Plenary paper

Selective elimination of leukemic CD34$^+$ progenitor cells by cytotoxic T lymphocytes specific for WT1

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Hematologic malignancies such as acute and chronic myeloid leukemia are characterized by the malignant transformation of immature CD34$^+$ progenitor cells. Transformation is associated with elevated expression of the Wilm's tumor gene encoded transcription factor (WT1). Here we demonstrate that WT1 can serve as a target for cytotoxic T lymphocytes (CTL) with exquisite specificity for leukemic progenitor cells. HLA-A0201–restricted CTL specific for WT1 kill leukemia cell lines and inhibit colony formation by transformed CD34$^+$ progenitor cells isolated from patients with chronic myeloid leukemia (CML), whereas colony formation by normal CD34$^+$ progenitor cells is unaffected. Thus, the tissue-specific transcription factor WT1 is an ideal target for CTL-mediated purging of leukemic progenitor cells in vitro and for antigen-specific therapy of leukemia and other WT1-expressing malignancies in vivo. (Blood. 2000;95:2198-2203)

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Introduction

Cells of the hematopoietic system are derived from hematopoietic stem cells (HSC) capable of self-renewal and differentiation. Transplantation experiments in humans and mice have shown that CD34$^+$ cell populations contain HSC capable of reconstituting the erythroid, myeloid, and lymphoid lineages in myeloablated recipients.[1] In addition, HSC capable of reconstituting murine hosts were recently demonstrated in a rare population of CD34$^-$/lin$^-$ bone marrow cells.[2]

There is strong evidence that the critical transformation events in CML and acute myeloid leukemia (AML) affect immature CD34$^+$ progenitor cells. Because the majority of leukemic blast cells have limited proliferative capacity, the malignant disease must be maintained by a subpopulation of leukemic progenitor cells with extensive proliferative and self-renewal capacities.[3,4] Transplantation studies with purified cells from patients with AML showed that only immature CD34$^+$ cells were capable of initiating leukemia in immunocompromised murine recipients.[5] Similarly, purified CD34$^+$ cells from patients with CML efficiently initiated leukemia in murine recipients.[6,7]

The molecular events leading to uncontrolled progenitor cell proliferation are not fully understood. Although BCR/ABL fusion proteins associated with the t(9;22) chromosomal translocation is the hallmark of CML, BCR/ABL transcripts can also be found in healthy persons, indicating that additional factors are required for leukemia to develop.[8] The Wilm's tumor gene (WT1) transcription factor is a candidate protein contributing to leukemogenesis. This transcription factor is normally expressed in immature CD34$^+$ progenitor cells, and differentiation is associated with WT1 down-regulation.[9,10] Elevated levels of WT1 expression have been observed in unseparated mononuclear cells and in purified CD34$^+$ cells from patients with AML and CML.[11,12] In vitro studies showing that increased WT1 expression can block normal differentiation and enhance proliferation of hematopoietic progenitor cells provide an explanation for the potential of WT1 to contribute to leukemogenesis.[13,14]

The results of a recent study suggested that T lymphocytes specific for CD34$^+$ progenitor cells are critically important in mediating antileukemic effects in patients with CML.[15] However, there is no information concerning the nature of T-cell recognized target antigens expressed by CD34$^+$ cells. In this study we explored the possibility of exploiting WT1 as a target molecule to direct cytotoxic T lymphocytes against leukemic progenitor cells. We tested the hypothesis that CML, but not normal CD34$^+$ progenitor cells, express sufficient WT1 protein to trigger a cytotoxic T lymphocyte (CTL) attack.

Materials and methods

Cell lines

The K562 cell line was established from the pleural effusion of a female patient with CML in blast crisis.[16] The BV173 cell line was established from the peripheral blood of a male patient with CML in blast crisis.[17] The cell line 697 was established from the bone marrow of a 12-year-old boy with acute lymphoblastic leukemia.[18] The C1R cell line is a HLA-A0201–negative Epstein-Barr virus (EBV) transformed lymphoblastoid cell line.[19] The T2 cell line has been selected for loss of the genes encoding TAP (transporter associated with antigen processing), resulting in inefficient loading of human leukocyte antigen (HLA) class I molecules with endogenous peptides.[20] As a consequence, the HLA-A0201 molecules of T2 cells can be efficiently loaded with exogenous peptides. *Drosophila* cells transfected with HLA-A0201, human β-2 microglobulin, B7.1, and ICAM-1 were a kind gift from Dr M. Jackson.

Synthetic peptides. A peptide derived from human Wilms tumor antigen 1 P126 (RMFPNAPYL) and a control HLA-A02 01-binding peptide derived from the E7 protein of human papillomavirus type 16 were

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synthesized by the central peptide synthesis laboratory of the Imperial College Medical School using fluorenylmethoxycarbonyl chemistry. The quality of the peptides was assessed by high-performance liquid chromatography analysis, and the expected molecular weight was observed using matrix-assisted laser desorption mass spectrometry. The peptides were dissolved in phosphate-buffered saline (PBS; pH 7.4) to give a concentration of 2 mmol/L and were stored at $-20°C$.

Generation of allo–HLA–restricted CTL lines. Peripheral blood mononuclear cells (PBMC) were separated from buffy coat packs using Ficoll gradient centrifugation and were stained with monoclonal antibodies HB54 (anti–HLA-A2, B17) and HB117 (anti–HLA-A2, A28). A2-negative PBMCs were used as responders. Peptide-coated *Drosophila* cells transfected with HLA-A0201, human β2-microglobulin, B7.1, and ICAM-1 were used as initial stimulators. *Drosophila* cells were induced in 100 mmol/L $CuSO_4$ for 48 hours, washed 3 times with medium, and loaded with peptide at a concentration of 100 μmol/L for 4 hours. Each well of a 24-well plate received 2×10^6 responder PBMC and 2×10^5 stimulator cells in 2 mL T-cell medium. On day 5, T cells were harvested and plated in fresh T-cell medium at a density of 5×10^5 per well, with the addition of 2×10^6 autologous irradiated PBMC as feeders, 2×10^5 irradiated peptide-coated T2 cells, 10% QS4120 culture supernatant (containing anti-CD4 antibodies), and 10 U/ml hu-rIL-2 (Roche Diagnostics, Lewes, UK). The cultures were restimulated weekly using T2 cells coated with the immunizing peptide as stimulators. After 2 to 3 cycles of stimulation, the bulk cultures were cloned in 96-well plates at densities of 1, 10, and 30 cells per well; 10^4 peptide-coated T2 cells, 2×10^5 HLA-A2 negative PBMC feeders, and 2 U/mL IL-2 were added to each well. The cytotoxicity of each well was tested against T2 target cells coated with the immunizing peptide or a control HLA-A0201–binding peptide. Peptide-specific microcultures were expanded and restimulated weekly in 24-well plates by adding 2×10^6 feeders, 2×10^5 stimulator cells, and 10 U/mL IL-2. The T-cell line 77 (Figure 1B) was maintained for more than 1 year in culture and served as a source of CTL for most experiments in this study. Because this line consisted of CD4+ and CD8+ T cells, CD8+ subclones were used to show that the specific killing activity was mediated by CD8+ CTL. Unlike the parental 77 line, the in vitro lifespan of CD8+ subclones was limited to a few months.

CTL assays. Cytotoxic T lymphocyte assays were performed as described. Briefly, 10^6 T2 cells were incubated for 1 hour in 200 μL assay medium (RPMI 1640 with 5% heat inactivated fetal calf serum) with 100 μmol/L synthetic peptide at 37°C. Peptide-coated T2 cells or tumor cells were labeled with chromium 51 for 1 hour, washed, and added to serial 2-fold dilutions of effector cells in round-bottom, 96-well plates to obtain a total volume of 200 μL/well. Assay plates were incubated for 4 hours at 37°C, 5% CO_2, and 100 μL supernatant was harvested and counted using a Wallac Gamma Counter, Wallac, Milton Keynes, UK. The specific lysis was calculated by the equation (experimental release − spontaneous release)/ maximum release − spontaneous release) × 100%.

Purification of hematopoietic CD34+ cells. As a source of normal CD34+ cells, we used human bone marrow from adult healthy donors (n = 5), leukapheresis products of stem cells mobilized from solid-tumor patients in disease remission (n = 2), and cord blood (n = 1). Samples of cord blood were obtained from discarded placental and umbilical tissues by drainage of the blood into sterile collection tubes. Informed consent for use of these cells was obtained from donors or parents as appropriate. As a source of leukemic CD34+ cells, peripheral blood was obtained from patients who had CML in chronic phase and who had not been treated with interferon in at least 3 months.

Samples were diluted 1:2 in Hanks balanced salt solution and enriched for mononuclear cells by density-gradient centrifugation (Lymphoprep 1.077 g/mL; Nycromed Pharma AS, Oslo, Norway), and the recovered mononuclear fraction was subject to magnetic microbead selection for the isolation of CD34+ fraction using the Minimacs system and following the manufacturer's instructions (Miltenyi Biotec, Bisley, UK). The purity of the cell population ranged from 80% to 95% as estimated by FACS analysis using an antihuman CD34 phycoerythrin (PE) mouse monoclonal antibody (Becton Dickinson, Cowley, UK).
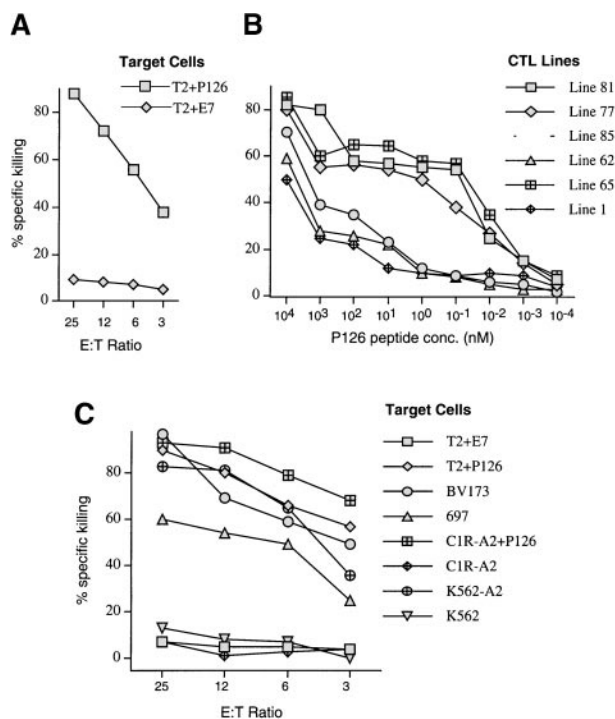

Figure 1. Specificity of allo-restricted CTL generated against the WT1-derived peptide P126. CTL were isolated by limiting dilution cloning of T-lymphocyte bulk cultures from HLA-A0201− donors stimulated with HLA-A0201+ stimulator cells coated with P126 peptide. (A) Isolated CTL lines killed the TAP-deficient T2 target cells coated with the immunizing P126 peptide but not T2 cells coated with the HLA-A0201–binding E7 control peptide. (B) Peptide titration experiments showing that 3 anti-P126 CTL lines were of high avidity recognizing low picomolar concentrations of P126, and that 3 CTL lines were of low avidity because nanomolar P126 concentrations were required for target cell recognition. T2 cells coated with the indicated concentrations of P126 were used as CTL targets. High-avidity CTL were used for all subsequent experiments because low-avidity CTL did not recognize target cells expressing WT1 endogenously. (C) High-avidity CTL killed the HLA-A0201+ leukemic cell lines BV173, 697 but not the HLA-A0201+, EBV-transformed B-lymphoid cells C1R-A2. Coating of C1R-A2 with P126 resulted in efficient CTL killing. The HLA-A0201− leukemia cell line K562 was not killed by the CTL unless transfected with the HLA-A0201 gene.

RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR). Total RNA of 10^6 cells was isolated according to RNAzol B protocol (AMS Bio, Witney, UK). cDNA synthesis of whole RNA pellet was performed in a 40μL reaction. The dissolved RNA pellet was first incubated with 2 μg oligo-dT 12-18 primer (Life Technologies, Paisley, UK) at 65°C for 10 minutes, followed by a 1-hour incubation at 42°C with a mixture of 50 U murine leukemia virus reverse transcriptase, 10 mmol/L dithiothreitol, 1 mmol/L dNTP (Roche Diagnostics), and 40 U RNase inhibitor (Promega, Southampton, UK). Five microliters cDNA preparation was used for polymerase chain reaction (PCR) amplification in a 50-μL volume of final reaction mixture containing 2.5 U Taq DNA polymerase (Qiagen, Crawley, UK), 1 mmol/L dNTP, and 20 OD/mL primer.

Amplification of the human WT1 coding region was achieved using sense primers located in exon 7 (21 mer 5'-ggc atc tga gac cag tga gaa-3') and antisense primers in exon 10 (22 mer 5'-gag agt cag act tga aag cag t-3'). The expected size for the WT1 PCR product was 482 bp. RNA integrity was verified by amplifying the human c-ABL gene in every sample using intron-spanning primers: 22 mer sense 5'-ccc aac ctt ttc gtt gca ctg t-3'; 22 mer antisense 5'-cgg ctc tcg gag gag acg atg a-3'. The expected size of the c-ABL PCR product was 385 bp. Hot-start PCR was performed for 35 cycles with a thermal cycler (Techne Genius, Cambridge, UK) under the following conditions (same for ABL and WT1 amplification): denaturing at 95°C for 1 minute, primer annealing at 56°C for 1 minute, and chain elongation at 72°C for 2 minutes. The cycling was initiated by a 5-minute denaturation step at 95°C to heat inactivate the reverse transcriptase, and it was terminated by a 10-minute final extension

at 72°C. All reverse transcription (RT)-PCRs were performed at least twice, and negative control (no cDNA) and positive control (cDNA from the WT1-expressing leukemic cell line BV173) were included in every experiment. PCR products were electrophoresed through 1.5% agarose gels.

Western blot analysis. Separated CD34$^+$ cells (2×10^5 cells) were washed in PBS and lysed in Laemmli buffer. The cell lysate was fractionated by a 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, Little Chalfont, UK) by wet transfer. The membrane was then blocked in PBS containing 0.01% Tween 20 and 5% nonfat dry milk for 1 hour at room temperature and incubated first with rabbit antihuman WT-1 C19 polyclonal antibody (1:200 in blocker; Santa Cruz Biotech, Santa Cruz, CA) overnight at 4°C and then with rabbit anti-actin polyclonal serum (1:500 in blocker; Sigma, Gillingham, UK) for 30 minutes at room temperature. The signal was revealed by incubating the membrane with horseradish peroxidase-conjugated swine antirabbit antibody (1:1000; DAKO, Cambridge, UK) and ECL reaction (Amersham) according to the manufacturer's instructions.

Progenitor (CFU) assays. Colony-forming unit (CFU) assays were performed by plating 1000 to 3000 CD34$^+$ cells in methylcellulose medium supplemented with the following recombinant human growth factors (Stem Cell Technologies, Northampton, UK): stem cell factor (50 ng/mL), IL-3 (20 ng/mL), IL-6 (20 ng/mL), granulocyte macrophage colony-stimulating factor (20 ng/mL), and granulocyte colony-stimulating factor (20 ng/mL). The cultures were incubated for 14 days at 37°C in humidified atmosphere at 5% CO_2 to allow the development of granulocyte macrophage colony-forming units.

Results

Generation of WT1-specific CTL

Expression of the WT1 transcription factor in adults is detectable in renal podocytes, testicular Sertoli cells, ovarian granulosa cells, and CD34$^+$ bone marrow cells.[21] To avoid possible immunologic tolerance to WT1, we used a previously described approach of generating peptide-specific CTL from major histocompatibility complex-mismatched donors. This approach is suitable for generating CTL against any protein overexpressed in tumor cells, independent of immunologic tolerance.[22,23] A 9-amino acid-long WT1-derived peptide epitope, P126 (RMFPNAPYL), was selected as the CTL target because it bound to HLA-A0201 class I molecules, the most frequent class I allele found in white populations (not shown). Responder lymphocytes from HLA-A0201$^-$ donors were cultured in vitro with HLA-A0201$^+$ stimulator cells presenting the P126 peptide, and limiting dilution cultures were used to isolate peptide-specific CTL lines. Experiments with peptide-coated T2 target cells showed that the CTL were highly specific for the P126 peptide (Figure 1A). Peptide titration indicated that the CTL could be divided into high-avidity lines capable of recognizing low picomolar peptide concentrations and low-avidity lines recognizing low nanomolar peptide concentrations (Figure 1B). High-avidity CTL lines were selected for further experiments.

WT1-specific CTL kill leukemia cell lines

Analysis of a panel of leukemia cell lines revealed that P126-specific CTL killed the HLA-A0201$^+$ cells BV173 and 697 (Figure 1C). The HLA-A0201$^-$ leukemia cell line K562 was only killed after transfection with HLA-A0201. In contrast, the HLA-A0201$^+$ EBV-transformed B cell line C1R-A2 was not killed unless cells were coated with P126 peptides (Figure 1C; similar results were seen with other EBV-transformed cells). The expression of WT1 in the CTL target cells was analyzed at the RNA and protein levels. RT-PCR demonstrated that the leukemia cell lines, but not the EBV-transformed C1R-A2 cells, expressed WT1 RNA (Figure

2A). Similar results were obtained by Western blot analysis showing that WT1 protein was only expressed in leukemia cells but not in C1R-A2 cells (Figure 2C). Together the data indicated that the CTL recognized A0201$^+$ leukemia cell lines and that CTL killing correlated with WT1 expression.

WT1-specific CTL kill fresh leukemic CD34$^+$ cells

PBMCs from patients with chronic-phase CML were separated into immature CD34$^+$ and mature CD34$^-$ populations. As expected, cells isolated from HLA-A0201$^-$ patients were not recognized by P126-specific CTL (Figure 3A). When cells from HLA-A0201$^+$ patients with CML were analyzed, the CTL selectively recognized the CD34$^+$ cell population, whereas no killing of the more mature CD34$^-$ population was observed (Figure 3A). Cold target competition experiments showed that the killing of CD34$^+$ CML cells was inhibited by the leukemia cell line BV173 but not by EBV-transformed C1R-A2 cells (Figure 3B). This indicated that CD34$^+$ cells from patients with CML and BV173 cells share the CTL-recognized target antigen and that this antigen is absent in C1R-A2 cells.

Lack of killing of CD34$^-$ cells most likely resulted from the insufficient expression of the WT1-derived target peptide because the coating of these cells with exogenous P126 peptide resulted in CTL killing (Figure 3A). RT-PCR analysis revealed strong WT1 RNA expression in CD34$^+$ cells and low expression in CD34$^-$ cells (Figure 2B). WT1 protein expression detectable by Western
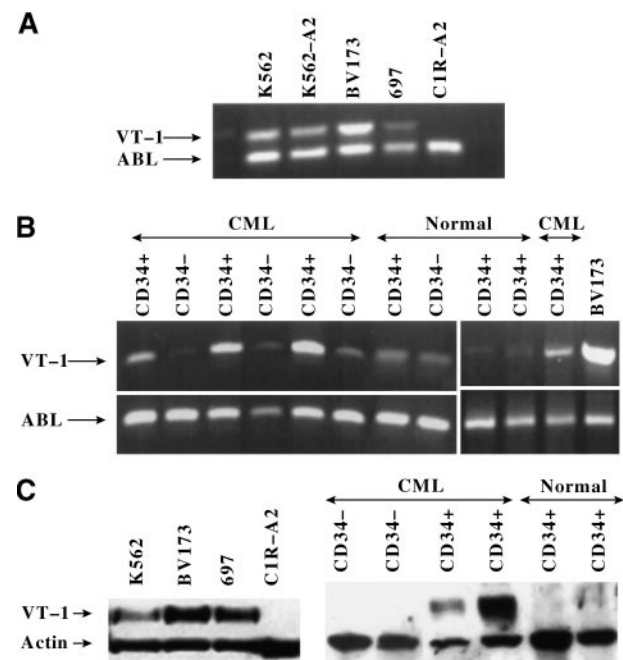

Figure 2. WT1 RNA and protein expression in leukemic cell lines and in CD34$^+$ and CD34$^-$ cell populations freshly isolated from patients with leukemia and normal donors. (A) RT-PCR to measure WT1 RNA in leukemic cell lines and in the B-lymphoid cell line C1R-A2. The same cell lines were used as CTL targets in Figure 1C. The amplified WT1 product is 482 bp long. The RNA of the housekeeping ABL gene was amplified to indicate the amount of RNA in each sample. The ABL product is 385 bp long. (B) RT-PCR to measure WT1 RNA expression in purified CD34$^+$ and CD34$^-$ cell populations from 4 patients with CML and 3 normal donors. The leukemic cell line BV173 served as a positive control for WT1 expression. Similar results were obtained with samples from 6 additional patients with CML. (C) Western blotting to measure WT1 protein expression in leukemia cell lines and in purified CD34$^+$ and CD34$^-$ cell populations from 2 patients with CML and 2 normal donors. The expression of the housekeeping actin protein was used as an indicator to control for the amount of protein in each sample. The WT1 protein measures approximately 54 kd and the actin protein approximately 42 kd.
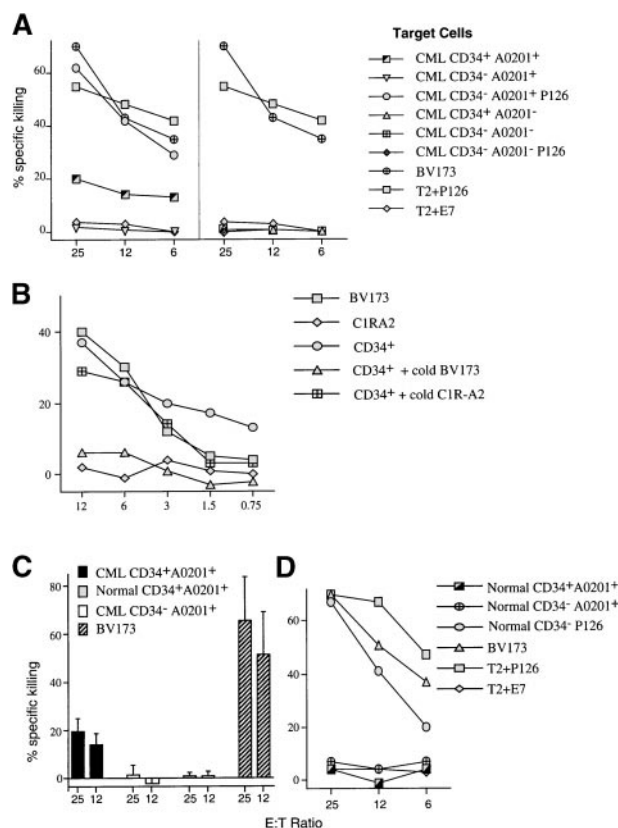
Figure 3. Analysis of CTL-mediated killing of CD34+ cell populations purified from patients with leukemia and normal donors. (A) Representative experiment showing the level of killing by anti-P126 CTL against purified CD34+ and CD34− cell populations isolated from patients with CML who were either HLA-A0201+ or A0201−. CD34−/A0201+ cells were not recognized by the CTL unless coated with P126 peptide. The leukemic cell line BV173 and the TAP-deficient T2 cells coated with P126 or the control E7 peptide were used as positive and negative controls in all experiments. (B) Cold target competition experiment. Shown is the killing by anti-P126 CTL against chromium-labeled CD34+ targets from an A0201+ patient with CML in the absence or presence of a 30-fold excess of cold BV173 and C1R-A2 targets. The killing of chromium-labeled BV173 and C1R-A2 is shown for comparison. (C) Average of the level of specific CTL killing of purified CD34+ cells from 11 different HLA-A0201+ patients with CML and from 6 normal donors. The level of killing of CD34− cells purified from patients with CML and against the positive control cells BV173 is also shown. The figure shows the mean level and standard deviation of specific CTL killing. (D) Representative experiment showing the level of killing by anti-P126 CTL of purified CD34+ and CD34− cell populations isolated from HLA-A0201+ normal donors. No CTL killing was detectable unless target cells were coated with P126 peptide.

blotting was restricted to CD34+, and no protein was detectable in CD34− cells (Figure 2C). Both RT-PCR and Western analysis showed variation in the level of WT1 expression in leukemic CD34+ cell populations. Thus, we explored whether the observed variation in the level of WT1 expression resulted in a variation in the level of CTL killing. However, analysis of 11 different patients with CML showed that the CTL consistently lysed approximately 20% (SD, 5%) of the CD34+ population (Figure 3C). This result raised the possibility that WT1 expression was restricted to a subpopulation of approximately 20% of CD34+ cells and that the expression level in all 11 patients with CML was sufficient to render most of these cells susceptible to CTL killing. We explored whether the subpopulation recognized by CTL included the clonogenic progenitor cells that can give rise to colonies of the granulocyte, macrophage, and erythroid lineages. When CD34+ populations isolated from 9 patients with CML were treated with P126-specific CTL, this resulted in 80% to 100% inhibition of colony formation (Figure 4A). This indicated that the majority of

colony-forming progenitor cells were removed by P126-specific CTL. The "escape" colonies seen in CTL-treated samples were small when compared to the colonies in untreated samples. This is consistent with the possibility that the escape colonies were derived from progenitor cells that had already initiated differentiation toward the granulocyte/myeloid lineage associated with the down-regulation of WT1 expression. Such partially differentiated progenitors might escape recognition by WT1-specific CTL, and the small size of the colonies observed in the CFU assay might reflect the reduced clonal burst size of these progenitors.

Importantly, colony formation by CD34+ cells from HLA-A0201− patients with CML was unaffected, indicating that the elimination of progenitors with colony-forming activity was dependent on HLA-restricted antigen recognition by the CTL. Furthermore, the elimination of colonies of the granulocyte, macrophage, and erythroid lineages in CD34+ cells from HLA-A0201+ patients with CML was dependent on high-avidity recognition of the P126 peptide. Only high-avidity CTL eliminated the progenitors of CFU-GM and BFU-E, whereas low-avidity CTL had no effect (Figure 4B).

Finally, we explored whether high-avidity CTL discriminated between leukemic and normal CD34+ progenitor cells. Normal CD34+ cells were isolated from bone marrow, peripheral blood, or umbilical cord blood of HLA-A0201+ donors and were used as CTL targets. Independent of the source of CD34+ cells, anti-P126 CTL did not inhibit colony formation by these cells (Figure 4A). Furthermore, normal CD34+ cells were not killed when used as targets in cytotoxicity assays (Figures 3C and 3D). The selective CTL killing of leukemic versus normal CD34+ cells can be explained by differences in WT1 expression. WT1 RNA expression was higher in leukemic than in normal CD34+ cells (Figure 2B),
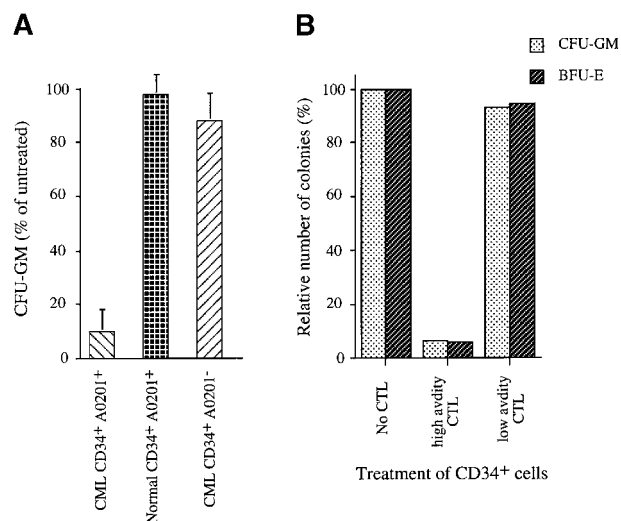

Figure 4. Analysis of CTL-mediated inhibition of colony formation of CD34+ cell populations purified from patients with leukemia and normal donors. (A) CTL-mediated inhibition of colony formation by purified CD34+ cells cocultured for 4 hours with CTL at an effector/target cell ratio of 10:1. Untreated control CD34+ cells were cultured under the same conditions without CTL. CTL treated and untreated control cells were then plated in methylcellulose, and after 14 days the numbers of granulocyte macrophage colony-forming units (GM-CFU) were counted. Shown is the percentage of GM-CFU after CTL treatment using the GM-CFU observed in the untreated controls as 100% reference. This figure shows the mean and standard deviation of independent experiments with CD34+ cells from 9 HLA-A0201+ patients with CML and 7 normal donors and with CD34+ cells from 5 HLA-A0201− patients with CML. (B) Colony formation by A0201+/CD34+ CML cells that were untreated or treated for 4 hours with high-avidity P126-specific CTL (line 81) or with low-avidity CTL (line 85) before plating. Shown are GM-CFU and BFU-E using the number of colonies observed in the untreated controls as 100% reference.

and Western blot analysis detected WT1 protein only in leukemic but not in normal CD34+ cell populations (Figure 2C).

Discussion

The infusion of donor lymphocytes to patients in relapse after previous allogeneic stem cell transplantation can engender strong graft-versus-leukemia effects.[24,25] A recent study showed that complete remission in patients with CML undergoing donor lymphocyte infusion was associated with an increased frequency of T cells recognizing leukemic CD34+ progenitor cells.[15] In contrast, T-cell recognition of more mature CD34− cells in patients with CML was not associated with a favorable clinical response. This suggested that T lymphocytes with specificity for CD34+ CML progenitor cells were critically important in mediating antileukemic effects in vivo. However, there is no information concerning the nature of target antigens that can direct T-cell responses selectively against leukemic CD34+ progenitor cells.

To identify such antigens, we used the allo-restricted CTL approach that, independent of immunologic tolerance, is suitable for raising CTL against any protein expressed at elevated levels in transformed cells.[22,23,26] Because the triggering of the cytotoxic effector function is a threshold phenomenon, it is possible to select CTL that are only triggered by elevated target protein levels in transformed cells but not by physiological levels of protein in normal cells.[23]

The transcription factor WT1 was chosen as a candidate target protein for several reasons. There is evidence of overexpression in leukemic CD34+ cells,[12] elevated WT1 expression can contribute to transformation,[13,14] normal WT1 expression is restricted to a small number of cells in postnatal life,[21] and, in addition to leukemia, elevated WT1 expression has been observed in renal cell carcinoma, ovarian cancer, advanced breast cancer, and melanoma.[27-30] Therefore, CTL selectively recognizing WT1 overexpressing malignant cells are invaluable reagents for antigen-specific therapy of leukemia and other more common malignancies. Furthermore, tumor escape by down-regulation of WT1 expression is unlikely to occur if overexpression is required to maintain the transformed phenotype.[31-33]

The allo-restricted CTL described here were isolated from HLA-A0201− donors, and they were specific for leukemic progenitor cells presenting the WT1-derived P126 peptide in the context of HLA-A0201 class I molecules. The P126 peptide was highly immunogenic because in vitro stimulation of lymphocytes from different HLA-A0201− donors consistently induced peptide-specific, HLA-A0201–restricted CTL. Therefore, P126-specific CTL are novel reagents for antigen-specific therapy of HLA-A0201+ patients with leukemia undergoing stem cell transplanta-tion from donors displaying a 1-locus HLA-mismatch involving the HLA-A0201 allele. A 1-locus HLA mismatch is clinically acceptable, as demonstrated in a recent study showing comparable prognoses in patients with leukemia receiving transplants from 1-locus–mismatched and HLA-matched unrelated donors.[34] Thus, a 1-locus–mismatch transplant provides an ideal setting for antigen-specific therapy with allo-restricted CTL derived from the donor. The in vitro stimulation protocol described here, in combination with the selection of relevant CTL by staining with HLA-A0201 tetramers containing P126 peptides, will allow rapid isolation of P126-specific CTL for adoptive therapy.

In addition, it is possible that WT1 can be exploited for antigen-specific therapy in the autologous setting. This is supported by our observation that P126-specific CTL can be isolated from HLA-A0201+ donors (unpublished data). Because WT1 expression in adults is restricted to a relatively small number of cells (eg, CD34+ bone marrow cells, renal podocytes, testicular Sertoli cells, and ovarian granulosa cells), tolerance of autologous T lymphocytes to WT1 is probably incomplete. Therefore, it may be possible to exploit the identified P126 epitope for the design of anti-WT1 vaccine preparations aimed at stimulating CTL responses against leukemia and other malignancies with elevated WT1 expression, such as renal cell carcinoma, ovarian cancer, melanoma, and breast cancer.

In addition to in vivo therapy, the WT1-specific CTL provides a tool for in vitro purging of autologous bone marrow cells harvested from patients with leukemia. The CTL removed leukemic progenitors of the granulocyte/macrophage lineage (Figure 4A) and also progenitors of the erythroid lineage (Figure 4B). In contrast, the CTL did not recognize normal progenitors of the 3 lineages. The selective removal of transformed CD34+ progenitor cells should reduce the risk for reinfusing leukemic progenitor cells, thus overcoming a major limitation of autologous stem cell transplantation.[35]

To date, tissue-specific minor histocompatibility antigens and lineage-specific antigens, such as proteinase 3, have been studied as potential targets for leukemia-reactive CTL.[36-39] The WT-1 transcription factor is the first target antigen capable of directing CTL responses selectively against leukemic progenitor cells.

Acknowledgments

References

1. DiGiusto D, Chen S, Combs J, et al. Human fetal bone marrow early progenitors for T, B, and myeloid cells are found exclusively in the population expressing high levels of CD34. Blood. 1994;84:421-432.

2. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat Med. 1998;4:1038-1045.

3. McCulloch EA. Stem cells in normal and leukemic hemopoiesis [Henry Stratton Lecture, 1982]. Blood. 1983;62:1-13.

4. Griffin JD, Lowenberg B. Clonogenic cells in acute myeloblastic leukemia. Blood. 1986;68:1185-1195.

5. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3:730-737.

6. Wang JC, Lapidot T, Cashman JD, et al. High level engraftment of NOD/SCID mice by primitive normal and leukemic hematopoietic cells from patients with chronic myeloid leukemia in chronic phase. Blood. 1998;91:2406-2414.

7. Dazzi F, Capelli D, Hasserjian R, et al. The kinetics and extent of engraftment of chronic myelogenous leukemia cells in non-obese diabetic/severe combined immunodeficiency mice reflect the phase of the donor's disease: an in vivo model of chronic myelogenous leukemia biology. Blood. 1998;92:1390-1396.

8. Bose S, Deininger M, Gora Tybor J, Goldman JM, Melo JV. The presence of typical and atypical BCR-ABL fusion genes in leukocytes of normal individuals: biologic significance and implications for the assessment of minimal residual disease. Blood. 1998;92:3362-3367.

9. Maurer U, Brieger J, Weidmann E, Mitrou PS, Hoelzer D, Bergmann L. The Wilms' tumor gene is expressed in a subset of CD34+ progenitors and downregulated early in the course of differentiation in vitro. Exp Hematol. 1997;25: 945-950.

10. Baird PN, Simmons PJ. Expression of the Wilms' tumor gene (WT1) in normal hemopoiesis. Exp Hematol. 1997;25:312-320.

11. Inoue K, Sugiyama H, Ogawa H, et al. WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood. 1994;84:3071-3079.

12. Inoue K, Ogawa H, Sonoda Y, et al. Aberrant overexpression of the Wilms tumor gene (WT1) in human leukemia. Blood. 1997;89:1405-1412.

13. Inoue K, Tamaki H, Ogawa H, et al. Wilms' tumor gene (WT1) competes with differentiation-inducing signal in hematopoietic progenitor cells. Blood. 1998;91:2969-2976.

14. Svedberg H, Chylicki K, Baldetorp B, Rauscher F Jr, Gullberg U. Constitutive expression of the Wilms' tumor gene (WT1) in the leukemic cell line U937 blocks parts of the differentiation program. Oncogene. 1998;16:925-932.

15. Smit WM, Rijnbeek M, van Bergen CA, Fibbe WE, Willemze R, Falkenburg JH. T cells recognizing leukemic CD34(+) progenitor cells mediate the antileukemic effect of donor lymphocyte infusions for relapsed chronic myeloid leukemia after allogeneic stem cell transplantation. Proc Natl Acad Sci U S A. 1998;95:10,152-10,157.

16. Lozzio CB, Lozzio BB. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood. 1975;45:321-334.

17. Pegoraro L, Matera L, Ritz J, Levis A, Palumbo A, Biagini G. Establishment of a Ph1-positive human cell line (BV173). J Natl Cancer Inst. 1983;70: 447-453.

18. Findley HW Jr, Cooper MD, Kim TH, Alvarado C, Ragab AH. Two new acute lymphoblastic leukemia cell lines with early B-cell phenotypes. Blood. 1982;60:1305-1309.

19. Zemmour J, Little AM, Schendel DJ, Parham P. The HLA-A, B "negative" mutant cell line C1R expresses a novel HLA-B35 allele which also has a point mutation in the translation initiation codon. J Immunol. 1992;148:1941-1948.

20. DeMars R, Chang CC, Shaw S, Reitnauer PJ, Sondel PM. Homozygous deletion that simultaneously eliminate expression of class I and class II antigens of EBV-transformed B-lymphoblastoid cells. I. Reduced proliferative responses of autologous and allogeneic T cells to mutant cells that have decreased expression of class II antigens. Hum Immunol. 1984;11:77-97.

21. Menke AL, van der Eb AJ, Jochemsen AG. The Wilms' tumor 1 gene: oncogene or tumor suppressor gene? Int Rev Cytol. 1998;181:151-212.

22. Stauss HJ. Immunotherapy with CTL restricted by non-self MHC. Immunol Today. 1999;20:180-183.

23. Sadovnikova E, Stauss HJ. Peptide-specific cytotoxic T lymphocytes restricted by nonself major histocompatibility complex class I molecules: reagents for tumor immunotherapy. Proc Natl Acad Sci U S A. 1996;93:13,114-13,118.

24. Kolb HJ, Mittermuller J, Clemm C, et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. Blood. 1990;76:2462-2465.

25. Dazzi F, Szydlo RM, Goldman JM. Donor lymphocyte infusions for relapse of chronic myeloid leukemia after allogeneic stem cell transplantation: where we now stand. Exp Hematol. 1999;27: 1477-1486.

26. Sadovnikova E, Jopling LA, Soo KS, Stauss HJ. Generation of human tumor-reactive cytotoxic T cells against peptides presented by non-self HLA class I molecules. Eur J Immunol. 1998;28:193-200.

27. Campbell CE, Kuriyan NP, Rackley RR, et al. Constitutive expression of the Wilms tumor suppressor gene (WT1) in renal cell carcinoma. Int J Cancer. 1998;78:182-188.

28. Viel A, Giannini F, Capozzi E, et al. Molecular mechanisms possibly affecting WT1 function in human ovarian tumors. Int J Cancer. 1994;57: 515-521.

29. Silberstein GB, Van Horn K, Strickland P, Roberts CT Jr, Daniel CW. Altered expression of the WT1 Wilms tumor suppressor gene in human breast cancer. Proc Natl Acad Sci U S A. 1997;94:8132-8137.

30. Rodeck U, Bossler A, Kari C, et al. Expression of the wt1 Wilms' tumor gene by normal and malignant human melanocytes. Int J Cancer. 1994;59: 78-82.

31. Algar EM, Khromykh T, Smith SI, Blackburn DM, Bryson GJ, Smith PJ. A WT1 antisense oligonucleotide inhibits proliferation and induces apoptosis in myeloid leukaemia cell lines. Oncogene. 1996;12:1005-1014.

32. Yamagami T, Sugiyama H, Inoue K, et al. Growth inhibition of human leukemic cells by WT1 (Wilms tumor gene) antisense oligodeoxynucleotides: implications for the involvement of WT1 in leukemogenesis. Blood. 1996;87:2878-2884.

33. Osaka M, Koami K, Sugiyama T. WT1 contributes to leukemogenesis: expression patterns in 7,12-dimethylbenz[a]anthracene (DMBA)-induced leukemia. Int J Cancer. 1997;72:696-699.

34. Petersdorf EW, Gooley TA, Anasetti C, et al. Optimizing outcome after unrelated marrow transplantation by comprehensive matching of HLA class I and II alleles in the donor and recipient. Blood. 1998;92:3515-3520.

35. O'Brien SG, Goldman JM. Current approaches to hematopoietic stem-cell purging in chronic myeloid leukemia [editorial]. J Clin Oncol. 1995;13: 541-546.

36. den Haan JM, Meadows LM, Wang W, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. Science. 1998;279:1054-1057.

37. Simpson E, Roopenian D, Goulmy E. Much ado about minor histocompatibility antigens. Immunol Today. 1998;19:108-112.

38. Warren EH, Gavin M, Greenberg PD, Riddell SR. Minor histocompatibility antigens as targets for T-cell therapy after bone marrow transplantation. Curr Opin Hematol. 1998;5:429-433.

39. Molldrem JJ, Clave E, Jiang YZ, et al. Cytotoxic T-lymphocytes specific for a nonpolymorphic proteinase-3 peptide preferentially inhibit chronic myeloid-leukemia colony-forming units. Blood. 1997;90:2529-2534.