Immunoelectron microscopy of acute graft versus host disease of the skin after allogeneic bone marrow transplantation

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Abstract

Aims—To clarify the pathological mechanisms of acute cutaneous graft versus host disease (GvHD) following allogeneic bone marrow transplantation.

Methods—Skin biopsy specimens from five patients were examined by immunoelectron microscopy. A panel of monoclonal antibodies against T cell and natural killer cell subpopulations was used, including anti-CD4, -CD8, -CD16b, -CD56, -CD57, and -TCR $\delta 1$ antibodies.

Results—All the specimens contained CD8+ cells, CD4+ cells, and CD56+ cells infiltrating the epidermis. Cells stained with anti-CD16b, -CD57, or -TCR $\delta 1$ were very sparse or absent. Most of the CD8+ cells in the epidermis displayed morphological features of activated cytotoxic T lymphocytes and apposition of such cells to degenerating keratinocytes was shown. CD4+ cells outnumbered CD8+ cells in the epidermis in all five cases. Noticeable intercellular as well as intracellular oedema of keratinocytes was observed at the site of prominent CD4+ cell infiltration, suggesting that these also have a role as actual effector cells by secreting cytotoxic cytokines. CD56+ cells infiltrating the epidermis did not exhibit the characteristic ultrastructural morphology of the natural killer cells thus far examined, and their lineage remained uncertain.

Conclusions—These data provide direct evidence that CD8+ cytotoxic T cells attack keratinocytes, and further suggest that CD4+ cells as well as CD56+ cells participate in the cellular pathogenesis of acute cutaneous GvHD.

Acute graft versus host disease (GvHD) is a major complication of allogeneic bone marrow transplantation. Although donor T lymphocytes are thought to have an important role in the development of acute GvHD, the precise nature of the effector cells and the mechanisms of tissue injury in target organs remain unknown.^{1,2}

Skin is one of the major target organs in acute GvHD, and the pathological mechanisms involved have been intensively investigated.³ Early histopathological studies have shown epidermotropic migration of lymphocytes and hyalinised necrosis of keratinocytes, often associated with adjacent lymphocytes—that is, satellite cell necrosis—as characteristic features of acute cutaneous GvHD, leading to the concept of the “aggressor lymphocyte” as a primary effector cell.^{4,5} Recent immunohistological analyses have shown that most infiltrating cells in the affected skin are CD3+ T cells, with a predominance of CD8+ suppressor/cytotoxic T cells⁶⁻⁹ or CD4+ helper/inducer T cells.¹⁰⁻¹² The surface phenotype and the functional characteristics of effector cells, however, are still unclear.

The purpose of this study was to investigate the phenotype and ultrastructural morphology of migrating mononuclear cells in the epidermis which may actually cause epithelial injury, and to clarify the cellular pathogenesis involved in acute cutaneous GvHD.

Methods

Five patients who developed acute GvHD after allogeneic bone marrow transplantation from HLA-identical sibling donors were included in this study. The clinical characterisation of these five cases is summarised in table 1. All the patients received cyclosporin in association with short-term methotrexate for the prophylaxis of the GvHD. Skin biopsy specimens were taken from the various sites within four days of the onset of skin rash. According to the histopathological grading proposed by Lerner *et al*,¹³ two patients (cases 1 and 2) had grade II histological changes and three (cases 3, 4, and 5) had grade III changes. None of the patients developed extracutaneous manifestations.

The skin tissue from each biopsy specimen was fixed in freshly prepared periodate-lysine-paraformaldehyde (PLP), embedded in OCT compound (Miles Scientific, Elkhart, Indiana), and frozen in liquid nitrogen. Cryosections (6 μ m) were stained using the

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Table 1 Clinical characterisation of patients with acute GvHD after allogeneic bone marrow transplantation

Case No	Age/sex (years)	Original disease*	Onset of skin rash†	Time of biopsy†	Histological grade‡
1	22/M	CML	30	32	II
2	17/M	AMoL	19	21	II
3	27/M	CML	20	24	III
4	17/F	ALL	24	27	III
5	23/M	CML	31	36	III

*CML, chronic myelocytic leukaemia; AMoL, acute monocytic leukaemia; ALL, acute lymphocytic leukaemia.

†Days after bone marrow transplantation.

‡According to the classification of Lerner *et al*.¹³

Table 2 Monoclonal antibodies for immunohistochemistry

Antibody*	Cluster of differentiation	Predominant reactivity
Leu 3a + 3b	CD4	Helper/inducer T cells
Leu 2a	CD8	Suppressor/cytotoxic T cells
Leu 11b	CD16b	Natural killer cells, granulocytes
Leu 19	CD56	Natural killer cells, activated T and B cells
Leu 7	CD57	Natural killer cells, some CD8 + T cells
TCR $\delta 1$		$\gamma\delta$ T cells

*Leu series monoclonal antibodies were purchased from Becton Dickinson Immunocytometry Systems, Sunnyvale, California, and TCR $\delta 1$ antibody from T Cell Science, Cambridge, Massachusetts.

Table 3 Antigenic phenotype of infiltrating cells in the epidermis

Case No	Number of cells/mm*		
	CD4	CD8	CD56
1	28.2	6.4	NE
2	50.0	30.0	NE
3	28.3	14.0	35.7
4	33.3	3.6	4.0
5	34.3	13.3	17.8

*Actual number of positively labelled cells per linear millimetre length of epidermis. NE, not examined.

biotin-streptavidin method, as described before.¹⁴ Briefly, the sections were first reacted overnight at 4°C with various primary monoclonal antibodies listed in table 2, followed by the sequential incubations with biotinylated goat antimouse immunoglobulins and peroxidase-conjugated streptavidin (Biogenex Laboratories, San Ramon, California). After having been refixed with 1% glutaraldehyde solution the sections were

treated with 0.02% diaminobenzidine (DAB) solution containing 0.01% hydrogen peroxide. They were then postfixed with 2% osmium tetroxide, dehydrated, and embedded in Epoxy resin. Semithin sections (1 μ m) were cut, counterstained with methylene blue, and observed by light microscopy. Finally, ultrathin sections were prepared from the appropriate Epon blocks, stained with 1% uranyl acetate, and examined with an electron microscope.

Results

LIGHT MICROSCOPY

The profiles of the proportion of cells labelled with various monoclonal antibodies were similar among all five cases studied. All had CD4+, CD8+, and CD56+ cells as main cellular infiltrates. CD57+ cells and TCR $\delta 1$ + cells were either absent or detected only occasionally within the dermal perivascular infiltrates. None of the specimens contained CD16b+ cells. Table 3 gives the actual number of CD4+, CD8+, as well as CD56+ cells observed in the epidermis. CD4+ cells outnumbered CD8+ cells in all cases and the CD4:CD8 ratio ranged from 1.7 to 9.2. CD56+ cells constituted a significant proportion of the epidermal infiltrates in three of the cases examined. CD56 was the most predominant phenotype in case 3. In the remaining two cases (cases 4 and 5) the numbers of CD56+ cells slightly exceeded those of CD8+ cells. Further electron microscopic observations were carried out in the specimens stained with CD4, CD8, and CD56 antibodies.

ELECTRON MICROSCOPY

Most of the CD8+ lymphocytes found in the epidermis had an eccentrically located euchromatic nucleus and an abundant cytoplasm at one pole of the cell. These CD8+ lymphocytes possessed microvilli-like projections on the cell surface, which were often observed congruent with the cell membrane of adjacent keratinocytes (fig 1). Such contacts between CD8+ lymphocytes and keratinocytes were found in all the cases. Target keratinocytes exhibited various degrees of cytoplasmic damage. Figure 2 shows a CD8+ lymphocyte that was found apposed to a basal layer keratinocyte undergoing apparent filamentous degeneration. The lymphocyte showed polarity with an eccentrically located nucleus and abundant cytoplasm facing the degenerating keratinocyte. The cytoplasm contained many cell organelles such as mitochondria and multivesicular bodies.

All the specimens contained numerous CD4+ cells in the epidermis. Most such CD4+ cells were lymphocytes, but some macrophages with indented nuclei and cytoplasmic autophagosomes were weakly stained with CD4 antibody. The ultrastructural morphology of the CD4+ lymphocytes was variable, and many of them had elongated cytoplasm which contained fewer cell organelles than the CD8+ lymphocytes. In

Figure 1 A CD8+ lymphocyte infiltrating in the epidermis which has a euchromatic nucleus, abundant cytoplasm, and cell surface microvilli-like projections. Cytoplasmic projections of other CD8+ cells (arrowheads) are also seen in close contact with adjacent keratinocytes (case 2; bar = 1 μ m).

Figure 2 A CD8+ lymphocyte apposed to a colloid body (CB) in the basal layer of the epidermis. The lymphocyte has an eccentrically located nucleus and a cytoplasm containing many cell organelles such as mitochondria and multivesicular bodies. The enclosed area is magnified in the inset. Arrowheads indicate the basal lamina (case 3; bar = 1 µm; inset: bar = 0.1 µm).

contrast to CD8+ cells, any contiguity between CD4+ lymphocytes with keratinocytes was rare. At the site of prominent infiltration of CD4+ lymphocytes, however, the intercellular connection of keratinocytes was disrupted, leading to the complete disappearance of desmosomes from the cell surface. Some keratinocytes showed intracytoplasmic oedema and vacuolisation (fig 3).

Figure 3 A site of numerous CD4+ cell infiltration in the epidermis. Note pronounced intercellular as well as intracellular oedema of keratinocytes (asterisk) with partial or complete loss of desmosomes from the cell surface (case 1; bar = 5 µm).

In three cases CD56+ cells were mainly distributed in the intercellular spaces of the lower epidermis. Most of the CD56+ cells possessed a centrally located nucleus and a small rim of cytoplasm with a relatively high nuclear:cytoplasmic ratio (fig 4). Some of them had cytoplasmic projections, but contiguity between CD56+ cells and adjacent keratinocytes was rarely seen. Although multivesicular bodies were common, electron dense granules could not be identified in the cytoplasm of CD56+ cells thus far examined.

Discussion

The ultrastructural observations of the affected skin in acute GvHD disclosed prominent migration of lymphocytes in the epidermis, disruption of intercellular connections of keratinocytes, and a close association between migrating lymphocytes and degenerating keratinocytes.^{5,15} Subsequent immunohistological studies of acute GvHD showed infiltration of CD8+ suppressor/cytotoxic T cells into the epidermis.^{6,10} These ultrastructural and immunohistochemical findings strongly suggested that migrating lymphocytes in the epidermis are cytotoxic T cells which mediate epidermal cell injury.

In the present study we have shown that most of the intraepidermal CD8+ cells exhibit morphological features of stimulated cytotoxic T lymphocytes^{16,17}—that is, they had cell surface microvilli, euchromatic nuclei, and cell organelle-rich cytoplasm containing multivesicular bodies. Furthermore, the apposition of a CD8+ lymphocyte to a keratinocyte undergoing filamentous degeneration was shown. Our observations therefore provide more direct evidence that intraepidermal CD8+ cells are activated cytotoxic T cells and cause epidermal cell necrosis by direct cell to cell contacts.

Although most of the previous immunohistological studies of acute cutaneous GvHD have identified CD4+ helper/inducer T cells within the dermal infiltrate,⁷⁻¹² only two studies have described the infiltration of the epidermis by CD4+ cells. Sloane *et al.*¹⁰ observed CD4+ T cells in the epidermis in five out of eight cases, but precluded the participation of these cells in the pathogenesis of GvHD, because there was no correlation between the intensity of the epidermal changes and the presence of epidermal CD4+ cells. Beschorner *et al.*¹⁸ who examined the epithelial HLA-DR antigen expression and surface phenotype of infiltrating lymphocytes, noted a mixed infiltrate containing more CD4+ cells than CD8+ cells in the epidermis in specimens with positive epithelial HLA-DR expression. We also showed numerous CD4+ cells infiltrating the epidermis on the electron microscopic level. CD4+ lymphocytes were mostly found in the widened intercellular spaces in the epidermis without apposition to adjacent keratinocytes. The role of these intraepidermal CD4+ cells in the pathogenesis of acute GvHD is still

Figure 4 Two CD56+ cells infiltrating in the basal layer of the epidermis (*). CD56+ lymphocytes and macrophages (M) are also present (case 5; bar = 1 μ m).

unclear. In animal experiments, however, Shiohara *et al*¹⁹ and Murphy *et al*²⁰ showed that a certain type of helper T cell clone or purified donor CD4+ T cell inoculated into the appropriate recipients induces histological changes consistent with cutaneous GvHD, and have indicated that CD4+ T cells act as actual effector cells of acute GvHD. These authors suggested that cytotoxic cytokines, such as tumour necrosis factor- α and interferon- γ released from CD4+ helper T cells are responsible for the epithelial injury. It is therefore possible that CD4+ T cells found in the epidermis of human acute GvHD could cause epidermal cell necrosis by secreting these cytotoxic mediators.

Participation of natural killer cells in the pathogenesis of acute GvHD is a matter of controversy. Dokhelar *et al*²¹ reported a strong association between high peripheral blood natural killer activity and the development of GvHD during the first month after a transplantation, and suggested that natural killer cells may be involved in the development of acute GvHD. In experimental models of murine acute GvHD, infiltrating mononuclear cells in the skin had phenotypic and morphological features of natural killer cells.^{22,23} In spite of these findings immunohistochemical studies in humans using CD57 and CD16b antibodies as natural killer cell markers did not detect those cells in the skin lesions of acute GvHD.^{7,12} Very recently, however, Acevedo *et al*,²⁴ using several different monoclonal antibodies recognising a natural killer cell subset, have detected a substantial number of cells displaying a natural killer cell associated phenotype, such as CD56 and Kp43 antigens in the skin infiltrates of acute GvHD. In the present study

we also identified a considerable number of CD56+ cells migrating into the epidermis. Ultrastructurally, however, we were not able to show the presence of cytoplasmic electron-dense granules in these CD56+ cells which are characteristic of natural killer cells and are closely associated with cytotoxic activity.²⁵ Furthermore, close contact between CD56+ cells and adjacent keratinocytes was rare. Therefore, we have no evidence that these CD56 cells are natural killer cells and act as actual effector cells. As it has been recently shown that the expression of CD56 antigen is not restricted to natural killer cells, but is seen on CD4+ or CD8+ interleukin-2 dependent T cell clones maintained on long term cultures,²⁶ the possibility remains that the CD56+ cells identified in the skin lesion of acute GvHD are activated T cells rather than natural killer cells. Further ultrastructural studies as well as investigations using double immunohistochemical staining with T cell markers are needed to confirm the lineage of the CD56+ cells.

The CD3+ $\gamma\delta$ T cells are distributed mainly in the epithelia and have natural killer cell-like non-major histocompatibility complex-restricted cytotoxic activity.²⁷ Ferrara *et al*²³ showed that cells showing high natural killer activity are important effector cells mediating tissue injury in their experimental model of murine GvHD. They proposed that CD3+ $\gamma\delta$ T cells may be the primary effector cells. This seems unlikely in human GvHD, however, because in concordance with the recent study of Norton *et al*²⁸ very few TCR δ 1+ cells were detected in the skin of acute GvHD.

This immunoelectron microscopic study has shown the direct cytotoxic activity of CD8+ lymphocytes against epidermal keratinocytes in acute GvHD of the skin. The pathogenetic participation of CD4+ helper/inducer T cells and of CD56+ cells is also apparent, although their precise role remains uncertain. CD4+ lymphocytes seem to act as actual effector cells. Furthermore, cytokine mediated interaction of these phenotypically different subsets of lymphocytes might be important in the cellular pathogenesis of acute GvHD.

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