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Difference in the expression of Fas/Fas-ligand and the lymphocyte subset reconstitution according to the occurrence of acute GVHD

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Summary:

Acute graft-versus-host disease (aGVHD) remains a major barrier to a wider application of allogeneic bone marrow transplantation (BMT). Although this complication is mainly dependent on donor-derived T lymphocytes, very little information is available concerning the mechanism of lethality. In this study, we investigated both the expression of Fas/Fas-ligand (FasL) and lymphocyte subset reconstitution in patients who underwent HLA-matched related allogeneic BMT ($n = 16$) and normal donors ($n = 10$), and several distinct features were observed. First, the reconstitutions of CD3$^+$ and CD56$^+$ cells were different between the aGVHD$^+$ and aGVHD$^-$ group. In particular, the percentage of CD3$^-$CD56$^+$ cells was significantly decreased in patients with aGVHD ($P < 0.01$). Second, the expansion of CD8$^+$ ($P = 0.01$) and CD8$^+$ CD28$^-$ T cells ($P = 0.03$) was a characteristic finding in patients with aGVHD. Finally, we found that the percentages of Fas$^+$CD8$^+$, Fas$^+$HLA-DR$^+$ and FasL$^+$ CD8$^+$ cells were significantly increased. Fas antigen was highly coexpressed on most of the lymphocyte subsets, especially on CD8$^+$ cells ($P < 0.01$), and also, significantly higher coexpression of FasL on CD8$^+$ cells was found in patients with aGVHD ($P < 0.01$). In summary, an increase in the percentage of CD8$^+$ cells which express Fas and its ligand in patients with aGVHD after BMT points to a possible role for the Fas/FasL pathway in the effector phase of aGVHD.
Keywords: acute graft-versus-host disease; CD8$^+$ cells; Fas; Fas-ligand

Acute graft-versus-host disease (aGVHD) remains a major complication following allogeneic bone marrow transplantation (BMT). Although the tissue damage is caused by donor-derived T lymphocytes that recognize antigenic disparities between donor and recipient, the lytic mechanism leading to lesion formation has not been fully ascertained but probably involves several concurrent processes. These include direct interactions between donor cytotoxic T lymphocytes (CTL) and host target cells, or more indirect mechanisms mediated by cytokines released from donor-derived T cells.[1,2] Therefore, more detailed identification of T cell subsets can be an exceptionally good way to elucidate the immune mechanism responsible for aGVHD.

Recently accumulating evidence indicates that the Fas (APO-1; CD95)/Fas-ligand (FasL) system represents an important cellular pathway responsible for T cell-mediated cytotoxicity and the induction of apoptosis in various tissues.[3–5] Fas antigen, which is a 45-kDa cell-surface type I membrane protein member of the tumor necrosis factor (TNF)/nerve growth factor receptor family, is expressed not only on activated T cells, but also on other tissues such as liver, heart, lung, skin and gastrointestinal tract.[3–6] On the other hand, FasL (known as a 40-kDa TNF-related type II membrane protein) expression is restricted to the activated mature T cells after various stimuli, except in the testis, and binding of FasL to Fas induces apoptosis, indicating that FasL is a death factor, while Fas is its receptor mediating the apoptotic signal.[3,4,7,8] Several reports[9,10] have suggested that Fas mutations result in a massive upregulation of FasL and could explain the *lpr*-induced GVHD-like wasting syndrome observed when *lpr* bone marrow derived cells were adoptively transferred to wild-type mice. However, Fas and FasL expression in aGVHD has not been further evaluated, and its clinical significance remains to be elucidated in humans.

Therefore, we evaluated both the lymphocyte subset reconstitution and the expression of Fas and FasL on each lymphocyte subset in order to explore the pathogenesis of aGVHD based on Fas/FasL pathway.

Materials and methods

Patients and donors

Between March 1994 and December 1995, 16 consecutive patients who underwent HLA-matched related allogeneic BMT for acute leukaemia at first complete remission were enrolled into this study. All patients were given a myeloablative therapy consisting of fractionated total body irradiation (TBI; 165 cGy twice daily for 4 consecutive days (total 1320 cGy)) combined with cyclophosphamide (60 mg/kg once daily i.v. for 2 days (total dose 120 mg/kg)). Cyclosporin A and short-course methotrexate were used for GVHD prophylaxis to all patients. Diagnosis of aGVHD was made by clinicians with the usual criteria of the disease.[11] GVHD scores were assigned at the time of blood collection and represented the overall score of

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skin, gut and liver GVHD. All cases of aGVHD were pathologically proven by skin biopsies which were taken from the affected sites before any steroid therapy was given. Because direct cytotoxic effects of chemoradiotherapy may be difficult to distinguish from the findings of aGVHD in the first 3 weeks after BMT,[12] we defined aGVHD when patients showed clinical and pathological findings including both epidermal basal-cell apoptosis with lymphoid infiltration and satellitosis. Also, serial biopsies and observations were performed for increasing diagnostic confidence in patients who developed aGVHD in the first 3 weeks after BMT. Prednisolone (60 mg/m^2/day) was used for the treatment of aGVHD. Characteristics of patients with aGVHD (aGVHD$^+$ group) and without aGVHD (aGVHD$^-$ group) are shown in Table 1. There were six males and 10 females, ages ranged from 18 to 44 years (mean 28 years). Six patients (37.5%) were aGVHD$^+$ and 10 (62.5%) were aGVHD$^-$. We could not detect any significant differences with respect to gender, age and total infused cell dose. We did not observe any case of viral infection after transplantation. All patients had successful engraftment without any significant treatment-related complications.

Surface phenotyping by flow cytometry

To compare the percentages of lymphocyte subsets and the levels of Fas and FasL expression, venous peripheral blood sampling was performed immediately after the appearance of the first sign of aGVHD in the aGVHD$^+$ group, and at day 14 or 21 after BM infusion in the aGVHD$^-$ group. Normal values were also established by testing 10 healthy donors. Peripheral blood lymphocytes (PBL) were isolated by standard Ficoll–Hypaque density gradient centrifugation (1.077 g/ml) and were adherence depleted twice on plastic in serum-free medium (AIM V, GIBCO, Grand Island, NY, USA). Freshly isolated PBL were labelled with monoclonal antibodies against the surface determinants CD3, CD45 (all FITC-conjugated), CD4, CD8, CD14, CD56, HLA-DR (all phycoerythrin-conjugated) from Becton Dickinson (Mountain View, CA, USA). The FITC-conjugated Fas and CD28 were purchased from Immunotech (Marseille, France), and affinity-purified mouse anti-human FasL antibody was also used (Transduction Lab, Lexington, KY, USA). Quantitative fluorescent analysis of the cells was performed using a FACScan flow cytometer (Becton Dickinson) equipped with an argon laser. Green (FITC) or red (phycoerythrin) fluorescence was selected using 530- and 585-nm filters, respectively. Events (at least 30 000) were recorded in list mode on a Consort 30 program. Cells falling into the lymphocyte gate were >98% CD14$^-$ CD45$^+$ population and a correction for this was made in subsequent analyses. Cells stained with isotype-matched FITC- and phycoerythrin-conjugated antibodies were used as negative controls. Using dual-color flow cytometry, we also investigated Fas and FasL expression on each lymphocyte subset.

Statistical analysis

Comparisons between the aGVHD$^+$ and aGVHD$^-$ groups were based on Fisher's exact test for dichotomized variables and the non-parametric Mann–Whitney *U* test for continuous variables. A significant difference was defined as a P value <0.05.

Results

Different pattern in the reconstitutions of CD3$^+$ and CD56$^+$ cells

In the aGVHD$^+$ and the control groups, the percentages of CD3$^+$ and CD56$^+$ cells were similar, but we observed a

Table 1 Characteristics of patients receiving HLA-matched related BMT

UPN	*Sex/Age*	*Disease*	*Infused cells (× 10^8/R.kg)*	*CMV infection*	*Days of ANC >500/mm^3*	*aGVHD grade/Days of onset*	*Days of testing*	*Days of Tx onset*
aGVHD$^+$ patients (n = 6)								
1	F/20	ALL	4.12	—	12	II (Skin II; Liver I; Gut I)/23	23	24
2	F/18	AML	4.10	—	11	II (Skin II; Liver I; Gut I)/13	13	14
3	M/38	AML	3.99	—	18	I (Skin II; Liver 0; Gut 0)/19	19	26
4	F/35	AML	5.18	—	12	I (Skin II; Liver 0; Gut 0)/17	17	25
5	M/44	AML	2.44	—	13	II (Skin III; Liver I; Gut I)/28	28	29
6	F/26	AML	3.57	—	12	I (Skin II; Liver 0; Gut 0)/15	15	24
aGVHD$^-$ patients (n = 10)								
7	M/26	ALL	6.73	—	11	0	21	
8	F/24	AML	5.18	—	10	0	14	
9	F/33	AML	3.79	—	11	0	21	
10	F/26	AML	5.47	—	11	0	21	
11	M/22	AML	3.46	—	10	0	14	
12	F/27	AML	2.79	—	12	0	21	
13	F/21	AML	3.05	—	12	0	21	
14	M/20	AML	3.99	—	15	0	21	
15	M/40	ALL	2.08	—	12	0	21	
16	F/24	AML	4.07	—	11	0	21	

UPN = unique patient number; CMV = cytomegalovirus; ANC = absolute neutrophil count; aGVHD = acute graft-versus-host disease; Tx = treatment; M = male; F = female; AML = acute myeloid leukaemia; ALL = acute lymphoblastic leukaemia; R = recipient.

significant increase in the percentage of $CD3^+$ cells in the $aGVHD^+$ group (65.6 ± 2.7 *vs* 42.7 ± 11.0, $P < 0.01$). Also, the percentage of $CD56^+$ cells was lower in the $aGVHD^+$ group (24.3 ± 8.2 *vs* 45.4 ± 4.7, $P < 0.01$). By dual-color flow cytometry, we found that most of the $CD56^+$ cells were $CD3^-$, and the percentage of $CD3^-CD56^+$ cells was significantly decreased in patients with aGVHD (16.1 ± 6.7 *vs* 40.2 ± 5.8, $P < 0.01$) (Figure 1).

Expansion of $CD8^+$ and $HLA\text{-}DR^+$ cells in patients with aGVHD

There was no significant difference between the $aGVHD^+$ and $aGVHD^-$ groups in the percentage of $CD4^+$ cells ($22.3 \pm 17.8\%$ *vs* $28.9 \pm 6.8\%$, $P > 0.05$). When compared with the control group, the proportion of $CD4^+$ cells remained at a relatively low level after BMT regardless of the occurrence of aGVHD. But there was a high percentage of $CD8^+$ cells in the $aGVHD^+$ group compared to that in the $aGVHD^-$ group ($43.3 \pm 11.9\%$ *vs* $29.4 \pm 6.2\%$, $P = 0.01$), and also a significantly increased percentage of $HLA\text{-}DR^+$ cells was shown after BMT, especially in patients with aGVHD ($83.3 \pm 5.1\%$ *vs* $67.0 \pm 2.2\%$, $P < 0.01$) (Figure 1).

Difference in the subpopulation of $CD8^+$ cells

As mentioned above, an increase in the percentage of $CD8^+$ cells was observed in patients with aGVHD (43.3 ± 11.9 *vs* 29.4 ± 6.2, $P = 0.01$). We further analyzed the $CD8^+$ subpopulation based on the expression of the CD28 marker by using double staining. In our study, we evaluated the percentages of $CD8^+CD28^+$ and $CD8^+CD28^-$ T subsets which were all $CD8^{bright+}$ cells and excluded NK cells, which are $CD8^{dim+}$ cells. We checked that $CD8^{bright+}$ cells were $CD3^+$ (data not shown) and therefore belong to the T cell population. We observed a significant increase in the percentage of $CD8^+CD28^-$ cells in patients with aGVHD ($40.0 \pm 13.1\%$) when compared with patients without aGVHD ($26.2 \pm 5.9\%$) ($P = 0.03$). In contrast, we did not observe a significant difference in the percentage of $CD8^+CD28^+$ cells according to the presence of aGVHD. There was no statistical correlation between $CD8^+CD28^-$ and $CD8^+CD28^+$ cell reconstitution (Figure 1).

Absolute numbers of total leukocytes and lymphocytes, and each lymphocyte subset

We also examined whether there were any differences in the absolute numbers of leukocytes and lymphocytes between the $aGVHD^+$ and $aGVHD^-$ groups. As shown in Table 2, total leukocyte counts were significantly decreased in patients with aGVHD but with a wide range between individuals (2082 ± 834 *vs* 4373 ± 1646, $P = 0.04$). Among the lymphocyte subsets, the absolute numbers of $CD56^+$ (194 ± 119 *vs* 683 ± 381, $P = 0.01$) and $CD3^-CD56^+$ (128 ± 79 *vs* 605 ± 337, $P < 0.01$) cells were significantly decreased in the $aGVHD^+$ group, but we did not find any significant difference in other lymphocyte subsets.

Table 2 Absolute numbers of WBC, total lymphocytes and each lymphocyte subset according to the occurrence of aGVHD

	$aGVHD^+$ (mean ± s.d.) (n = 6)	*$aGVHD^-$ (mean ± s.d.) (n = 10)*	*P value*
WBC	2082 ± 834	4373 ± 1646	0.04
Lymphocyte	795 ± 491	1505 ± 839	NS
$CD3^+$	522 ± 322	643 ± 358	NS
$CD56^+$	194 ± 119	683 ± 381	0.01
$CD3^+CD56^-$	457 ± 282	566 ± 315	NS
$CD3^-CD56^+$	128 ± 79	605 ± 337	< 0.01
$CD4^+$	177 ± 109	435 ± 242	NS
$CD8^+$	345 ± 213	442 ± 247	NS
$CD8^+CD28^-$	318 ± 196	394 ± 220	NS
$CD8^+CD28^+$	43 ± 27	72 ± 40	NS
$HLA\text{-}DR^+$	662 ± 409	1008 ± 562	NS

NS = not significant.

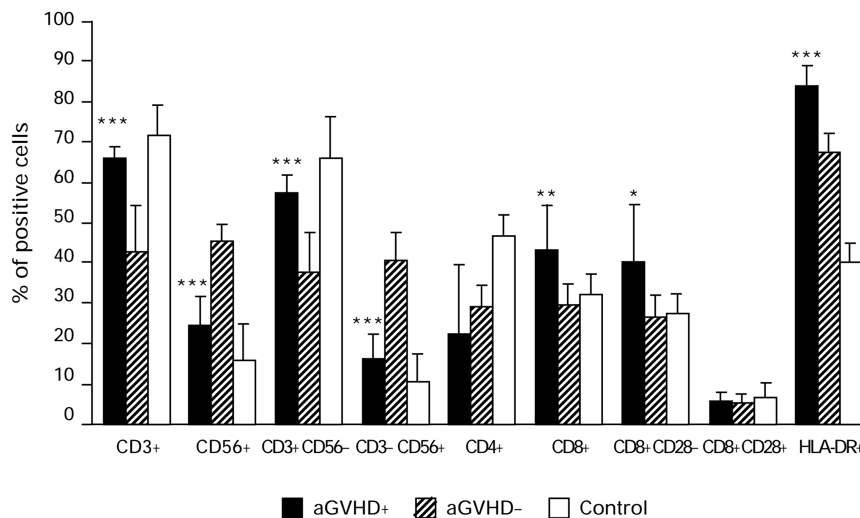

Figure 1 Different patterns of reconstitution of each lymphocyte subset according to the occurrence of aGVHD. aGVHD = acute graft-versus-host disease; * $P = 0.03$; ** $P = 0.01$; *** $P < 0.01$.

Higher expression of Fas and FasL in patients with aGVHD

To help elucidate the immune pathogenesis of aGVHD, we analyzed the coexpression of Fas or FasL together with the lymphocyte subset markers (CD4, CD8, CD56, HLA-DR). Here we found that Fas^+CD8^+ (35.0 ± 9.3 *vs* 13.6 ± 3.1, $P < 0.01$), $Fas^+HLA\text{-}DR^+$ (76.3 ± 2.5 *vs* 47.3 ± 1.2, $P < 0.01$), and $FasL^+CD8^+$ (32.2 ± 6.7 *vs* 11.4 ± 2.7, $P < 0.01$) cells were significantly increased in the $aGVHD^+$ patients (Figure 2). We also analyzed the proportion of these coexpressing cells on each lymphocyte subset (ie $Fas^+CD8^+/CD8^+$) according to the occurrence of aGVHD. Fas antigen was highly coexpressed on most of the lymphocyte subsets, especially on $CD8^+$ cells (81.2 ± 1.5 *vs* 47.8 ± 12.7, $P < 0.01$), and also, significantly higher coexpression of FasL on $CD8^+$ cells was shown in patients with aGVHD (77.1 ± 13.5 *vs* 40.4 ± 13.2, $P < 0.01$). However, Fas or FasL expression on $CD4^+$ cells was not different according to the presence of aGVHD (Figures 2 and 3).

Discussion

This study examined the pattern of lymphocyte subset reconstitution associated with the occurrence of aGVHD and elucidated the pathophysiology of aGVHD based on Fas/FasL pathway. We evaluated both the percentages and the absolute numbers of each lymphocyte subset. Since the absolute numbers of total leukocytes and lymphocytes were highly variable between individuals and not informative, we showed and analyzed our results as percentages. Our data revealed that the percentage of $CD56^+$ cells was significantly decreased in patients with aGVHD compared with patients without aGVHD, and most of these cells expressed $CD3^-$ by detailed analysis using double immunofluorescence staining. Therefore, most of these $CD56^+$ cells belong to the classical NK cell population. This finding was consistent with other reports[13,14] which suggested that the percentage of $CD3^-CD56^+$ cells was closely associated with the occurrence of aGVHD. Therefore, the evaluation of the percentage of $CD3^-CD56^+$ cells may be a reliable marker in the diagnosis of aGVHD.

Another characteristic finding was the close correlation between the occurrence of aGVHD and the expansion of $CD8^+$ cells. This finding was similar to other reports.[14–16] However, at present, only a few studies have evaluated the subsets of $CD8^+$ cells after BMT.[14,17,18] To help elucidate the role of $CD8^+$ cells, we further analyzed the $CD8^+$ population based on the expression of the CD28 marker by using double staining. Our data showed that most of the $CD8^+$ cells were composed of $CD8^+CD28^-$ T cells and the percentage of these cell populations was significantly higher in $aGVHD^+$ patients. On the other hand, we did not find any significant difference in the percentage of $CD8^+CD28^+$ cells according to the presence of aGVHD. Also, there was no correlation between $CD8^+CD28^-$ and $CD8^+CD28^+$ cells in our $aGVHD^+$ and $aGVHD^-$ patients. Thus, we suggest that the expansion of $CD8^+$ cells is closely correlated with the increased percentage of $CD8^+CD28^-$ cells and the evaluation of these cell populations can also be a reliable marker closely associated with aGVHD.

We cannot explain why the percentage of $CD3^- CD56^+$ NK cells was significantly decreased in $aGVHD^+$ patients. However, based on our results, we can postulate that the increase of $CD8^+$ and $CD8^+CD28^-$ T cells may be responsible for the relative decrease of $CD3^- CD56^+$ cells, since these cells are nonoverlapping subsets. Given the apparent importance of CD28 in T cell costimulation,[19,20] the differential role of $CD8^+CD28^-$ and $CD8^+CD28^+$ T cells in the immune response is still under discussion.[21–24] Koide and Engleman[22] first hypothesized that $CD8^+CD28^-$ T cells could act as suppressor cells and $CD8^+CD28^+$ T cells as

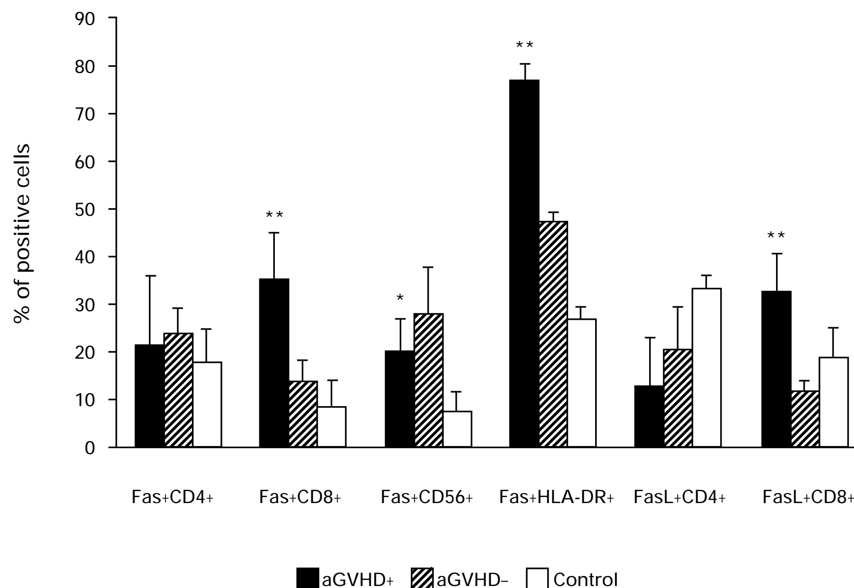

Figure 2 Simultaneous expression of Fas/Fas-ligand and CD4/CD8/HLA-DR according to the occurrence of aGVHD. aGVHD = acute graft-versus-host disease; FasL = Fas-ligand; * $P = 0.04$; ** $P < 0.01$.

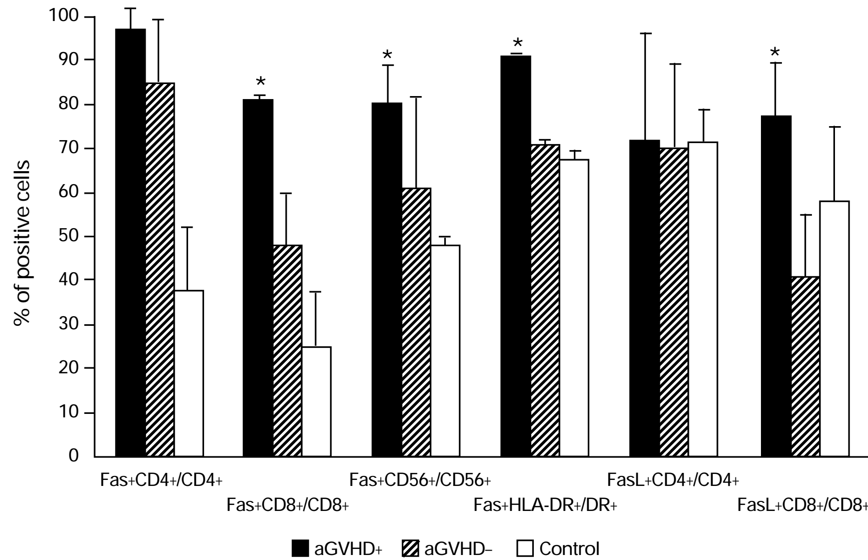

Figure 3 Coexpression of Fas or Fas-ligand on each lymphocyte subset according to the occurrence of aGVHD. aGVHD = acute graft-versus-host disease; FasL = Fas-ligand; * $P < 0.01$.

cytotoxic cells. However, some recent reports suggested that $CD8^+CD28^-$ T cells had a more potent cytolytic activity than $CD8^+CD28^+$ T cells.[21,23,24] Also, the role of NK cells in human GVHD or graft-versus-leukaemia effect is controversial.[25–29] Dokhelar *et al*[25] reported that a strong correlation between high NK values and aGVHD occurrence existed. Conversely, according to some reports,[27–29] NK cells did not directly mediate aGVHD but had the potential to suppress the growth of clonogenic leukaemia and when activated by cytokines could be directly lytic to leukaemia cells. From this point of view, it appears reasonable to suggest that $CD8^+$ $CD28^-$ T cells are one of the most important effectors in aGVHD.

According to Garin *et al*,[14] a decrease in the percentages of HLA-DQ^+ and DR^+ monocytes and HLA-DQ^+ lymphocytes was closely associated with the occurrence of aGVHD but the percentage of HLA-DR^+ lymphocytes was similar between the $aGVHD^+$ and $aGVHD^-$ groups. However, in our study, an increase in the percentage of HLA-DR^+ lymphocytes was a characteristic finding in patients with aGVHD. Although we did not evaluate the HLA-DR and DQ expression on monocyte population, we considered that the reason for this different pattern could be due to either heterogeneity of underlying diseases and conditioning regimens or difference of sampling period. To confirm the significance of the alternation of these parameters, further cumulative studies will be needed.

Since the alloreactive activated T cells express FasL as an effector molecule,[3,4] it may be of interest to study whether FasL is also involved in aGVHD induced by allogeneic BMT in humans. Here, we showed that the percentages of Fas^+CD8^+ and Fas^+HLA-DR^+ cells were significantly increased and Fas antigen was highly coexpressed on most of the lymphocyte subsets, especially on $CD8^+$ cells in patients with aGVHD. Also, the percentage of $FasL^+CD8^+$ cells and the proportion of coexpressed cells on $CD8^+$ cells ($FasL^+CD8^+/CD8^+$) were significant increased in $aGVHD^+$ patients. Since HLA-DR has been usually suggested to indicate T cell activation[15,16,30] and Fas or FasL are mainly expressed on activated lymphocyte populations,[3–6] our data suggest that the activated lymphocyte population may be significantly increased after BMT, especially in $aGVHD^+$ patients, and the activated $CD8^+$ cells may play a possible role as an effector through the Fas/FasL pathway in human aGVHD. To elucidate the precise role of Fas/FasL pathway and its effectors, confirmation of the expression of FasL on $CD8^+CD28^-$ T cells and Fas on target issues is required, since the expansion of $CD8^+$ cells was closely correlated with the increased percentage of $CD8^+CD28^-$ cells in this study.

In conclusion, an increase in the percentage of $CD8^+$ lymphocytes that express Fas and its ligand in patients with aGVHD after BMT points to a possible role for the Fas/FasL pathway in the effector phase of aGVHD.

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