TEL/PDGFβR Induces Hematologic Malignancies in Mice That Respond to a Specific Tyrosine Kinase Inhibitor

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Despite significant advances in the therapy of human leukemia, most adults still die of their disease or complications of therapy. Chronic myelomonocytic leukemia (CMML) is exemplary of the problem: although indolent at presentation, CMML progresses in most cases to acute myeloid leukemia (AML), which is frequently a fatal complication of the disease. There are currently no therapies for CMML or other of the myelodysplastic syndromes that are known to prolong survival apart from bone marrow transplant for selected patients. There is therefore a need to develop more effective therapies for these diseases.

The TEL/PDGFβR fusion protein is expressed as the consequence of a recurring t(5;12) translocation associated with chronic myelomonocytic leukemia (CMML). Unlike other activated protein tyrosine kinases associated with hematopoietic malignancies, TEL/PDGFβR is invariably associated with a myeloid leukemia phenotype in humans. To test the transforming properties of TEL/PDGFβR in vivo, and to analyze the basis for myeloid lineage specificity in humans, we constructed transgenic mice with TEL/PDGFβR expression driven by a lymphoid-specific immunoglobulin enhancer-promoter cassette. These mice developed lymphoblastic lymphomas of both T and B lineage, demonstrating that TEL/PDGFβR is a transforming protein in vivo, and that the transforming ability of this fusion is not inherently restricted to the myeloid lineage. Treatment of TEL/PDGFβR transgenic animals with a protein tyrosine kinase inhibitor with in vitro activity against PDGFβR (CGP57148) resulted in suppression of disease and a prolongation of survival. A therapeutic benefit was apparent both in animals treated before the development of overt clonal disease and in animals transplanted with clonal tumor cells. These results suggest that small-molecule tyrosine kinase inhibitors may be effective treatment for activated tyrosine kinase-mediated malignancies both early in the course of disease and after the development of additional transforming mutations.

Materials and Methods

Construction of transgenic mice. The backbone plasmid pBSVE6βK containing EμVHβ and the β-globin splice acceptor and poly A sequences was obtained as a gift from Dr Fred Alt, Harvard Medical School, Boston, MA. The TEL/PDGFβR cDNA cloned by Golub et al8 was inserted 3′ of the EμVHβ cassette (Fig 1).4,16 The prokaryotic plasmid sequences were removed from the above constructs by restriction enzyme digestion with BstHII and gel purification. Each purified construct was diluted to a concentration of 1.5 to 3.0 ng/μL and 1 μL was microinjected into the pronucleus of a FVB strain mouse oocyte. The injected eggs were implanted into the oviduct of a pseudopregnant female. At 2 weeks of age, founder mice were identified by Southern

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spleen, lymph nodes, blood, and thymus were prepared. Red blood cells were deparaffinized and stained with hematoxylin and eosin (H&E). Single-cell suspensions were prepared by passing spleen and lymph node tumor tissue through nylon mesh (Falcon, Lincoln Park, NJ) for 4 hours before processing. The 3-µm sections were an additional decalcification step in RDO (Apex Engineering Products, Plainfield, IL) for 4 hours before processing. The 3-µm sections were subjected to 35 cycles of the polymerase chain reaction (94°C for 60 seconds, 56°C for 60 seconds, and 72°C for 60 seconds) using primers: TPy3F, 5'-TAC AAA AAG TAC CAG CAG-3' and HBG1, 5'-GGC AGC TTA GTG A TA CTT GT-3'. Each sample was reverse-transcribed using AMV reverse transcriptase (TRIzol; GibcoBRL, Gaithersburg, MD) and processing according to the manufacturer’s instructions. A 4-µg quantity of total mRNA from each sample was reverse-transcribed using AMV reverse transcriptase (GibcoBRL) at 41°C for 1 hour. One microliter of cDNA solution was subjected to 35 cycles of the polymerase chain reaction (94°C for 60 seconds, 56°C for 60 seconds, and 72°C for 60 seconds) using primers: TPy3F, 5'-TAC AAA AAG TAC CAG CAG-3' and HBG1, 5'-GGC AGC TTA GTG A TA CTT GT-3'. Cells from representative tissues were washed twice with PBS and lysed in 1% NP40, 150 mmol/L NaCl, 20 mmol/L Tris pH 7.4, 10% glycerol containing 1 mmol/L phenylmethylsulfonylfluoride, 20 µg/mL aprotinin, and 1 mmol/L sodium orthovanadate at 5 × 10^7 cells/mL. Equal amounts of lysates (100 µg) were analyzed by denaturing polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and Western blotting with polyclonal rabbit anti-βPDGFR tail (Pharmingen, San Diego, CA) as described.17

Histology. Murine tissues were fixed for 24 hours in 10% neutral buffered formalin and embedded in paraffin. Femurs were subjected to an additional decalcification step in RDO (Apex Engineering Products, Plainfield, IL) for 4 hours before processing. The 3-µm sections were deparaffinized and stained with hematoxylin and eosin (H&E).

Flow cytometric analysis. Single-cell suspensions of bone marrow, spleen, lymph nodes, blood, and thymus were prepared. Red blood cells were lysed in ammonium chloride solution (150 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA, pH 7.4) for 5 minutes at room temperature. The cells were washed in PBS with 0.1% Na₂O₃ and 0.1% bovine serum albumin (BSA; staining buffer). To block nonspecific Fc receptor-mediated binding, the cells were preincubated with supernatant from the 2.4G2 hybridoma line (anti-CD16/CD32; cell line obtained from American Type Culture Collection, Rockville, MD) for 20 minutes on ice. Aliquots of 0.5 to 1.0 × 10^9 cells were then stained for 20 minutes on ice with monoclonal antibodies specific for B220 (CD45R), CD24 (heat-stable antigen), CD117 (c-kit), IgM, CD3, CD2, CD4, CD8, CD25 (IL-2Rα chain), CD40, CD43, CD48, and Ly-51 (BP-1) (Pharmingen) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or biotin. Binding of biotinylated primary antibodies was detected using PE-conjugated streptavidin (Immunotech, Westbrook, ME) or FITC-conjugated avidin (Southern Biotechnology, Birmingham, AL). Cells were washed once in staining buffer followed by two-color flow cytometric analysis with a FACScan (Becton Dickinson, San Jose, CA). A minimum of 10,000 events was acquired and the data were analyzed using CellQuest software (Version 3.1; LCC International, McLean, VA). The results are presented as contour plots showing FITC and PE fluorescence signals of viable cells gated on the basis of forward and side scatter signals.

Immunoglobulin gene rearrangement. Genomic DNA was prepared from single-cell suspensions of lymph node tumor cells, and control DNA was isolated from the tail of an unaffected littermate as described.18,19 A 10-µg quantity of genomic DNA was digested with the appropriate restriction endonucleases overnight, subjected to electrophoresis on a 1% agarose gel, and transferred to nylon membranes (Hybond N+; Amersham, Arlington Heights, IL) using alkaline transfer as described.20 A 1.5-kb Pur 1 IgH fragment, a 2.4-kb HindIII-BamHI IgK fragment, and 2.0-kb EcoRI βTCR fragment were used as probes (a gift of Dr Benjamin Rich), random-labeled using 32PdCTP (Boehringer Mannheim, Indianapolis, IN). Hybridization was performed at 65°C for 16 hours and membranes were washed with 2× SSC, 0.1% SDS for 20 minutes at room temperature; 1× SSC, 0.1% SDS at 65°C for 20 minutes; and exposed to photographic film (BioMax; Eastman Kodak, Rochester, NY) at −80°C overnight.

Tyrosine kinase inhibitor treatment. CGP57148 was diluted in PBS to a concentration of 3 mg/mL; 50 mg/kg was injected intraperitoneally (IP) every day for 30 days. Control mice were injected IP with 0.5 mL of PBS alone at the same time. P values for statistical significance were calculated by the log-rank (Mantel-Cox) method. Statistical calculations and Kaplan-Meier survival analyses were performed using the program Statview (SAS Institute, Cary, NC).

RESULTS

Construction of Eµ-TEL/PGDFBR transgenic mice. The ability of TEL/PGDFBR to transform lymphoid cells in vivo was tested using a transgenic mouse model in which the immunoglobulin heavy-chain enhancer/promoter EµV₅P was used to direct TEL/PGDFBR expression to the lymphoid compartment. EµV₅P would be expected to result in expression predominantly to the B-cell compartment, but expression in T cells has also been well described.21,22 A total of 10 founder mice were identified as having integrated the TEL/PGDFBR construct. Germline transmission allowed the establishment of eight transgenic lines. One founder failed to breed successfully, and another developed a mediastinal mass and died at 65 days old before analysis or breeding could be completed. Two of the eight lines (I and N lines) consistently developed lymphoid malignancies. The most extensive analysis of tumors (40 separate tumors) was performed on mice from the I line, all of which developed diffuse lymphadenopathy and massive hepatosplenomegaly at a median age of 4 months (Fig 2A). N line
mice also consistently developed tumors at the same median age (seven tumors analyzed) that presented in several different patterns (see below).

**Microscopic and flow cytometric analysis of the malignant phenotype.** Histopathologic examination of I line mice showed tumor with features of lymphoblastic lymphoma, including intermediate-sized lymphoid cells with scant cytoplasm, dispersed nuclear chromatin, and a brisk mitotic rate. All lymph node groups were affected, as well as peritonal tissue in the liver, diffuse involvement of the spleen with effacement of normal splenic architecture, and of bone marrow with lymphoblastic lymphoma (Fig 2A through C, and data not shown). Flow cytometry of cell suspensions from bone marrow, spleen, blood, and lymph nodes was performed to determine cell lineage of the tumors. All I-line tissues analyzed contained a dominant population of B220⁺, CD3⁻, CD40⁻, CD43⁺, HSA⁺, and BP-1⁺ cells (Fig 3A), consistent with hardy fraction C lymphoblastic lymphoma.²³,²⁴ This phenotype corresponds most closely with a late pro-B- or early pre-B-cell lymphoma in humans. However, while all mice of this line showed early B-cell phenotype, other individual mice from the same line had variable staining for CD43 and CD117 (c-kit). These different immunophenotypes show some variability in the stage of differentiation of these tumors, an observation that has been made in several other mouse models of lymphoid malignancy.²⁵-²⁸

**TEL/PDGFβR lymphoid tumors are clonal.** Clonality of tumors was assessed in transgenic mice by Southern blot analysis with probes for the immunoglobulin heavy-chain, kappa light-chain, and βTCR loci (Fig 3B through D). Clonal rearrangements were detected in I-line tumor tissue with probes for the immunoglobulin heavy-chain locus, but not for the kappa light-chain or βTCR loci, consistent with the histopathologic and flow cytometry data. Furthermore, these data show that in addition to expression of TEL/PDGFβR, tumorigenesis requires additional mutations to give rise to the full malignant phenotype.

**TEL/PDGFβR is expressed in involved tissues from transgenic mice.** Expression of TEL/PDGFβR in tissues involved with lymphoma was confirmed by both reverse-transcriptase polymerase chain reaction (RT-PCR) and by Western blot analysis in I-line (Fig 4) and N-line mice (data not shown). RT-PCR used primers flanking an intron in the TEL/PDGFβR construct (Fig 1) so that amplimers resulting from contaminat-
ing genomic DNA could be distinguished from those arising from cDNA on the basis of size. TEL/PDGFβR transcript was detected in marrow, spleen, and nodes of transgenic animals, but not in marrow or spleen of controls (Fig 4A). TEL/PDGFβR protein was detected by Western blotting with anti-PDGFβR antibody in nodes and bone marrow, but not in kidney of transgenic mice or marrow of control mice (Fig 4B).

I-line Hardy C lymphoblastic lymphoma is transplantable. To confirm the malignant phenotype of lymphoma cells, 10⁴ tumor cells in single-cell suspensions derived from I-line lymph
node tumors were transplanted by intravenous injection from an affected transgenic animal into sublethally irradiated syngeneic mice. Tumors developed in these transplanted mice 3 to 4 weeks after transplantation and affected liver, lymph node, marrow, and spleen tissues. Histopathologic and flow cytometric analysis was consistent with B-cell lymphoblastic lymphoma, demonstrating that the transplanted tumors retained the same morphology and immunophenotype as the primary B-cell lymphoblastic lymphoma (data not shown). Tumors also developed in unirradiated recipient mice that received $10^6$ lymph node cells (data not shown).

Other transgenic lines of TEL/PDGFRβ mice also develop lymphoblastic lymphoma. The N-line founder developed a massive mediastinal mass with involvement of pulmonary parenchyma and spleen. Histopathologic examination was consistent with lymphoblastic lymphoma (Fig 2D and E). Flow cytometric analysis of tumor cells (Fig 3A) showed CD3$, CD2^+$, CD19$, CD13$, CD14$, CD15$, and B220$^+$ cells consistent with T-cell lymphoblastic lymphoma. Southern blot analysis confirmed a clonal rearrangement of the TCRβ locus, but not of immunoglobulin heavy-chain or kappa chain loci (Fig 3B through D). RT-PCR confirmed the presence of TEL/PDGFRβ transcript (data not shown). Five F1 progeny of the N-line founder have developed a distinct clinical phenotype consisting of massive splenomegaly, weight loss, and eventually hind limb paralysis. The spleen and bone marrow of these animals was involved with lymphoblastic lymphoma (Fig 2F and G) with an identical immunophenotype to that seen in I-line animals, and there were tumor cells circulating in the blood (data not shown). The phenotype of splenomegaly with bone marrow involvement by Hardy C lymphoblastic lymphoma cells is most analogous to human pre-B-cell ALL. Last, one mouse from the N line developed massive lymphadenopathy that was histologically and immunophenotypically identical to that seen in I-line mice.

**Treatment with a specific tyrosine kinase inhibitor.** Transgenic I-line mice were treated with daily IP injections of CGP57148 (50 mg/kg). CGP57148 is a specific inhibitor of the PDGFβR and ABL kinases at concentrations as low as 1 µmol/L, but has no effect on a broad spectrum of other tyrosine and serine/threonine kinases at concentrations as high as 100 µmol/L. Mice were treated for 30 consecutive days, and did not display any obvious toxicity from the treatment. Control mice were treated with PBS for the same duration. There was a statistically significant prolongation of survival in mice treated with CGP57148 compared with PBS controls (Fig 5A). Latency was prolonged by approximately the same duration as CGP57148 therapy in this experiment, suggesting that CGP57148 may have inhibited growth of these cells or prevent tumor progression, but did not eradicate TEL/PDGFRβ-expressing cells at the concentration and dosing schedule used in this experiment.

In a separate experiment, the effect of CGP57148 was tested in a transplant model. A total of $10^4$ tumor cells derived from affected lymph nodes of transgenic animals were injected intravenously into 19 sublethally irradiated animals; 10 animals then received daily IP CGP57148 as above and nine received PBS. Again, animals treated with the specific kinase inhibitor had a statistically significant inhibition of development of lymphoblastic lymphoma and survived longer than control animals ($P = .0040$, Fig 5B). These data suggest that even after acquisition of clonal disease through additional mutations, CGP57148 is capable of inhibiting lymphoblastic lymphoma cell growth. Prolongation of latency was approximately equivalent to the duration of CGP57148 therapy.
of CGP57148 or PBS.

vival. In both figures, an open bar represents the duration and timing with CGP57148 had a statistically significant improvement in sur-
thally irradiated syngeneic mice. Ten were treated with CGP57148. (B) Tumor transplant model: tumor
ment of overt malignancy is delayed and survival is improved in animals treated with CGP57148. (A)
mice between 5 and 7 weeks old without evidence of malignancy on examination were treated with
daily IP injections of CGP57148 for 30 days (—). Concurrently, 9 similar
without evidence of malignancy on examination were treated with the specific tyrosine kinase inhibitor CGP57148. (A)
mice with massive lymphadenopathy were put
malignant mice model: 6 I-line mice between 5 and 7 weeks old characterized by dysplastic monocytosis, variable bone marrow
CMML is a subtype of myelodysplastic syndrome and is
tons associated with progression of disease to AML. For example, in
secondary lymphoid lymphomas. TEL/PDGFβR observed in human leukemias is not due to an inherent inability
malignancy to (1) characterize the basis for myeloid lineage specificity of TEL/PDGFβR in
tions that favor acquisition of the TEL/PDGFβR 
transgenic mouse model of TEL/PDGFβR-mediated malignancy to (1) characterize the basis for myeloid lineage specificity of TEL/PDGFβR in humans, (2) test the transforming properties of the fusion protein in vivo, (3) develop a model system for studying progression of disease, and (4) test therapeutic interventions targeted at the PDGFβR kinase.

Expression of TEL/PDGFβR was directed to the lymphoid
t the immunoglobulin heavy-chain enhancer-promoter, Eµ. TEL/PDGFβR was capable of transforming primary lymphoid lineage cells, as evidenced by the development of B and T lymphoblastic lymphomas in different transgenic lines of mice. Expression of TEL/PDGFβR was assayed in mice that developed lymphomas. Tumor, but not unaffected tissues, expressed the TEL/PDGFβR mRNA and protein. Eµ is known to direct expression primarily to the B-cell compartment, but low levels of expression have been documented in other cell types, including T cells. In fact, Eµ-E2A/ PXB transgenic mice developed T-cell neoplasms exclusively.30 Because Eµ-TEL/PDGFβR transgenic mice develop lymphoid malignancy, the myeloid lineage specificity of TEL/PDGFβR observed in human leukemias is not due to an inherent inability of the fusion protein to transform lymphoid lineage lymphoid cells. These data contrast with transforming proteins such as TAN-1, which has T-cell lineage specificity both in humans and in murine BMT models, or BCR/ABL, which causes both

Ba/F3 cells by TEL/PDGFβR is dependent on PDGFβR tyrosine kinase activity. A kinase inactive point mutant of TEL/PDGFβR is not transforming,10 and the PDGFβR kinase–specific inhibitor CGP57148 inhibits the growth of TEL/ PDGFβR-transformed Ba/F3 cells.15 t(5;12)(q31;p13) is exclusively associated with a myeloid leukemia phenotype; it is never seen in association with lymphoproliferative disorders or lymphoid malignancy. We have developed an animal model of TEL/PDGFβR-mediated malignancy to (1) characterize the basis for myeloid lineage specificity of TEL/PDGFβR in humans, (2) test the transforming properties of the fusion protein in vivo, (3) develop a model system for studying progression of disease, and (4) test therapeutic interventions targeted at the PDGFβR kinase.

**DISCUSSION**

CMML is a subtype of myelodysplastic syndrome and is characterized by dysplastic monocytosis, variable bone marrow fibrosis, and progression to AML. AML is frequently a fatal complication of CMML, but the molecular genetic basis for progression of disease is poorly understood and there are no known therapies to prevent progression.

t(5;12)(q31;p13), a recurring cytogenetic abnormality associated with CMML, results in fusion of the amino terminus of TEL, containing the PNT oligomerization domain, to the tyrosine kinase domain of PDGFβR. The transformation of

Fig 5. Improved survival of Eµ-TEL/PDGFβR transgenic mice treated with the specific tyrosine kinase inhibitor CGP57148. (A) Premalignant mice model: 6 I-line mice between 5 and 7 weeks old
Gene rearrangement was also analyzed by Southern blotting. TEL/PDGFβR mRNA and protein expression in the Eµ-TEL/PDGFβR transgenic mice was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) analysis, which showed the presence of TEL/PDGFβR mRNA in both lymphoid and myeloid lineages. Immunohistochemical analysis of tumor sections confirmed the presence of TEL/PDGFβR protein in T-cell and B-cell lymphomas.

**Southern blot analysis** showed the presence of TEL/PDGFβR mRNA in tumor tissue from the BALB/c nude mice. TEL/PDGFβR was detected in both B and T lymphoid lineages, indicating that this fusion protein is capable of transforming cells of both lymphoid and myeloid lineages.

**Conclusion**

The results presented in this study suggest that TEL/PDGFβR is a potent oncogene in both lymphoid and myeloid lineages. The development of a preclinical model of TEL/PDGFβR-mediated malignancy in the BALB/c nude mouse system provides a unique opportunity to study the biology of this fusion protein and to test therapeutic interventions aimed at targeting the PDGFβR kinase. These findings may have important implications for the development of new therapeutic strategies for the treatment of CMML and other myeloid malignancies.
PDGFβR and ABL kinases, with negligible effect on other tyrosine and serine/threonine kinases at concentrations in the 0.1 to 1 µmol/L range.15,31,32 Furthermore, CGP57148 inhibits the growth of TEL/PDGFβR-transformed Ba/F3 cells, and inhibition can be rescued by addition of IL-3, demonstrating that the effect of CGP57148 is specific for PDGFβR.35

To determine whether CGP57148 could prolong disease latency in the TEL/PDGFβR transgenic model, drug was administered by daily IP injections for 30 days. Although the compound can inhibit both native PDGFβR and c-ABL kinases, there was no observed toxicity. Furthermore, there was statistically significant prolongation of latency of disease of approximately the same duration as administration of drug. CGP57148 has previously been shown to be active in vivo against BCR/ABL.31 Our data suggest that CGP57148 may also be useful clinically in prolonging disease latency in humans with TEL/PDGFβR-mediated disease. Drug efficacy could potentially be improved by testing modulations in dose, schedule, or route of administration in this animal model. For example, the half-life of CGP57148 in vivo measured in rats is approximately 4 hours (B. Druker, unpublished observation); thus, single-day dosing might not be expected to give maximal efficacy.

The effect of CGP57148 was then tested on tumor cells in a transplant model in which tumor cells were introduced into a syngeneic recipient. There was also a statistically significant prolongation of disease latency in this context as well, demonstrating that CGP57148 is able to inhibit the growth of tumor cells expressing TEL/PDGFβR even after the acquisition of additional mutations. However, tumor cells were apparently not killed by the drug treatment. It is plausible that CGP57148 inhibits the proliferation of cells mediated by TEL/PDGFβR, but that after cessation of therapy, tumor cells are again able to proliferate. This hypothesis could be further explored by testing the effect of CGP57148 ex vivo on tumor cells before transplantation.

The present study shows that TEL/PDGFβR can cause hematopoietic malignancy in a transgenic mouse model, and is capable of causing lymphoid malignancy in this context, despite myeloid lineage specificity in humans. CGP57148, a PDGFβR-specific tyrosine kinase inhibitor, is nontoxic in mice under the conditions used, and prolongs disease latency both in transgenic mice and in transplanted tumor cells. Although these pilot experiments used a small number of animals, the results were statistically significant. Further analysis of CGP57148 and other compounds in this animal model may identify novel therapies to treat CMML in humans and prevent disease progression.

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