Inflammatory Cytokines Predominate in Cases of Tumor Regression after Hematopoietic Stem Cell Transplantation for Solid Cancer

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ABSTRACT
Allogeneic hematopoietic stem cell transplantation (SCT) has recently been presented as promising immunotherapy against renal cell, colon, ovarian, breast, and primary liver cancer. Because clinical results demonstrate a variable effect on metastases, we studied whether there is an association between the clinical response and free cytokines in serum. Two patients with metastatic colorectal and 4 with renal cell cancer underwent allogeneic SCT. Conditioning included fludarabine (30 mg/m²) for 3 or 5 days, using sibling or matched unrelated donors, respectively, followed by 2 Gy total body irradiation (n = 5) or cyclophosphamide (60 mg/kg) for 2 days (n = 1). Antithymoglobuline (4 mg/kg) was given to patients with matched unrelated donors (n = 3). Immunosuppression was cyclosporin A, combined with mycophenolate mofetil (n = 5) or methotrexate (n = 1). The tumor load was examined by computer tomography of the thorax and abdomen before and 3, 6, 9, and 12 months after SCT. Free cytokines in serum were analyzed using enzyme-linked immunosorbent assay. In each patient, the ratio between inflammatory (I) and anti-I cytokines was calculated. No statistical significance was found between the cytokine ratio in correlation to the tumor load according to international response evaluation criteria in solid tumors criteria. In contrast, tumor regression was found to correlate with dominating I cytokine levels in 5/7 occasions, compared with 1/12 of cases with anti-I cytokines using our local method focusing on metastases in lungs, lymph nodes, and liver (P = .01). Thus, an increased level of I cytokines possibly mirrors tumor killing induced by type 1 T-cell response. Furthermore, anti-I cytokines might inhibit cytotoxic cells from exerting the antitumor effect of allogeneic SCT.

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KEY WORDS
Allogeneic stem cell transplantation • Cytokines • Renal cell cancer • Colon cancer • Tumor response

INTRODUCTION
Allogeneic hematopoietic stem cell transplantation (SCT) with nonmyeloablative or reduced intensity conditioning (RIC) is one immunologic treatment with promising results to achieve reduction of tumors in patients with metastatic solid cancer. A graft-versus-leukemia effect has been demonstrated after SCT in patients with myeloid and lymphoid leukemia, lymphoma, and multiple myeloma [1-7]. A comparable allogeneic graft-versus-tumor (GVT) effect has been reported in metastatic breast cancer, colon cancer (CC), ovarian cancer, and renal cell cancer (RCC) [8-12]. The graft-versus-leukemia and GVT effects seem to be mediated by alloreactive donor T lymphocytes recognizing target antigens on major histocom-
patibility complex class I of leukemic and tumor cells. Furthermore, donor lymphocyte infusion (DLI) is an established posttransplantation immunotherapy with antitumor potential for both chronic myeloic leukemia and metastatic RCC [3,10,12,13].

Childs et al. [10] presented the first clinical study on GVT effect on solid malignancy showing complete or partial regression (R) of metastases at different localizations in patients with RCC. Complete or partial R of metastatic lesions has also been documented in patients with CC and breast, ovarian, prostate, and nonsmall-cell lung carcinoma [9,14-19]. Interestingly, disparate responses of metastases in different localizations have been demonstrated in one patient with CC and two patients with RCC, who had progression in the liver but R in the lungs [11].

There is evidence for involvement of inflammatory (I) cytokines, especially tumor necrosis factor (TNF)-α, as important mediators of graft-versus-host disease (GVHD) [20]. Velardi et al [21] showed production of interferon (IFN)-γ, another I cytokine, in T-cell clones isolated from patients with GVHD. Furthermore, a recent study showed a moderate to severe acute GVHD (aGVHD) with higher levels of TNF-α and IFN-γ, but lower levels of interleukin (IL)-10 and transforming growth factor (TGF)-β1, 2 weeks after SCT in patients with myeloid leukemia as compared with during the period of conditioning [22]. Different subsets of T-cell clones are responsible for cytokine production. The type 1 T-cell response consists mainly of IFN-γ, TNF-β, and IL-2, which promote cell-mediated responses and cytotoxicity. IL-4, -5, -6, -10, and -13 mediate a type 2 T-cell response, inducing a specific humoral immunity that involves the production of IgG1 and IgE.

In this study, we demonstrate an association between free cytokines in serum and variable response in terms of tumor burden in 6 patients with solid cancer after RIC and SCT.

PATIENTS AND METHODS

Patients

Six patients with metastatic solid tumors were included in the study: two patients had adenocarcinoma in the colon (C4, C7) and 4 had RCC (R2, R4, R7, R13). Patients were treated with RIC and allogeneic peripheral SCT at our institution between March 2000 and August 2002. All 6 patients had been considered to have tumors that were incurable with any conventional therapy. All patients had undergone debulking of the primary tumor, and in two patients, reduction of metastases was also performed. Two patients had been given additional prior therapy, such as cytostatics or interferon. The characteristics of the patients are presented in Table 1. Our research ethics committee approved the study.
Donors

Patients and donors were HLA antigen typed with a high-resolution polymerase chain reaction-sequence-specific primers (SSP) method for HLA antigen classes I and II [23]. All donors were at least HLA antigen-A, -B and -DRB1 compatible with the recipient. An HLA antigen–identical sibling donor (n = 3) was given priority but, if not available, an HLA antigen–matched unrelated donor (n = 3) was accepted (Table 1). From all donors, peripheral blood stem cells were collected after stimulation with granulocyte-colony stimulating factor (Neupogen, Amgen, Stockholm, Sweden) [11].

Conditioning

The RIC consisted of fludarabine (30 mg/m²/d) for 3 or 5 days, in HLA antigen–identical sibling donor or matched unrelated donor, respectively (n = 6), followed by 2 Gy of total body irradiation (n = 5). Antithymocyte globulin (Thymoglobuline, Genzyme, Cambridge, Mass) (2 mg/kg/d) was given for 2 days to recipients with matched unrelated donor. After May 2001, total body irradiation was replaced by cyclophosphamide (60 mg/kg/d) for 2 days (n = 1) [11,18].

Immunosuppression

Posttransplantation immunosuppression consisted of cyclosporin A (Sandimmun Neoral, Novartis Pharma AG, Stein, Switzerland) in all patients for up to 3 months. In addition, mycophenolate mofetil (Cellcept, Hoffman LaRoche, Basel, Switzerland) (0.5-1 g twice a day) was given for 1 to 2 months. After May 2001, mycophenolate mofetil (n = 5) was replaced by methotrexate (n = 1). The cyclosporin A doses ranged between 3 to 12 mg/kg/d to achieve a through level of 100 ng/mL in patients with a sibling donor, or 200 to 300 ng/mL in patients with an unrelated donor. Grade I aGVHD was treated with prednisolone (2 mg/kg/d) to prevent more severe GVHD [11,18,24,25].

Supportive Therapy and Treatment

The supportive therapy against bacterial, viral, and fungal infections was given according to the SCT protocol of the center [24,25]. Asymptomatic cytomegalovirus infection, diagnosed by testing peripheral blood leukocytes for cytomegalovirus DNA by polymerase chain reaction, was treated with pre-emptive therapy using ganciclovir by mouth or intravenously according to an ongoing randomized study.

DLIs

DLIs were given in escalating doses, 1, 5, 10, and 100 × 10⁶ CD3⁺ cells/kg recipient body weight. The therapy usually started at 3 to 4 months after SCT and after the immunosuppressive therapy had been discontinued. The indications for DLI were tumor progression and/or mixed chimerism in the absence of GVHD (Table 1).

GVHD

aGVHD was graded from 0 to IV according to published criteria [26]. Chronic GVHD (cGVHD) was graded as limited or extensive. aGVHD and cGVHD were diagnosed from the clinical symptoms and/or

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<td>CT indicates computer tomography; gr, grade; GVHD, graft-versus-host disease; Lim, limited; NA, not available; PD, progressive disease; PR, partial response; Q, ratio between inflammatory and anti-inflammatory cytokines; SD, stable disease.</td>
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biopsy specimens from skin, oral mucosa, liver, and gut (Tables 1, 2a, and 2b).

Evaluation of Tumor Status

The tumor load was examined by computed tomography (CT) of the thorax and abdomen before SCT and 3, 6, 9, and 12 months after SCT (Tables 2a and 2b). Evaluation of tumor response was based on two methods, the international response evaluation criteria in solid tumors (RECIST) [27] and our local method [11,28].

According to RECIST, a partial response (PR) was defined as at least 30% decrease in the sum of the longest diameter of metastatic lesions compared with tumor load before SCT. Progressive disease (PD) indicated at least 20% increase in the same metastatic lesions or the appearance of one or more new lesions. Stable disease (SD) was defined as neither sufficient decrease to qualify for PR nor sufficient increase to qualify for PD (Table 2a). Each CT examination was compared with the CT examination performed before SCT.

According to our local method, evaluation of tumor load was based both on the number and the size of the metastatic lesions on CT. R, PD, and SD were defined separately for each metastatic localization (lung tissue, pleura, lymph nodes, and liver). R was defined as a decreased size of all metastases in the localization relative to the tumor load before SCT or previous CT examination. PD indicated increased tumor load or appearance of one or more lesions. SD was defined as neither sufficient decrease to qualify for R nor sufficient increase to qualify for PD. Based on this local evaluation an assessment of the total metastatic load was performed as follows: (1) if more than or equal to 50% of metastatic localizations were characterized as R, the total load was defined as mixed response (MR); (2) if at least one localization showed PD and at the same time all other localizations indicated SD, the total load was defined as PD; and (3) an indication of SD in all localizations lead to the total assessment of SD (Table 2b). Each CT examination was compared with the nearest preceding CT examination.

Cytokine Analyses

Blood samples were collected within 1 month before (n = 6) and 1, 3, 6, 9, and 12 months after SCT (n = 30). The compliance of sample collection was 88.9% (32/36). Fresh blood samples were centrifuged and the serum was frozen in aliquots at −20°C for later analysis. An automated chemiluminescence immunoassay (Immulite, DPC, Los Angeles, CA) was used for analyzing TNF-α and IL-10 levels. IFN-γ and TGF-β1 levels were determined by enzyme-linked immunosorbent assay kits (Quantikine, R and D Systems, Minneapolis, Minn). Sensitivity of the assay for TNF-α, IL-10, IFN-γ, and TGF-β1 was 1.7, 1.0, 8.0, and 7.0 pg/mL, respectively. Intra-assay coefficient of variation was 3.2%, 3.1%, 3.4%, and 5.3%, respectively. Cytokine analyses were performed according to the manufacturer’s instructions.

Table 2b. Status of Metastases Determined by Computer Tomography according to our Local Method and Evaluation of Cytokines in Patients with Solid Tumor after Hematopoietic Stem Cell Transplantation

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CT indicates computer tomography; gr, grade; GVHD, graft-versus-host disease; Lim, limited; MR, mixed response; PD, progressive disease; Q, ratio between inflammatory and anti-inflammatory cytokines; R, regression; SD, stable disease.
Statistical Analysis

The 2-sided Fischer exact test was used to compare the cytokine balance to tumor response. All data were computed by using software (Statistica, Statsoft Inc., Tulsa, OK). \( P \) less than .05 was chosen to show a statistical significant difference.

RESULTS

DLIs

Of 6 patients, 5 were given DLIs (Table 1) after withdrawal of immunosuppressive treatment: one patient (R7) received one dose, one (R13) received two doses, two (C7, R2) received 4 doses, and one patient (C4) received 5 doses of DLI (Figures 1 and 2). DLIs were administered between days 105 and 333 after SCT. One patient (R4) did not receive DLI because of fluctuating GVHD after SCT.

GVHD

aGVHD, cGVHD, or both developed in 5 patients and was treated with corticosteroids in combination with cyclosporine with or without other immunosuppressive therapy. Two patients (R2, R13) had aGVHD grade I to II early (<100 days) after SCT, which recurred as aGVHD or limited cGVHD after DLI (Figure 1, Tables 2a and 2b). One patient (C4) without aGVHD developed limited cGVHD after DLI. One patient (R7) developed aGVHD before receiving DLI. One patient (R4) who did not receive DLI had early aGVHD that recurred as aGVHD.

Tumor Status and Cytokine Analyses

The tumor response after allogeneic SCT and DLI determined by CT is shown in Tables 2a and 2b. For each patient, blood samples were analyzed for I cytokines, TNF-\( \alpha \), and IFN-\( \gamma \), and for anti-I (A-I)
ones, TGF-β1, and IL-10 (Figures 1 and 2). The ratio between I and A-I cytokines for each individual was compared with the tumor load (Tables 2a and 2b). A ratio of I or greater corresponded with I status, whereas a ratio less than 1 was associated with A-I status.

According to RECIST and using the evaluation of target lesions, 4/7 of occasions with PR or SD correlated with I cytokines (Table 2a). This was compared with 3/12 of occasions where A-I cytokines were found with PR or SD. Thus, progressive status was found in 3 and 9 occasions with I and A-I, respectively (not significant). If both target and nontarget lesions were evaluated for the best overall response according to RECIST criteria, 2/7 of occasions with PR or SD correlated with I cytokines (Table 2a). None of 12 occasions with PR or SD was found with dominating A-I cytokines. Thus, progressive status was found in 5 and 12 occasions with I and A-I, respectively (not significant).

According to our local method, we found MR or SD in 5/7 of occasions correlated with I cytokines (Table 2b). This was compared with 1/12 of evaluations where A-I cytokines were found with MR or SD. Thus, progressive status was found in 2 and 11 occasions with I and A-I, respectively (P = .01).

DISCUSSION

Allogeneic hematopoietic SCT is a promising immunotherapy for patients with metastatic solid cancer [8-12]. However, clinical experience has demonstrated a variable antitumor effect on metastases in different organ localizations [10,11]. Despite tumor R that was seen in some metastatic lesions and at some time, the GVT effect was only transient. Therefore, additional immunotherapy seems to be needed to control tumor growth. Other tumor load reducing tools such as stereotactic irradiation and radiofrequency ablation should also be taken into account. Especially in the liver, metastases are difficult to control and the reason might involve escape mechanisms of metastases. According to our evaluation method, all patients but one, who were classified as MR, had PD in the liver, whereas in all other localizations R or SD were found. This is in accordance with previous findings on unsatisfied tumor response on liver metastases in parallel with an antitumor effect in other localizations in patients with metastatic RCC, CC, and melanoma [11,29,30]. The liver metastases probably need additional treatment, such as DLI by the hepatic artery with or without radiofrequency ablation therapy [30]. A delayed immunologic metastases reducing effect on the tumor in patients with breast cancer, RCC, and ovarian cancer has been reported 6 months after SCT [8,10,14,15].

In this study according to RECIST, no statistical significance was found between the ratio of I and A-I cytokines as correlated with the tumor load. In contrast, according to our local method, we show, to our knowledge for the first time, a correlation between GVT effect and free cytokines in serum. Tumor R was found to correlate with I cytokine levels in 5/7 of occasions, which was statistically significant, if compared with 1/12 of occasions with predominating A-I cytokines.

The RECIST criteria might not be applicable to our patients. It is appropriate for solid tumor patients, where the main emphasis is on the effect of chemoradiotherapy with necrotic cells as a result of the treatment. In allogeneic SCT, however, the transplantation is applied as an immunotherapy. We, therefore, are more interested in immunologic effects, which are delayed in comparison with other traditional anticancer treatments. The patient’s new immune system develops slowly allowing late R of metastases [31].

There are both advantages and disadvantages with RECIST and our local method, respectively. One disadvantage of RECIST is the possibility to choose between the evaluation of target lesions or evaluation of both target and nontarget lesions (i.e., the best overall response). Another disadvantage with RECIST is the choice of the 5 largest metastases per organ (and 10 largest in total), which implies that smaller metastases in other organs are missed. Therefore, in some patients, the evaluation of the largest target lesions may occur only in one organ totally passing the changes of other metastases (e.g., patients C4 and C7; Table 2a). In contrast, our local method includes 4 main metastatic localizations independent of their initial size. Furthermore, an appearance of a new metastasis independently of the organ localization will be evaluated as PD using RECIST, despite the R of other metastases in the same or other organs (e.g., patient R2). Our evaluation method mirrors the dynamics of the metastases over time because it compares the changes with the previous CT examination instead of with the pretransplantation one as according to RECIST (e.g., patient R7).

Secretion of TNF-α and IFN-γ from the recipient’s activated T and natural killer cells occurs as early as during conditioning before SCT [20,22]. Donor T cells of the graft, activated by antigen-presenting cells of the host, will augment this I cytokine production [32]. Upon donor T-cell activation, HLA antigen classes I and II or minor histocompatibility antigen differences stimulate CD8+ and CD4+ T cells creating the platform for GVHD. These T-cell populations also mediate the graft-versus-leukemia/GVT effect with or without association to HLA antigen, although the association to minor histocompatibility antigen may be stronger [33,34]. In mouse and human studies on GVHD and GVT, cytokine (TNF-α, IL-1) mediated toxicity and antitumor effect, respectively,
were associated with host antigen-presenting cells alone, without requiring alloantigen presentation on host target cells [34,35]. Visentainer et al. [36] reported higher levels of TNF-α in patients with leukemia and aGVHD than those without, 2 weeks and 2 months after SCT. Similarly, increased amounts of serum cytokines TNF-α and IFN-γ were found in 3 of the 6 patients in association with conditioning and aGVHD early after SCT (patients R2, R7, R13).

Elevated TNF-α and IFN-γ levels found in this study toward the end of the first year after SCT may contribute to an antitumor effect and are in line with observations in animals and human beings. Mice deficient in IFN-γ and IFN-γ receptor, or cytokines IL-13 and IL-23, which stimulate IFN-γ production, are more sensitive to carcinogens and show enhanced tumor development as compared with normal mice [37]. Double-knockout mice lacking T and B cells, and a transcription factor required for IFN-γ signaling, spontaneously develop adenocarcinomas of the colon, breast, and lung. IFN-γ may contribute to an antitumor effect, as it can up-regulate major histocompatibility complex I expression in the tumor, catalyzing an immune response [37]. Thus, an increased tumor development mediated by IFN-γ deficiency may occur because of diminished control of target cell growth and apoptosis. An increased TNF-α production has been found in association to successful chemotherapy of patients with colorectal cancer [38]. In addition, DLI containing natural killer and T cells may contribute to an antitumor effect by enhanced IFN-γ production as was seen in some of our patients who showed R of metastases after DLI.

Elevated levels of TGF-β1 protein in serum and TGF-β1 messenger RNA in tumor tissue of colorectal cancer were correlated with disease progression [39]. In addition, increased levels of TGF-β1 in serum, in combination with a reduced amount of circulating dendritic cells, were found in patients with colorectal cancer [40]. This might mirror decreased antigen presentation and immune activation. Patients with breast, lung, prostate, ovarian, colon, and hepatocellular carcinoma have been shown to have elevated TGF-β1 levels in plasma in connection with loss of response to TGF-β1 as a growth inhibitor [41]. Several in vivo models of breast and prostate cancer have demonstrated a connection between up-regulated TGF-β1 expression and enhanced tumorigenicity, increased tissue invasion, and drug resistance. Secretion of TGF-β1 from malignant cells suppresses immune responses against the tumor growth and enhances angiogenesis by signaling to nontransformed stroma cells in the tumor [41]. Decreased serum levels of TGF-β1 have been found in patients with leukemia in association with aGVHD after engraftment of hematopoietic SCT. Maintaining this immunologic status would be in line with the allogeneic antileukemia effect. Serum TGF-β1 levels were accordingly low in our patients early after conditioning and during aGVHD. In contrast, patients whose tumor load continued to increase had increased TGF-β1 levels.

Another immunosuppressive cytokine, IL-10, reduces the antigen-presenting capacity of antigen-presenting cells and is present in large amounts in tumor biopsy specimens from patients with ovarian cancer [42]. IL-10 inhibits IFN-γ synthesis, production of TNF-α, and IL-1, and type 1 T cells differentiation in the mouse model. Furthermore, this cytokine suppresses T-cell mediated lysis by down-regulating major histocompatibility complex class I expression on tumor cells [43]. The levels of IL-10 have shown to increase up to 4 months after SCT in patients with leukemia who develop aGVHD [44]. This may reflect an attempt by the immune system to control the immune reaction.

In this article, we describe the mass effect of cytokines by calculating a ratio between I and A-I cytokines. This ratio mirrors the immune response against tumor growth mediated by various activation levels of types I and 2 T-cell populations, and is in line with the same mechanism suggested for GVHD by Visentainer et al. [36]. One should take into account the kinetics of these cytokines over time (i.e., the ratio measured at each time point) would be expected to predict the developing immune response to allogeneic SCT, which would be measured some weeks or months later using CT assay. Serial cytokine analyses are recommended to guide the early tapering of immune suppressive therapy. Later this monitoring might be useful to guide infusion of donor lymphocytes or adoptively transferred tumor specific T or natural killer cells of stem cell donor origin to intensify the antitumor effect after allogeneic SCT against solid cancer.

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