculopapular skin lesions of acute GVHD included CD4⁺ and CD8⁺ lymphocytes as well as ICAM-1⁺ and HLA-DR⁺ epidermal keratinocytes. These results are similar to those of Leskinen et al.⁹ and Takata et al.¹⁰ who found an increase of CD2⁺, CD4⁺, and CD8⁺ T lymphocytes in skin biopsy samples from patients with acute GVHD. However, the results disagree with those of Guyotat et al.,¹¹ who reported a predominance of CD8⁺ T cells in cutaneous lesions of GVHD.

GVHD was initially described as a T cell-mediated disease. Cellular injury in GVHD was thought to be caused by the infiltration of effector cells into target tissues, resulting in tissue destruction. However, several immunohistochemical studies have shown that many, but not all, of these cells have a phenotype that is more consistent with that of natural killer cells rather than with that of mature T cells.¹²

Immunohistologic examination of skin biopsy specimens may be useful in the differential diagnosis of skin lesions after BMT, because it is difficult to clearly identify lymphoid cell infiltrates by ordinary staining methods. The recent suggested role of cytokines in GVHD,^{13,14} and the presence of ICAM-1-positive epidermal keratinocytes in the papillary layer during early acute GVHD should be explored further to determine the influence of cytokines on the epidermis.

Maeda et al.¹⁵ recently reported high serum levels of ICAM-1 in acute GVHD, and we found that a large proportion of T cells in the skin expressed this molecule. Thus, a high serum ICAM-1 level and presence of ICAM-1-positive cutaneous T lymphocytes may be useful indicators for the initiation of immunosuppressive therapy in acute GVHD. We also detected LFA-1-positive infiltrating cells around the skin appendages. Similar findings were observed in the oral mucosa in chronic GVHD.

The epithelial cells of the epidermis, or epidermal keratinocytes, produce several cytokines, including IL-1 and tumor necrosis factor-α¹⁶. After activation by T lymphocyte-derived interferon-γ, these cells secrete ICAM-1^{17,18} and HLA-DR.¹⁹ Histologic changes in the epidermis are common after BMT. Cyclophosphamide and total body irradiation can combine to produce

dyskeratosis with or without satellitosis, but these changes disappear by 3 weeks after transplantation. Sometimes the epithelial damage caused by chemotherapy and irradiation cannot be distinguished from the changes caused by acute GVHD. Therefore, the diagnosis of acute GVHD should also include analysis of dermal lymphoid cell infiltrates.²⁰ Our results indicate that the cellular composition of the skin infiltrates in acute GVHD is characteristic and that immunostaining can assist in the diagnosis of atypical skin lesions caused by acute GVHD.

A model has been proposed for the interactions of cytokines in the development of GVHD.²¹ Our study suggests that many of the manifestations of GVHD are caused directly by the cytokines themselves or by secondary activation of bystander cells such as natural killer cells, which then inflict much of the cytotoxic damage that occurs. Accordingly, cytokines would seem to be involved in mediating many of the clinical and experimental manifestations of GVHD.

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