ORIGINAL ARTICLE

Skin CD30$^+$ T cells and circulating levels of soluble CD30 are increased in patients with graft versus host disease

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Abstract

Objective To determine serum soluble CD30 (sCD30) levels in patients with graft versus host disease (GVHD).

Methods Serum soluble CD30 levels and IgE levels were assayed by a sensitive ELISA in 57 patients with bone marrow transplantation, and in 44 healthy controls. We analyzed the type of effector T cells in patients with GVHD.

Results Serum levels of sCD30 and serum IgE levels were significantly higher (*p* values <0.05) in patients with acute and chronic GVHD than in healthy controls. We found that CD30$^+$ T-cells are present in the skin of patients with GVHD.

Conclusion These results suggest that serum sCD30 levels may be helpful for the management of patients with bone marrow transplantation.

Keywords CD30 · Cytokines · Graft versus host disease · Autoimmunity · Systemic sclerosis

Introduction

Clinical and experimental evidence highlighted that clinical manifestations for patients with chronic graft versus host disease (GVHD) overlap those for systemic sclerosis (SSc), including skin sclerosis and myofibroblast accumulation [1–5]. The sclerodermatous GVHD model is generated by transferring splenocytes into irradiated or immunodeficient mouse strains allogenic at multiple histocompatibility loci [6–10].

It has been demonstrated that CD30 is expressed in the skin of patients with systemic sclerosis and that serum levels of soluble CD30 (sCD30) are increased in patients with systemic sclerosis [11, 12]. Thus, we investigated the levels of soluble CD30 and IgE in patients that underwent bone marrow transplantation (BMT) and in healthy controls, as well as we analyzed the type of effector T cells. We found that both CD30$^+$ T-cells are present in the skin of patients with GVHD and serum levels of sCD30 and IgE are significantly increased in patients with acute and chronic GVHD.

Materials and methods

Patients

Fifty-seven BMT recipients (31 male and 26 female, average age 33 ± 11 years, range 17–58), after informed consent, were studied before and after transplantation (usually for 6 months). In 45 patients allogenic BMT was provided by HLA-A, -B, -C, and -D histocompatible siblings or matched unrelated donors, whereas 12 patients received autologous BMT. Forty-one patients were transplanted for acute leukemia, eight for lymphoma, four for chronic myelogenous leukemia, and four for aplastic anemia. Patients receiving BMT for leukemic disorders were conditioned with cytosine arabinoside, cyclophosphamide, and total body irradiation [13]. GVHD prophylaxis regimen included methotrexate (four doses) and/or CsA

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administered for 6 months (unless toxicity occurred). In the course of this study, 4 out of the 45 allogenic BMT and 3 out of the 12 autologous BMT recipients underwent relapse of their original disease. Twenty-nine allogenic BMT and 6 autologous BMT recipients suffered from one or more infection episodes of viral, bacterial or fungal origin. None of the 12 autologous BMT recipients underwent grade I or greater acute GVHD, whereas among the 45 allogenic BMT recipients, 23 had no acute GVHD (21 grade zero and 2 grade I), 12 had grade II, 6 grade III and 4 grade IV acute GVHD, which was diagnosed and scored according to standard criteria [14]. In 10 of these latter patients acute GVHD was followed by mild chronic GVHD. Other 5 allogenic BMT recipients developed only mild chronic GVHD. Patients developing grade 2 or greater acute GVHD were treated with prednisone and/or CsA. Six of the 22 patients who had acute GVHD gave their informed consent to a second biopsy of their skin lesions for immunohistochemistry and cell cultures. Serum samples for the measurement of sCD30 (CD30 ELISA, DAKO, Glostrup, Denmark) and total IgE (CAP System IgE FEIA, Pharmacia Diagnostics, Uppsala, Sweden) were obtained from all patients before and after BMT (usually twice per month). Serum samples were also obtained at the onset of, and during, complications, such as infections, GVHD or relapse. Forty-four normal subjects provided control serum samples.

Reagents

Recombinant human IL-2 was kindly provided by Eurocetus (Milano, Italy), recombinant human IL-5 was purchased by Amgen Biologicals (Thousand Oaks, CA, USA). Phytohemagglutinin (PHA) was purchased from Gibco Laboratories (Grand Island, NY, USA) and phorbol 12-myristate 13-acetate (PMA) from Sigma (St. Louis, MO, USA). Staphylococcal enterotoxins SEA, SEB, SED and SEE were purchased from Serva (Heidelberg, Germany). Anti-CD3 monoclonal antibody (mAb) was purchased from Ortho Pharmaceuticals (Raritan, NJ, USA), anti-CD4 and anti-CD8 mAbs were purchased from Becton–Dickinson (Mountain View, CA, USA). Anti-CD30 (HRS-4 and BerH2) mAbs were purchased from Immunotech (Marseille, France) and DAKO (Glostrup, Denmark), respectively.

Cell cultures

The skin biopsies were obtained from six patients undergoing acute GVHD (grade II or III). Tissue fragments were cultured for 7–9 days in 10 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, 2×10^{-5} M 2-ME (complete medium), 5 % heat-inactivated human serum and human recombinant IL-2 (50 U/ml) in order to preferentially expand in vivo activated T cells. Biopsy specimens were then disrupted and the T cells obtained were cloned in U-round-bottomed microwell plates containing 10^5 irradiated allogenic peripheral blood mononuclear cells (PBMC) as feeder cells, PHA (0,5 % vol/vol) and IL-2 (20 U/ml), as reported elsewhere in detail [15]. Fresh peripheral (PB) lymphocytes of the same patients underwent a similar cloning protocol. The frequency of proliferating clones (clonal efficiency), determined according to the minimum χ^2 method reported by Taswell [16], ranged between 68 and 92 %. Cell surface marker analysis of T cell clones was then performed by FACSCalibur cytofluorimetric analysis.

T-cell blasts (5×10^4) from all skin-derived CD4$^+$ clones were screened in triplicates cultures for their responsiveness to staphylococcal enterotoxins SEA, SEB, SED and SEE (5 ng/ml), because they act as super-antigens, in the presence of 5×10^4 irradiated allogenic antigen presenting cells (APC) by measuring ^{3}H-TdR uptake after 60 h stimulation. Mitogenic index (ratio between mean cpm of stimulated and unstimulated cultures) greater that 5 was considered as positive.

Frozen PBMC obtained from patients four and six before their pre-BMT conditioning regimen were thawed and B cell-enriched suspensions were prepared by double rosetting with sheep erythrocytes. To obtain EBV-transformed lymphoblastoid B-cell lines (EBV-B cells), B cells were incubated for 48 h with supernatant of the EBV-producing marmoset cell line B95.8 and subsequently expanded in complete medium supplemented with 15 % FCS.

To assess their anti-host reactivity, all CD4$^+$ clones derived from the skin of patients four and six were extensively washed and T-cell blast (5×10^4) were cocultured for 60 h with 5×10^4 irradiated EBV B-cell lines derived from either themselves or the other BMT recipients (as control), and ^{3}H-TdR uptake was measured. Mitogenic index greater than five was considered as positive.

Characterization of the cytokine profile of T-cell clones

To induce cytokine production by T-cell clones, 10^6 T-cell blasts were stimulated for 36 h with PMA (10 ng/ml) plus anti-CD3 mAb (200 ng/ml) in 1 ml complete medium, as detailed elsewhere [16]. To assess antigen-induced cytokine production, T-cell blasts (5×10^5) of host-reactive clones derived from the skin of patients 4 and 6 were cocultured for 48 h in 0.5 ml with 5×10^5 irradiated autologous or control EBV B-cells, and cytokine production was measured in culture supernatants. Culture supernatants were assayed for IFN-γ, IL-4, and IL-5 content. The quantitative determination of IFN-γ, IL-4 and IL-5 was performed by

commercial assays (Biosource International Inc., Camarillo, CA, USA or Quantikine R & D Systems, Minneapolis, MN, USA respectively). For the measurement of IL-5, the murine LyH7.B13 cell line was also used as source of indicator cells, as detailed elsewhere [17]. Supernatants showing IFN-γ, but not IL-4 or IL-5, were categorized as Th1; clones able to produce IL-4 and/or IL-5, but not IFN-γ, were categorized as Th2, and clones producing both IFN-γ and IL-4 or IL-5, were categorized as Th0.

Immunohistochemistry

Staining was performed on 10-μm cryostat sections fixed in 4 % paraformaldehyde for 20 min. Sections were then exposed to a 0.3 % hydrogen peroxide-methanol solution to quench endogenous peroxidase activity. After a 20-min preincubation with normal horse serum, sections were layered for 30 min with anti-CD4 (5 μg/ml) or anti-CD30 (5 μg/ml), followed by biotinylated anti-mouse immunoglobulin Ab and the avidin–biotin-peroxidase complex (vectastain avidin–biotin complex kit, Vector Laboratories, Milan, Italy), as decribed [11, 18].

Diaminobenzidinetetrahydrochloride (Sigma Immunochemical, Milan, Italy) was used as a peroxidase substrate. Sections were then counterstained with Gill's hematoxylin and mounted with permount. As a negative control, primary mAbs were omitted or replaced with mouse ascites fluid. On average, 20 sections for each skin biopsy were analyzed.

Statistical analysis

For statistical analysis a multivariate analysis of variance (MANOVA) was used. Briefly, sCD30 and IgE levels in each serum sample were transformed to their ^{10}log value and complications were coded in the following dichotomous variables: $0 =$ absence versus $1 =$ presence of relapse, $0 =$ grade 0-I versus $1 =$ grade II-IV acute GVHD, $0 =$ absence versus $1 =$ presence of bacterial, viral or fungal infections. For multivariate analysis of the effect of different complication (i.e. the effect of each complication on post-BMT sCD30 and IgE levels corrected for simultaneous occurrence of other complications), the following statement is used: independent factors (sCD30 or IgE) by patients with relapse, acute GVHD, infection. By taking 'patients' as a factor in the MANOVA, a correction for general patient levels is carried out. Since changes within patients and not between patient groups were analyzed, only data from patients who suffered from a given complication during at least one post-BMT time point measured were included in the MANOVA. This analysis can be seen as an extension of the well-known paired t test. P values <0.05 were accepted as significant.

Results and discussion

Cytokine profile of T-cell clones derived from the skin of patients with acute GVHD

Since skin involvement was almost invariably present in patients with grade II or greater acute GVHD, lesional skin was considered an appropriate source of in vivo activated T cells during acute GVHD. Four patients with grade II (#2, 3, 5 and 6) and two with grade III (#1 and 4) acute GVHD were asked for their informed consent to an additional blood sampling and biopsy of lesional skin to make possible the comparative assessment of the cytokine profile of T cell clones derived from their circulating or skin-infiltrating T cells. Each skin biopsy specimen was splitted into two parts: one frozen for immunohistochemistry and the other for immediate cell culture, respectively. Tissue fragments were cultured for 7–9 days in IL-2 conditioned medium to preferentially expand in vivo activated skin-infiltrating T cells. Growing T-cells blasts and freshly prepared PB T-cells were then cloned in the presence of irradiated feeder cells, PHA and IL-2 according to a highly efficient cloning procedure successfully used to generate clones from tissue-infiltrating human T-cells [15]. As shown in Table 1, a total number of 226 CD4$^+$ T-cells clones obtained from the skin samples could be compared with 256 CD4$^+$ T-cells clones derived from the PB of the same six patients. The frequency of proliferating clones (clonal efficiency), as determined according to the minimum χ^2 method (16), was sufficiently high and ranged from 67 to 89 % and from 70 to 99 % for cloning of skin and PB T-cells, respectively. However, in view of their small number (totally nine) of CD8$^+$ T-cell clones derived from the corresponding PB were not further analyzed. Since the great majority of T-cell clones showed individual patterns of proliferative response to staphylococcal enterotoxins A, B, D and E in the presence of allogenic APC (indicating that they had expressed different TCR Vβ rearrangement), we concluded that the series of T-cell clones obtained were polyclonal in origin and were adequately representative of the skin-infiltrating or PB T-cells in each patient.

To characterize their cytokine secretion profile, all clones were stimulated for 36 h with PMA plus anti-CD3 mAb and cytokine (IFN-γ, IL-4 and IL-5) secretion was assessed in their culture supernatants. As expected, no detectable cytokine production was found in any unstimulated T-cell clones, irrespective of their origin. Upon stimulation, the majority of both skin-derived and PB CD4$^+$ clones showed combined production of Th1- and Th2-type cytokines (Th0 pattern). However, in the series of skin-derived T-cell clones, the proportion of clones with Th2-like profile was remarkably higher (29 versus 6 %)

Table 1 Cytokine secretion profile of T-cell clones derived from skin biopsies and peripheral blood (PB) of patients with acute GvHD

Patients and source of T cells	No. of CD4 clones obtained	Cytokine profile		
		Th1	Th0	Th2
1. Skin	37	2 (5)	25 (68)	10 (27)
PB	41	14 (34)	25 (61)	2 (5)
2. Skin	33	1 (3)	13 (39)	19 (58)
PB	49	18 (37)	27 (55)	4 (8)
3. Skin	42	2 (5)	35 (83)	5 (12)
PB	37	14 (38)	22 (59)	1 (3)
4. Skin	34	0 (–)	28 (82)	6 (18)
PB	52	21 (41)	29 (56)	2 (4)
5. Skin	36	0 (–)	31 (86)	5 (14)
PB	41	16 (39)	22 (54)	3 (7)
6. Skin	44	1 (2)	22 (50)	21 (48)
PB	36	12 (33)	21 (58)	3 (8)
All cases				
Skin	226	6 (3)	154	66 (29)
PB	256	95 (37)	146	15 (6)
χ^2		86.02	6.30	46.78
P		<0.0001	<0.02	<0.0001

T-cell blasts (10^6/ml) of each clone were stimulated for 36 h with PMA (10 ng/ml) plus anti-CD3 MoAb (200 ng/ml) and culture supernatants were assayed for IFN-γ, IL-4, and IL-5 content. T-cell clones able to produce IFN-γ, but not IL-4 or IL-5, were categorized as Th1; clones able to produce IL-4 and/or IL-5, but not IFN-γ, were categorized as Th2, and clones producing both IFN-γ and IL-4 or IL-5 were categorized as Th0

Percent values in parenthesis

than those found in the series of PB-derived clones (Table 1). Accordingly the proportion of skin-derived clones producing IFN-γ, but not IL-4 or IL-5 (Th1 pattern) was much lower (3 versus 37 %) than those found in the series of PB-derived clones (Table 1).

In the subsequent experiments, an attempt was made to assess whether the series of skin-derived T-cell clones included a proportion of anti-host alloreactive clones and whether stimulation with host alloantigen-bearing EBV-B cells resulted in cytokine secretion. This assay was possible in two GVHD patients only (#4 and 6), whose pre-BMT B-cells could be successfully transformed with EBV. When cocultured for 60 h with irradiated EBV B-cells from the appropriate host, 7 out of the 34 CD4$^+$ skin-derived clones of patient #6 showed significant proliferation and cytokine production (data not shown). All the 17 host-reactive clones confirmed their ability to produce IL-4, as they did in response to PMA plus anti-CD3, and 4 of them confirmed their inability to produce IFN-γ (Th2 profile). In the 13 Th0 clones, at equal number of T-cell blasts in culture, the levels of antigen-induced IL-4 production were similar to those obtained in response to mitogen stimulation,

whereas in 9 clones IFN-γ production was 12–68 % lower (data not shown).

In concomitant experiments, T-cell blasts of alloantigen-stimulated clones were assessed for co-expression of CD4 and CD30 molecules. As expected in view of their IL-4 production, all clones showed CD30 expression, which was higher in Th2 clones (53, 75, 88 and 90 %) and tended to correlate ($p = 0.05$) with the ratio between IL-4 and IFN-γ production.

CD30 expression in vivo by skin-infiltrating CD4$^+$ T-cells of patients with acute GVHD

To have an in vivo correlate with in vitro data, sequential sections of the frozen aliquots of lesional skin biopsies from the six GVHD patients were analyzed by immuno-histochemistry for CD3, CD4, and CD30 expression. In all specimens, most of infiltrating cells were CD3$^+$CD4$^+$ lymphocytes located in perivascular infiltrates of different size, as well as scattered through the dermis (data not shown). In the skin of all GVHD patients, remarkable numbers of CD4$^+$ cells stained positive for CD30 (range 45–78 %). High numbers of CD4$^+$CD30$^+$ cells were also found in the skin of systemic sclerosis patients (59–87 %), whereas these cells were rare (<10 %) in the skin of contact dermatitis patients.

Increase of individual sCD30 and IgE serum levels in acute GVHD episodes

Since sCD30 is the proteolytically cleaved 88kD soluble form of CD30, which is released from the membrane of CD30$^+$ cells activated either in vitro or in vivo, [11], and since both CD30 expression and IgE antibody production are two IL-4-dependent phenomena [11], serum levels of both sCD30 and IgE were sequentially measured in the course of 4 months in each of the 57 BMT recipients enrolled in this study.

The multivariate analysis of variance (MANOVA) was applied to assess the individual effect of either BMT or the various post-BMT complications (i.e. relapse, infection episodes and GVHD) on the serum levels of sCD30 and IgE in single patient. In the 12 autologous BMT recipients, mean (±SD) pre-BMT levels of sCD30 and IgE (12.6 ± 1.8 U/ml and 11.5 ± 3.5 kU/l) were not significantly different from those found in post-BMT samples (9.1 ± 1.5 U/ml and 12.0 ± 2.1 kU/l). Nor substantial changes were found in pre-versus post-BMT sCD30 and IgE serum levels of the 45 allogenic BMT recipients (8.9 ± 1.7 U/ml and 19.1 ± 3.4 ku/l versus 8.1 ± 1.8 U/ml and 16.6 ± 2.5 kU/l, respectively). Likewise, relapse of the original disease (which occurred in seven patients) was

Table 2 Increase of sCD30 and IgE serum levels in acute and chronic GVHD, but not in infection episodes in BMT patients

Type of complication	No. of samples tested	Mean (±SD) serum levels	
		sCD30 (U/ml)	IgE (kD/l)
Infection			
+*	49	21.0 ± 3.5	27.2 ± 4.8
−	192	13.9 ± 2.8	23.0 ± 3.9
		($p = 0.310$)	($p = 0.184$)
Grade II-IV acute GVHD			
+	42	41.1 ± 2.6	59.0 ± 4.4
−	199	14.6 ± 2.5	24.3 ± 3.5
		($p < 0.001$)	($p < 0.001$)
Chronic GVHD			
+	32	33.2 ± 2.6	53.3 ± 4.4
−	20	13.5 ± 2.9	21.6 ± 3.9
		($p < 0.001$)	($p = 0.002$)

A multivariate analysis of variance (MANOVA) was used to assess the effect of each complication on post-BMT sCD30 and IgE levels corrected for simultaneous occurrence of other complications. By taking 'patients' as a factor, a correction for general patient levels is carried out. Since changes within patients and not between patient groups were analyzed, only data from patients who suffered from a given complication during at least one post-BMT time point measured were included in the MANOVA. P values <0.05 were accepted as significant

* +/− Indicates the presence or absence of the clinical complication at the thime of sampling

not associated with significant changes of sCD30 and IgE ($p = 0.910$ and $p = 0.783$, respectively).

In the course of this study, 35 BMT recipients suffered from one or more infection episodes of viral, bacterial or fungal origin. When the 49 serum samples obtained during the various infections episodes were compared for their sCD30 and IgE levels with the 192 samples obtained in infection-free periods, no major differences were found (Table 2). Interestingly, the lack of correlation between infection and elevation of sCD30 and/or IgE was also observed in autologous BMT recipients, where none developed GVHD. In contrast, in the 42 samples obtained from 22 allogenic BMT recipients at the onset and/or during episodes of grade II-IV acute GVHD, both sCD30 and IgE levels were significantly higher ($p < 0.001$) than those measured in the 199 samples obtained in acute GVHD-periods (Table 2). Likewise, in the 32 samples obtained from 15 patients during their chronic GVHD, both sCD30 and IgE levels were significantly higher ($p < 0.001$ and $p < 0.002$, respectively) than those found in the 209 samples obtained in GVHD-free periods (Table 2). A remarkable increase of sCD30 and IgE serum levels (mean fold ± SD: 12.6 ± 7 and 19.8 ± 9, respectively) at the time of, or immediately before, the onset of acute GVHD was detectable in 18 out of 22 patients,

whereas the other 4 patients (18 %) with acute GVHD showed poor (less than threefold) or no increase with respect to their pre-BMT sCD30 and IgE levels (9.3 ± 3 and 20.2 ± 12 mean fold increase, respectively). On the other hand, 2 out of the 18 patients (11 %), who had neither acute or not chronic GVHD, showed transient increase of their sCD30 and IgE (6–8 fold and 4–20 fold, respectively) during this study, whereas the other 16 GVHD-free patients had only negligible fluctuations of these parameters (1.7 and 2.4 mean fold increases, respectively).

In conclusion, multivariate analysis of data from serial blood samples revealed that, when corrected for individual patients' levels, increased sCD30 and IgE levels were significantly associated with either grade II-IV acute or chronic GVHD, but not with other complications, such as infection episodes or relapse, which occur in BMT patients.

Overall, the results obtained so far indicated that both CD30+ T-cells are present in the skin of patients with GVHD and serum sCD30 and IgE are significantly elevated in patients with acute episodes of GVHD, thus suggesting the usefulness of measuring those serums sCD30 and IgE for the management of patients undergoing bone marrow transplantation.

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