Cytolytic Effector Mechanisms and Gene Expression in Autologous Graft-versus-Host Disease: Distinct Roles of Perforin and Fas Ligand

Yuji Miura, Christopher J. Thoburn, Emilie C. Bright, Allan D. Hess

The Sidney Kimmel Comprehensive Cancer Research Center at Johns Hopkins University, Baltimore, Maryland

Correspondence and reprint requests: Allan D. Hess, PhD, The Sidney Kimmel Comprehensive Cancer Research Center at Johns Hopkins University, 1650 Orleans St., Room 484, Baltimore, MD 21231 (e-mail: adhess@jhmi.edu).

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ABSTRACT
The administration of cyclosporin A (CsA) after autologous stem cell transplantation (SCT) paradoxically elicits a systemic autoimmune syndrome that resembles graft-versus-host disease (GVHD); this is termed autologous GVHD (autoGVHD). Although dominated by activated CD8+ cytotoxic T lymphocytes, the complex cellular reaction also includes CD4+ T cells and involves multiple effector mechanisms. To determine the temporal development and relative importance of these mechanisms in autoGVHD, perforin/granzyme, Fas ligand (FasL), interferon-γ (IFN-γ), tumor necrosis factor (TNF-α), and interleukin-18 gene expression in peripheral blood mononuclear cells was examined in 36 patients treated with CsA after SCT. Quantitative real-time polymerase chain reaction analysis revealed that perforin/granzyme B, TNF-α, and interleukin-18 messenger RNA (mRNA) levels in peripheral blood mononuclear cells from patients in whom autoGVHD developed were markedly higher (and temporally associated with the onset of autoaggression) compared with the levels detected in healthy individuals and in control, non–CsA-treated SCT patients. It is interesting to note that patients in whom autoGVHD did not develop also demonstrated increased mRNA levels for these cytokines; however, expression was substantially lower compared with that in patients with autoGVHD. It is important to note that IFN-γ mRNA levels were selectively increased in CD8+ cells only from patients in whom autoGVHD developed. The development of autocytoytic T cells in autoGVHD correlated with increased expression of perforin, IFN-γ, and TNF-α mRNA. Furthermore, enhanced autoreactive T-cell activity and the induction of autoGVHD was also concordant with perforin and TNF-α mRNA upregulation in CD4+ cells. Surprisingly, FasL mRNA levels were significantly decreased, with a progressive loss of FasL mRNA expression as autocytoytic activity increased. These findings suggest that IFN-γ/perforin–based CD8+ cytotoxic T lymphocytes seem to play a dominant role in autoGVHD and that TNF-α/perforin–based CD4+ cells may amplify this autoaggressive syndrome. The FasL pathway may play an important role in the regulation of this immune syndrome.

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KEY WORDS
Autologous GVHD • Perforin • Fas ligand • IFN-γ • TNF-α

INTRODUCTION
Induction of autologous graft-versus-host disease (autoGVHD) after autologous stem cell transplantation (SCT) has been a potential therapeutic modality for patients with hematologic malignancies and for patients with breast cancer. AutoGVHD can be induced by the administration of cyclosporin A (CsA) for a limited period after SCT [1-7]. Initial clinical trials in patients with hematologic malignancies suggest that the induction of autoGVHD can reduce the rate of relapse [8-11]. The therapeutic benefits are derived not only from the high doses of chemotherapy or irradiation used in the preparative regimen, but also from the eradication of residual tumor cells by autoreactive T lymphocytes.

The induction of autoGVHD is a 2-tiered process that requires both the active inhibition of the thymic-dependent clonal deletion of autoreactive T cells and the elimination of a peripheral immunoregulatory system by the preparative regimen; this provides a per-
immune environment for the autoreactive T cells to manifest autoaggression [2,3]. Reconstitution of the immunoregulatory system alters the course of autoaggressive disease. The onset of autoGVHD is characterized by a vigorous cell-mediated immune response with the proliferation of autoreactive CD8$^+$ cytotoxic T lymphocytes (CTLs) that promiscuously recognize major histocompatibility complex (MHC) class II determinants, a process that is dependent on presentation and recognition of the MHC class II invariant chain peptide [12-14]. The activity of the effector CD8$^+$ T cells is potentiated by an autoreactive CD4$^+$ T-cell subset [3,4]. Natural killer (NK) cells also seem to contribute significantly to the development of autoGVHD [15,16]. These autoreactive lymphocytes can lyse a variety of tumor cell lines derived from patients with myeloma, lymphoma, and breast cancer [12,13]. Together, these effector mechanisms primarily mediate dermal pathology with the epithelial cell destruction that is characteristic of GVHD after allogeneic SCT.

Immune-mediated cell killing can occur by several distinct mechanisms, including cytokine induction of cell death, cell-mediated lysis through the perforin/granzyme system, or Fas/Fas ligand (FasL) interactions [17,18]. CTLs, including CD4$^+$ T cells, CD8$^+$ T cells, and NK cells, can kill a variety of target cells (ie, virally infected cells and tumor cells) by using 1 or more of these pathways. Cytotoxic lymphocytes can secrete cytotoxic cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α with a direct local effect on the target cells [19]. Alternatively, CTLs can make direct contact with target cells by using their clonotypic T-cell receptor for antigen. Contact-dependent cytotoxic lymphocyte–induced death can occur by either the secretion of cytotoxic granules onto the surface of the target cells (perforin/granzyme pathway) or by the interaction of FasL on the CTLs and the Fas receptor on the target cells (Fas/FasL pathway), leading to apoptotic cell death [20]. Although several recent studies have demonstrated that both perforin and Fas/FasL pathways are important for the development of allogeneic GVHD (alloGVHD) [21-27], the molecular effector mechanisms used by the autoreactive T cells in the novel autoaggression syndrome, autoGVHD, remain unclear.

To evaluate the mechanisms involved in autoGVHD, gene expression for cytotoxic molecules (perforin, FasL, and granzyme A or B) and cytokotoxicity-associated cytokines (IFN-γ, TNF-α, interleukin [IL]-12, and IL-18) was examined temporally in peripheral blood mononuclear cells (PBMCs) from 36 patients undergoing autologous SCT with autoGVHD induction. Quantitative real-time polymerase chain reaction (PCR) analysis reveals that IFN-γ/perforin–based CD8$^+$ CTLs seem to play a dominant role in autoGVHD and that TNF-α/perforin–based CD4$^+$ cells may amplify this autoaggressive syndrome. However, FasL messenger RNA (mRNA) levels are decreased during autoGVHD and exhibit an inverse temporal relationship with autocrine/lytic T-cell activity and maintenance of NK function. These results suggest that the Fas/FasL pathway may play an important role in the regulation of this syndrome. The cytotoxic pathway in autoGVHD is complex and involves synergistic interactions between cellular and cytokine pathways and distinct subsets of effector cells. Understanding these pathways may not only promote more effective immunotherapeutic strategies to enhance the antitumor efficacy of autoGVHD, but also provide insight into the reconstitution of self tolerance after SCT.

MATERIALS AND METHODS

Patients

Women from 18 to 60 years of age with metastatic breast cancer and from a complete or partial response to chemotherapy were included in this study, which was approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Hospital, as previously described [13,28]. All patients provided informed written consent. Normal renal, cardiac, pulmonary, and hematopoietic reserves, in addition to an Eastern Cooperative Oncology Group performance status of 0 to 1, were required for all patients.

Preparative Regimens and Induction of AutoGVHD

The patients were prepared for autologous bone marrow transplantation by treatment with cyclophosphamide (1.5 g/m$^2$) and thiopeta (200 mg/m$^2$; 4 times daily) before autologous bone marrow rescue [13,28]. AutoGVHD was induced by the intravenous administration of CsA (2.5 mg/kg/d for 28 days; Novartis, Hanover, NJ) beginning on the day of transplantation. Recombinant IFN-γ (0.025 mg/m$^2$; National Cancer Institute, Bethesda, MD) was administered subcutaneously every other day from days 7 through 28 [29,30]. One group of control patients (n = 6) underwent autologous SCT with the same preparative regimens but without the administration of CsA and IFN-γ. The control patients elected not to receive the autoGVHD induction treatment. Four patients who underwent allogeneic SCT and experienced the development of grade III to IV acute GVHD were also compared with those patients in whom autoGVHD developed. Patients were evaluated daily for evidence of autoGVHD (erythematous rash). Each biopsy specimen was examined by one of the investigators in a blinded fashion. The histologic diagnosis of acute cutaneous GVHD was established when at least grade II

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changes were present, including basal vacuolar degeneration, spongiosis, dyskeratosis, and an infiltration of lymphocytes in the upper dermis and lower epidermis. Assays were conducted within 24 to 72 hours from the onset of the rash [13,28].

**Cell-Mediated Lympholysis Assay**

Cytotoxic activity was assessed sequentially after autologous SCT by using PBMCs isolated by Ficoll-Hypaque density centrifugation. Target cells used in these studies included autologous phytohemagglutinin-stimulated lymphoblasts (PHA-blasts) cryopreserved before transplantation, the breast cancer cell line T47D [31,32], and the NK target cell line K562. The pretransplant lymphocytes were thawed and stimulated with PHA for 72 hours (RPMI 1640; 20% normal human serum) before use as targets. The K562 cell line was grown in suspension culture in RPMI 1640 tissue culture medium supplemented with 10% fetal calf serum. T47D is an adherent breast cancer cell line grown in Dulbecco modified Eagle tissue culture medium supplemented with 10% fetal calf serum, glutamine, and sodium pyruvate [31,32]. Before assay, the cells were mildly trypsinized and grown in Nalgene Teflon flasks (Thomas Scientific, Swedesboro, NJ) for 24 hours to allow for the recovery of cell-surface antigens. The target cells were labeled with 250 μCi of chromium 51 (51Cr) for 1 hour at 37°C and washed 3 times before the assay. The effector lymphocytes were cocultured with 2.5 × 10^3 labeled target cells in triplicate in round-bottomed microtiter wells. After 4 hours of incubation, 51Cr release was assessed, and the percentage of specific lysis was determined from triplicate cultures as previously described [13,28].

**RNA Extraction**

Heparinized peripheral blood was collected from the patients after informed consent was obtained, and PBMCs were separated by using density-gradient centrifugation. Monocytes were isolated by plastic adherence and harvested by gentle scraping (>85% CD14+ by flow cytometry). Lymphocytes were separated into distinct T-cell subsets by immunomagnetic bead separation chromatography by using monoclonal antibodies to the CD4+ and CD8+ cell-surface determinants (Dynal Biotech, Oslo, Norway), as previously described [4,13]. The purity of the isolated CD4+ and CD8+ cells was typically >97%, as determined flow cytometrically. Skin biopsy samples (4-mm punch biopsies) were obtained, with informed patient consent, before transplantation and on the initial development of erythematous rash. The tissue was frozen in liquid nitrogen and pulverized with a mortar and pestle [33,34]. Total RNA was purified by dissolving the powder in Trizol reagent (Life Technologies, Gaithersburg, MD) with adequate mixing. Cell lysate was prepared from 5 × 10^6 PBMCs by adding 1 mL of Trizol reagent with adequate mixing. After 200 μL of chloroform was added, the solution was well mixed and centrifuged. The supernatant was collected and extracted once with chloroform. RNA was precipitated with 2-propanol and rinsed with 70% ethanol. Purified RNA was dissolved in 30 μL of diethylpyrocarbonate-treated distilled water.

**Quantification of Cytotoxic Molecule and Cytokine mRNA Levels by Real-Time PCR**

Reverse transcription was conducted as follows: 32 μL of water containing 1 μg of total RNA was added to 0.4 μg of random primers (Life Technologies) and incubated at 65°C for 10 minutes. Samples were chilled on ice, and complementary DNA was prepared with Ready-To-Go You-Prime First-Strand kit (Amersham Pharmacia Biotech, Piscataway, NJ), according to the manufacturer’s protocol. Real-time PCR reactions were performed by using the TaqMan assay (Applied Biosystems of PerkinElmer [ABI-PE], Foster City, CA), and PCR amplifications were performed in the ABI-PE Prism 7700 sequence detection system [35,36]. Briefly, a solution of TaqMan Universal PCR Master Mix (25 μL; ABI-PE) containing sense and antisense primers (300 nmol/L each) and dual-labeled fluorogenic probes (100 nmol/L) was prepared and aliquoted into individual MicroAmp Optical Plates (ABI-PE), and 5 μL of complementary DNA was added to give a final volume of 50 μL. Conditions for PCR reactions included 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing/extension). Data were analyzed with Sequencer Detector version 1.6 software. The threshold cycle (CT) during the exponential phase of amplification was determined by real-time monitoring of fluorescent emissions after cleavage of sequence-specific probes by the nuclease activity of Taq polymerase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control gene. Primers and fluorogenic probes for GAPDH, IFN-γ, TNF-α, FasL, IL-12, and IL-18 were from TaqMan kits (ABI-PE). The perforin and granzyme A and B reagents were generated by using the specific primer sequences (Integrated DNA Technologies, Coralville, IA) shown in Table 1. Messenger RNA levels were expressed as the absolute number of copies normalized against GAPDH mRNA. The difference in amplification was determined as follows: I/(CTtarget – CTGAPDH)^2. Although levels of the cytokine proteins were not determined in this study, recent studies in our laboratory have demonstrated that the level of cytokine mRNA quantified by real-time PCR directly correlates with cytokine production by PBMCs from patients with autoGVHD, as measured by enzyme-linked immunosorbent assays [16].
Maximum autolytic, anti-T47D, and NK cell activity observed during the course of treatment or at the onset of autoGVHD (day 12 through 33). Extensive analyses of lytic activity in patients with autoGVHD were also increased (1.8-fold) compared with that of patients without autoGVHD and with that of the control group of non-CsA-treated SCT patients. NK activity of patients with autoGVHD was not significantly different compared with the control group but was significantly (P = .03) greater than the NK activity detected in the patients who did not develop autoGVHD despite treatment with CsA and IFN-γ.

Statistical Analysis

Data were analyzed by the Fisher exact test or Pearson correlation (r) by using StatView software (SAS Institute, Cary, NC), with P values <.05 considered statistically significant.

RESULTS

Cytotoxic Activities and AutoGVHD

Cytotoxic molecule/cytokine gene expression and cytotoxic activity were evaluated in PBMCs from 36 patients treated with CsA after SCT. Clinical GVHD developed in 15 (41.6%) of 36 patients. Table 2 summarizes the data from patients with biopsy–confirmed autoGVHD, patients without clinical manifestations of autoGVHD, and control autologous SCT (non-CsA-treated) patients, evaluating maximal cytotoxic activity during the course of treatment or at the onset of autoGVHD (day 12 through 33). Extensive analyses of lytic activity in patients with autoGVHD were previously published and are succinctly summarized below [13,28]. Cytotoxicity mediated by posttransplantation lymphocytes of patients with confirmed autoGVHD was also significantly (P = .04; range, 7.4%-53.7% lysis) enhanced when compared with that of patients without autoGVHD and with that of the control group of non–CsA-treated SCT patients. NK activity of patients with autoGVHD was not significantly different compared with the control group but was significantly (P = .03) greater than the NK activity detected in the patients who did not develop autoGVHD despite treatment with CsA and IFN-γ.

PBMCs from the patients on the autoGVHD induction protocol were serially monitored for perforin, FasL, and granzyme A or B gene expression by real-time PCR during the course of treatment (days 12, 19, 25, and 33). Gene expression was determined in the PBMCs of the CsA-treated patients and compared with that of the control autologous SCT (non–CsA-treated) patients, healthy individuals, and allogeneic SCT patients. Figure 1A summarizes the data from patients evaluated at the onset of autoGVHD (appearance of erythrodermatous rash or biopsy–confirmed GVHD) and from patients who did not have any clinical manifestations of GVHD (data presented are the 2 highest levels measured during the course of treatment determined for each patient); perforin and FasL mRNA levels in PBMCs normalized against the housekeeping gene GAPDH were evaluated. Compared with healthy individuals, PBMCs obtained from patients at the onset of autoGVHD had significantly higher mRNA transcript levels for perforin (3.5-fold; P = .01). Perforin mRNA levels in PBMCs from patients who did not have any clinical manifestations of autoGVHD were also increased (1.8-fold) compared with the control groups. Expression was significantly (P < .01) less than the levels detected in patients with autoGVHD. FasL mRNA levels in PBMCs isolated from patients at the onset of autoGVHD, however, were 3.3-fold lower than the lev-
FasL mRNA levels in healthy individuals were 1.5-fold lower than the levels observed in patients who did not have any clinical manifestation of autoGVHD. FasL mRNA levels in this latter group of patients were still significantly (P < .01) less than the levels detected in PBMCs isolated from healthy individuals. Analysis of granzyme mRNA transcripts in PBMCs is summarized in Figure 1B. Granzyme B mRNA levels in PBMCs isolated from patients at the onset of autoGVHD and from patients in whom autoGVHD did not develop were increased compared with the levels detected in PBMCs from healthy individuals (3.1-fold and 2.2-fold, respectively; P < .01). However, levels of granzyme B mRNA in PBMCs from patients at the
onset of autoGVHD were marginally increased compared with the levels detected in patients in whom this syndrome did not develop. Granzyme A mRNA levels were comparable for all groups. Levels of mRNA expression for the cytotoxic molecules in the 6 control, non–CsA-treated SCT patients were similar, although FasL mRNA transcripts were increased (but not significantly) compared with the levels detected in healthy individuals. Perforin and granzyme B mRNA levels in patients at the onset of autoGVHD were significantly higher than the levels in control SCT (non–CsA-treated) patients (3.9-fold and 2.7-fold, respectively; \( P < .01 \)).

Figure 1, C and D, summarizes the temporal analysis of perforin and FasL mRNA expression for patients in whom autoGVHD developed and for patients who did not have this experimental autoaggression syndrome. Perforin mRNA levels in PBMCs from both groups of patients increased after SCT and reached maximal levels 26 to 33 days after transplantation. Levels of perforin mRNA transcripts in PBMCs from patients with autoGVHD were significantly \( (P = .03) \) higher than the levels detected in patients without GVHD at day 26. It is of interest to note that most (11 of 15) patients experienced the development of autoGVHD during this interval (between days 23 and 27) [13,28]. IFN-\( \gamma \), IL-18, and TNF-\( \alpha \) mRNA levels in patients at the onset of autoGVHD were significantly higher than the levels in control (non–CsA-treated) SCT patients (3.9-fold, 2.7-fold, and 6.4-fold, respectively; \( P < .05 \)). However, FasL mRNA levels in PBMCs isolated from both groups of patients on days 12, 19, and 26 were low compared with the levels detected in the PBMCs from healthy individuals but returned to near-normal levels by day 33. It is interesting to note that for patients with autoGVHD, FasL mRNA levels reached a nadir at day 26, the height of perforin mRNA expression. FasL mRNA expression at day 26 for patients with autoGVHD was significantly \( (P = .04) \) lower even compared with patients in whom this autoaggression syndrome did not develop. The onset and duration of autoGVHD (average time to onset, day 23; average duration, 6.4 days) were also concordant with decreasing levels of FasL and increasing levels perforin mRNA transcripts. Comparatively, mRNA levels for perforin and FasL in 4 patients with grade III to IV alloGVHD were significantly \( (P < .01) \) increased compared with those of healthy individuals (6.1-fold and 7.4-fold, respectively).

**Cytotoxicity-Associated Cytokine Gene Expression in PBMCs**

Although TNF-\( \alpha \) is critically involved in CTL effector function, IFN-\( \gamma \), IL-12, and IL-18 are also important for the maturation of CTLs and can modulate the effector function (perforin or FasL) of both T lymphocytes and NK cells. PBMCs from the patients on the autoGVHD induction protocol were serially monitored for cytotoxicity-associated cytokine gene expression by real-time PCR during the course of treatment (day 12 through 33). Analysis of IFN-\( \gamma \), TNF-\( \alpha \), and IL-18 mRNA transcripts in PBMCs is summarized in Figure 2A. TNF-\( \alpha \) mRNA levels in PBMCs isolated from patients at the onset of autoGVHD were 8.8-fold higher compared with those in healthy individuals \( (P < .01) \). TNF-\( \alpha \) mRNA levels (data presented report the highest level during the assessed time interval) in patients in whom autoGVHD did not develop were also increased (5.6-fold) compared with healthy individuals. TNF-\( \alpha \) mRNA levels in this group of patients were significantly \( (P < .01) \) lower compared with the levels detected in patients in whom autoGVHD developed. Levels of IL-18 mRNA were also significantly \( (P < .01) \) increased in the PBMCs from both groups of patients (autoGVHD positive, 2.8-fold; autoGVHD negative, 1.1-fold) compared with healthy individuals. Clearly, the highest levels of IL-18 were detected in the patients in whom autoGVHD developed. It is interesting to note that levels of IFN-\( \gamma \) mRNA were selectively increased only in the patients who experienced the development of autoGVHD, with a 3.6-fold increase compared with the levels detected in both healthy individuals and in patients who did not experience this autoaggression syndrome \( (P < .01) \). Because of very low transcription rates, levels of IL-12 could not be quantified in the patient population. However, low levels of IL-12 mRNA were detected at 14 of 17 time points for patients with autoGVHD compared with only 5 of 42 points for patients who did not experience the development of autoGVHD \( (P < .01; \) data not presented). Comparatively, levels of cytokine message in autoGVHD patients were lower than in patients for whom GVHD developed after allogeneic SCT (IFN-\( \gamma \), TNF-\( \alpha \), and IL-18 mRNA transcripts were detected at levels of 4.3-fold, 11.4-fold, or 3.2-fold higher compared with healthy individuals, respectively; \( P < .05 \)). Messenger RNA levels for IFN-\( \gamma \), TNF-\( \alpha \), and IL-18 in the PBMCs from autologous SCT control recipients (not treated with CsA and IFN-\( \gamma \)) were identical to the levels detected in healthy individuals.

Figure 2, B and D, summarizes the temporal analysis of IFN-\( \gamma \), TNF-\( \alpha \), and IL-18 mRNA expression for patients in whom autoGVHD developed and for patients in whom this experimental autoaggression syndrome did not develop. The temporal expression of IFN-\( \gamma \) and TNF-\( \alpha \) mRNA levels in the PBMCs from patients with autoGVHD was remarkably similar to the temporal expression of perforin mRNA. Levels of IFN-\( \gamma \) and TNF-\( \alpha \) mRNA detected in PBMCs after transplantation increased in a time-sequential manner and reached maximal levels on day...
26. Levels of these cytokine transcripts were significantly higher than the levels detected in patients without GVHD (this was particularly pronounced on day 26) or the levels detected in the PBMCs from healthy individuals and in PBMCs from control non–CsA-treated SCT patients \((P < .01)\). A similar pattern was observed for the temporal expression of IL-18 posttransplantation mRNA levels; there were only modest differences between patients who experienced the development of autoGVHD and those who did not.

**Correlation between Cytotoxic Activity and Cytotoxic Molecule/Cytokine Expression**

The correlations between perforin, FasL, IFN-\(\gamma\), and TNF-\(\alpha\) gene expression in PBMCs of autologous SCT recipients who were treated with CsA and IFN-\(\gamma\) and developed significant cytolytic killer cell activity is illustrated in Figure 3A to H. Killing of autologous PHA-blasts mediated by posttransplantation lymphocytes correlated with the level of perforin, IFN-\(\gamma\), and TNF-\(\alpha\) mRNA expression in PBMCs (Figure 3A, C, and D; \(P = .01\), \(P < .01\), and \(P = .02\), respectively). Similarly, the ability to kill the T47D breast cancer cell line also correlated with perforin, IFN-\(\gamma\), and TNF-\(\alpha\) gene expression (Figure 3E, G, and H; \(P < .01\), \(P = .01\), and \(P = .02\), respectively). The NK activity was proportioned to the levels of perforin, IFN-\(\gamma\), TNF-\(\alpha\), and IL-18 mRNA expression \((P < .05\); data not presented). However, there was a progressive loss of FasL mRNA expression with increasing cytotoxicity against PHA-blasts and T47D cells (Figure 3, B and F; \(P = .01\) and \(P = .08\), respectively). Attempts to correlate levels of IL-18 mRNA with the ability to kill autologous PHA blasts and the T47D cell line were unsuccessful.

Changes in the cytotoxicity and mRNA expression...
over time were assessed in PBMCs from patients with autoGVHD after transplantation. Representative experiments evaluating lymphocytoxicity and cytotoxic molecule expression for 2 patients are shown in Figure 4. NK activity and the ability of the PBMCs from patient A to kill T47D tumor cells (autologous PHA blasts were not available for this patient) paralleled increased perforin gene expression and decreased FasL gene expression (Figure 4A). Similarly, the ability of PBMCs from patient B to kill autologous PHA blasts and the NK cell line K562 exhibited an identical temporal relationship with increased TNF-α gene expression and decreased FasL gene expression (Figure 4B). Both patients experienced the development of clinical cutaneous GVHD concordant with the development of the killer cell activity.

**Cytotoxic Effector Molecule and Cytokine Gene Expression in Skin Lesions of GVHD**

To confirm the pathogenic involvement of cytokines and cytotoxic effector molecules in GVHD, gene expression was evaluated in the skin lesions of 2 patients with autoGVHD. Comparisons were also made of skin biopsy samples from 4 patients with alloGVHD by real-time PCR. Figure 5 summarizes the data evaluating mRNA levels normalized against GAPDH. Comparable levels of perforin and granzyme B cytotoxic effector molecules and IFN-γ, TNF-α, and IL-18 cytokine mRNA transcripts were detected in the skin lesions of patients with autoGVHD and alloGVHD. Although pronounced levels of FasL mRNA transcripts were detected in skin lesions of all patients with alloGVHD, FasL mRNA transcripts either were not detected or were marginally expressed in the skin lesions of patients with autoGVHD.

**Cellular Origin of Cytotoxic Effector Molecules and Cytokine Transcription**

To identify the cellular source of the IFN-γ, TNF-α, IL-18, perforin, and FasL mRNA transcripts, CD4+ and CD8+ T cells and monocytes were separated from PBMCs of 5 healthy individuals and 7 patients who received CsA after autologous SCT. Two patients had clinical autoGVHD with detectable cytotoxicity against autologous PHA blasts (14.2% or 15.5%, respectively; effector-target ratio, 100:1). Gene expression was determined in each population. The results in Figure 6 show that perforin mRNA levels in both the CD4+ and CD8+ subset in whom autoGVHD developed were markedly higher compared with the levels from both healthy individuals and patients in whom autoGVHD did not develop ($P < .05$). However, FasL mRNA levels were relatively lower in the CD4+ and CD8+ subsets of patients in whom autoGVHD developed compared with
Figure 4. Relationship between the induction of autoGVHD and cytotoxic activity/molecule expression. The changes in the cytotoxic activity and effector molecule expression over time were assessed in PBMCs from 2 representative patients (patient A, panel A; patient B, panel B) with autoGVHD (the interval of biopsy-confirmed erythematous rash is highlighted in the figure) after transplantation. Cytotoxicity against autologous PHA-blasts, K562 NK target cell lines, and the T47D breast cancer cell line were measured with a standard 51Cr release assay at a 100:1 effector-target ratio. The relative expression of perforin, FasL, and TNF-α mRNA levels normalized against GAPDH was determined by real-time PCR.

Figure 5. Expression of cytotoxicity-associated molecule and cytokine mRNA in skin lesions. RNA was harvested from skin lesions of 2 patients with autoGVHD or 4 patients with alloGVHD. Complementary DNA was analyzed for effector molecule and cytokine transcription, including perforin, FasL, granzyme B, IFN-γ, TNF-α, and IL-18, by real-time PCR. Data were normalized against GAPDH.
both healthy individuals ($P < .05$) and patients in whom this autoaggression syndrome did not develop. IFN-γ mRNA levels in the CD8⁺ subset from patients in whom autoGVHD developed were higher compared with the levels from healthy individuals ($P < .05$). TNF-α mRNA levels in the CD4⁺ subset from patients in whom autoGVHD developed were higher compared with the levels from healthy individu-
uals (P < .01), whereas the levels in the CD8+ subset were lower. IL-18 mRNA levels in both the CD4+ and CD8+ subset in whom autoGVHD developed were higher compared with the levels from healthy individuals (P < .01). Attempts to correlate these levels of mRNA in the monocytes subset were unsuccessful. Comparatively, these mRNA levels in both CD4+ and CD8+ cells from 4 patients with grade III to IV alloGVHD were significantly increased (P < .01) except for IL-18 in CD8+ cells.

**DISCUSSION**

This study examined the autocytoytic T cells associated with autoGVHD and their molecular pathways as assessed by the expression of cytokine- and cytotoxicity-associated genes. The results provide several new insights into the effector and regulatory mechanisms associated with this novel autoimmune syndrome. Levels of mRNA transcripts for the cytolytic molecules perforin and granzyme B and the cytotoxicity-associated cytokines IFN-γ, TNF-α, and IL-18 were markedly increased in the PBMCs from patients with autoGVHD compared with the levels detected in PBMCs from healthy individuals and from control, non-CsA-treated SCT patients. To a large extent, the results parallel the findings in alloGVHD, in which cytotoxicity-associated molecules and cytokines play an important role in the pathogenesis of GVHD [21-27]. Moreover, temporal posttransplantation analysis also revealed that peak expression of both cytolytic molecule– and cytotoxicity-associated cytokine mRNA transcripts was associated with the onset of clinical evidence of autoGVHD in most patients [13,28].

It is interesting to note that with the exception of IFN-γ, enhanced expression of the cytotoxicity-associated molecule and cytokine mRNA transcripts was not limited to patients in whom autoGVHD developed. PBMCs from SCT patients treated with CsA but who did not have autoGVHD also had increased levels of mRNA transcripts for perforin, granzyme B, TNF-α, and IL-18. Expression, however, was substantially lower compared with that in patients in whom autoGVHD developed. Because the levels of the cytolytic molecule– and cytotoxicity-associated cytokine mRNA in control non-CsA-treated SCT patients were comparable to those of healthy individuals, it seems likely that the expression of these mRNA transcripts in patients who did not have autoGVHD were due to the CsA plus IFN-γ treatment regimen. The reduced levels of these cytokines in patients who did not experience autoGVHD development may be due to the limited amplification of the effector mechanisms and may reflect a subclinical autoGVHD. However, there was a selective increase in mRNA levels for IFN-γ only in patients in whom autoGVHD developed. These results suggest that IFN-γ plays an essential role in the development of clinical disease. Because IFN-γ is administered exogenously in the treatment regimen, it seems likely that local production of this cytokine by the effector T cells in the patients who have clinical autoGVHD is critical. Local production of IFN-γ would lead to the upregulation of the MHC class II target antigen in the target tissue, allowing target cell recognition and destruction. In accord are the results from a rat model of autoGVHD, which showed that local upregulation of the MHC class II target antigen by IFN-γ is important for the onset of autoimmune pathology [37].

It is important to note that expression of the cytotoxicity-associated molecule and cytokine mRNA transcripts occurred during CsA treatment; this suggests that activation of the cells and the signaling pathways leading to the production of these transcripts may be relatively resistant to the effects of this immunosuppressive drug. The relative resistance of these pathways may have significant consequences. Failure to adequately suppress production of the cytolytic molecule and cytotoxicity-associated cytokine mRNA transcripts may compromise the efficacy of CsA-based immunosuppression in certain settings, including allogeneic SCT and the prevention of GVHD, that use these cytokines and cytolytic effector molecules [21-27].

The induction of autoGVHD correlates with the appearance of MHC class II–dependent cytotoxic activity against autologous PHA-blasts mediated by autoreactive CD8+ T cells. Consistent with this observation are the results demonstrating that expression of perforin and IFN-γ mRNA transcripts was increased in CD8+ cells and that autocytoytic killer cell function correlated with the increased expression of these mRNA transcripts. Enhanced expression of perforin and TNF-α mRNA in CD4+ T cells was also detected in patients in whom autoGVHD developed. It is interesting to note that NK activity recovered quickly after SCT and was maintained in the CsA-treated SCT patients: this finding is consistent with increased perforin, TNF-α, IFN-γ, and IL-18 gene expression. Recent studies, however, demonstrate that the CD8+ T-cell subset is the major effector mechanism in autoGVHD, although NK cells may contribute to the histologic damage observed [15,16]. However, there was a progressive loss of FasL mRNA expression in the context of increasing cytotoxicity against PHA-blasts and T47D cells. Messenger RNA levels for both perforin and FasL in the PBMCs from 4 patients with grade III to IV alloGVHD were markedly increased. It is interesting to note that perforin mRNA transcripts were detected in the skin lesions of both patients with autoGVHD and alloGVHD, whereas high levels of FasL transcripts were detected only in the
patients with alloGVHD. FasL mRNA transcripts in the patients with autoGVHD either were not detected or were marginally expressed—certainly a remarkable difference between alloGVHD and autoGVHD. Taken together, these findings imply that perforin/granzyme, rather than FasL, is crucial for the pathologic damage mediated by the autoreactive T cells in autoGVHD. This is in contrast to the relative importance of FasL in alloGVHD [21-27].

Therapeutic immune modulation has been explored in several clinical trials of autoGVHD in an attempt to enhance antitumor immunity after autologous SCT. Moreover, the ability to experimentally induce autoaggression may reveal unique insights into autoreactive and autoregulatory mechanisms. The induction of autoGVHD requires both the active inhibition of the thymic-dependent clonal deletion of autoreactive T cells and the elimination of peripheral immunoregulation [38-41]. Both CD4+ and CD8+ autoreactive T cells are clearly important and play distinct roles in the onset and progression of disease, as demonstrated in an adoptive transfer model of autoGVHD in the rat [4,5]. CD8+ autoreactive T cells can transfer the acute phase of autoGVHD that resolves within 2 weeks, whereas CD4+ cells are ineffective by themselves. Adoptive transfer of both subsets, however, results in autoaggressive disease with greater intensity that can progress into a sustained chronic phase. The autoreactive CD4+ T-cell subset certainly seems to play a critical role in autoGVHD. In accord are the results from this study, which show that IFN-γ/ perforin-based CD8+ CTLs seem to play a dominant role in autoGVHD and that TNF-α/ perforin-based CD4+ cells may amplify this autoaggressive syndrome.

Of additional importance are the findings that TNF-α mRNA transcripts were also upregulated in PBMCs, including CD4+ T cells, from patients in whom autoGVHD developed. TNF-α secreted by activated lymphocytes can act directly as an effector pathway in target cell killing [19,42]. The results from this study also demonstrate that cytotoxicity against autologous PHA-blasts mediated by autoreactive T cells correlated with increased expression of TNF-α mRNA. The increase in TNF-α mRNA expression also correlated with the clonal expansion of autoreactive T cells in patients in whom autoGVHD developed [7]. Because TNF-α is also required for optimal maturation of CTLs [43], it seems likely that this cytokine plays a role in the development of autoreactive CTLs in autoGVHD. Moreover, upregulation of both perforin and TNF-α mRNA in CD4+ T cells from patients with autoGVHD suggests that this subset can contribute to the pathology of autoGVHD and can amplify or mature CD8+ autoreactive effector T cells. In addition, TNF-α also induces IFN-γ production [44,45]. As described previously, IFN-γ plays a critical role in the development of autoGVHD [37]. Clearly, TNF-α, with its proinflammatory actions, can mediate direct damage and amplify the autoreactive T-cell response in this disease.

The most pronounced and unexpected finding from this study was the decrease in FasL mRNA transcripts detected in both the CD4+ and CD8+ cells of patients in whom autoGVHD developed. Levels of FasL mRNA transcripts were decreased even in the presence of increased levels of IL-18, a cytokine that promotes FasL expression [46,47]. One potential explanation is that there is a cytotoxic dysregulation that ensues with the onset of autoaggression. Alternatively, the autoreactive T cells associated with this syndrome may have aberrant signaling pathways. Certainly, CsA treatment alters T-cell differentiation and may disrupt the expression of classic cell-surface/intracellular signaling mechanisms [40,41]. However, the progressive loss of FasL mRNA expression as MHC class II-dependent autologous lymphocytotoxicity increased suggests that the FasL pathway may be relevant for the regulation of this immune syndrome. Previous studies indicate that the release of autoreactive T cells into the periphery by itself is not sufficient to induce autoGVHD; a peripheral autoregulatory system must be eliminated to provide a permissive environment for the autoreactive T cells to manifest autoaggression and allow for the development of autoGVHD [48,49]. Recent studies in the rat model suggest that the autoregulatory T cells mediate a peripheral clonal deletion of autoreactive T cells that is Fas/FasL dependent [50]. The results from this study showed a reduction in FasL in both CD4+ and CD8+ T cells in patients with autoGVHD; a progressive increase in autologous lymphocytotoxicity suggested the possibility that downregulation of FasL expressed by autoregulatory cells leads to the development of autoGVHD. Alternatively, there may be a failure to reconstitute the autoregulatory compartment. In this regard, recent studies indicate that CsA treatment impedes the development of autoregulatory T cells in the thymus [51].

IL-18, a multifunctional cytokine, was originally discovered as a factor that induced IFN-γ production from lymphocytes and monocytes [52,53]. IL-18 upregulates FasL expression on T cells and NK cells [46,47], facilitating killing activity and participating in the development of alloGVHD. In contrast, Reddy et al. [54] provided substantial evidence that IL-18 regulates alloGVHD by inducing Fas-mediated apoptosis of donor T cells, presumably mediated by reduced TNF-α production. However, in human autoGVHD, both IL-18 and TNF-α mRNA transcripts are increased, whereas FasL mRNA transcripts are paradoxically decreased in patients in whom autoGVHD develops. These results suggest that the development of autoGVHD and its respective pathology is not depen-
dent on IL-18 and Fas/FasL-mediated cytolysis. However, recent studies demonstrate that IL-18 augments perforin-mediated and IFN-γ-mediated NK lytic activity [55,56]; these findings are in accord with those of the present study, which show that the induction of autoGVHD correlates with perforin-, IFN-γ-, and IL-18–dependent NK activity. In addition, IL-12 (a cytokine that can augment perforin-dependent lytic activity collaboratively with IL-18) mRNA could be detected in most patients in whom autoGVHD developed [57].

Collectively, the results from this study indicate that the autoreactive T cells make differential use of cytotoxic pathways for the induction and regulation of autoGVHD, as schematically illustrated in Figure 7. In human autoGVHD, the perforin/granzyme B and TNF-α pathways are critically important for the autoreactive T-cell response and promote destruction of self. Essential to this process is IFN-γ, which may not only enhance CTL function but also induce local upregulation of the MHC class II target antigen. The FasL pathway, however, seems to play a central role in the regulation of this immune syndrome. In particular, it is controversial which pathway has greater relative contribution to alloGVHD or a graft-versus-tumor effect [25,26]. Recent experiments indicate that the perforin pathway, rather than the Fas/FasL pathway, is more important for the graft-versus-tumor effect separated from alloGVHD [54]; this is an expected advantageous benefit of such a cytotoxic pathway in autoGVHD. Understanding these pathways and the manipulation of autoreactive and autoregulatory activity through the effector pathways may facilitate the development of more effective immunotherapeutic strategies for enhancing the antitumor efficacy of autoGVHD, including the specific blockade of FasL to inhibit autoregulatory mechanisms.

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