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The biology of chronic myelogenous leukemia: mouse models and cell adhesion

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Chronic myelogenous leukemia (CML) is a biphasic neoplasm of the bone marrow that is precipitated by the Philadelphia chromosome, a t(9;22) balanced translocation that encodes a constitutively activated nonreceptor tyrosine kinase termed P210$^{BCR-ABL}$. This oncoprotein has several intracellular functions; however, the most important effect of P210$^{BCR-ABL}$ leading to cell transformation is phosphorylation of signaling molecules through a constitutively active tyrosine kinase domain. Despite extensive knowledge of the structure and functional domains of BCR-ABL, its precise function in transformation is not known. Progress has been hampered, in part, by the lack of relevant CML models, as cell culture and *in vitro* assays do not mimic the pathogenesis of CML. Recently, there has been significant progress toward improving murine models that closely resemble human CML. This has allowed researchers to evaluate critical functions of BCR-ABL and has provided a model to test the efficacy of therapeutic medications that block these pathways. Our laboratory has developed two intersecting research programs to better understand the functioning of P210$^{BCR-ABL}$ in leukemogenesis. In one approach, we have developed a murine CML model by transferring HSCs that express BCR-ABL from a retroviral vector. All recipients develop a rapidly fatal MPD that shares several important features with CML. This model has been extremely useful for studying the function of BCR-ABL in the pathogenesis of CML. A second approach utilizes a quantitative cell detachment apparatus capable of measuring small changes in cell adhesion to investigate the mechanism by which P210$^{BCR-ABL}$ causes abnormal cell binding. Altered cell adhesion may contribute to the imbalance between proliferation and self-renewal in the hematopoietic progenitor compartment. To better understand the role abnormal adhesion may play in the development of leukemia, we have attempted to correlate the effects of functional P210$^{BCR-ABL}$ mutants in regulating adhesion and oncogenicity.
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Introduction

The Philadelphia (Ph) chromosome, a shortened derivative of chromosome 22 formed by a balanced translocation with chromosome 9, was the first consistent genetic mutation to be associated with a human cancer (Nowell and Hungerford, 1960; Rowley, 1973). The formation of the Ph chromosome is commonly thought to be the initiating event leading to oncogenesis. It is present in 95% of patients who carry a diagnosis of chronic myelogenous leukemia (CML) and 5% of children and 15–30% of adults who develop acute lymphoblastic leukemia (ALL).

The characteristic presenting feature of CML is an elevated white blood cell count (typically 50 000 to 300 000 per μl) with a high percentage of mature neutrophils with metamyelocytes and myelocytes and few blasts. Basophils and eosinophils are also frequently present. Some patients exhibit splenomegaly; however, nearly 40% are asymptomatic. Bone marrow (BM) biopsy reveals a packed bone marrow characterized by replacement of adipose tissue with marked myeloid hyperplasia.

CML has an incidence of one to two cases per 100 000 each year with a median age at onset of 53 years. Between 12–30% of patients are 60 years of age or older, limiting the number of patients who are candidates for stem cell transplantation. The small number of leukemia patients who are eligible for bone marrow transplantation (BMT) and the limited pool of HLA-matched donors have driven the research for alternative therapies. After 3 to 5 years, CML progresses through an accelerated phase into a fatal blast crisis with a block in differentiation and greater than 30% blasts in the peripheral blood (PB). Blast crisis acute leukemias are most frequently myeloid (60%) or B-lymphoid (30%). T-cell blast crisis occurs infrequently. It is clear that the pathogenesis of CML originates at the level of a multipotential hematopoietic cell because the Ph chromosome can be identified in all hematopoietic lineages during the chronic phase, and

blast crisis has been found to occur in each of the hematopoietic lineages.

The precise mechanism underlying the abnormal accumulation of myeloid cells in the BM is unknown. It likely results from a shift from the self-renewal capacity of myeloid progenitor cells toward increased cell proliferation, decreased apoptosis, and abnormal adhesion to surrounding stromal cells (reviewed in Gordon and Goldman, 1996). Myeloid progenitor cells are released prematurely from the BM into the peripheral circulation and home to sites of extramedullary hematopoiesis such as the liver and spleen, possibly due to abnormal cell adhesion or trafficking.

The molecular consequence of the t(9;22) translocation is the approximation of the 5′ end of the c-*abl* gene onto the *bcr* gene of chromosome 22 in a head-to-tail manner (Melo, 1996a). Nearly all of c-*abl* is retained in *BCR-ABL* with the c-*abl* breakpoint commonly between the first alternate exon Ib and the second alternate exon Ia, but occurs less commonly upstream of alternate exon Ib or downstream of alternate exon Ia. Three forms of this molecular rearrangement have been identified which vary in the amount of *bcr* included in the chimeric gene. Expression of the fusion gene is driven by the *bcr* promoter. A fourth fusion protein involving c-*abl* has been identified in a translocation with the *tel* gene on chromosome 12 (Figure 1a).

Patients with CML predominantly express a 210 kd protein termed $P210^{BCR\text{-}ABL}$ which is created by fusion of c-*abl* onto *bcr* at exons 12–16 (commonly referred to as M-*bcr*) creating junctions at b2a2 or b3a2. *Bcr* breakpoints between exons e2′ and e2 (commonly referred to as m-*bcr)* when fused to c-*abl* result in translation of a 190 kd fusion protein termed $P190^{BCR\text{-}ABL}$ which leads to a more aggressive acute lymphoblastic leukemia. A rare BCR-ABL protein with a molecular weight of 230 kd ($P230^{BCR\text{-}ABL}$) results from fusion of c-*abl* onto *bcr* downstream of exon 19 (referred to as μ-bcr) (Pane *et al*., 1996). The original description of $P230^{BCR\text{-}ABL}$ suggested an association with neutrophilic CML (N-CML), which follows a more benign course than typical CML, characterized by lower WBC, mild splenomegaly, and failure to progress to blast crisis (Pane *et al*., 1996). The association of $P230^{BCR\text{-}ABL}$ with N-CML is not absolute as several patients with $P230^{BCR\text{-}ABL}$ have developed typical symptoms of CML and acute leukemia (Haskovec *et al*., 1998; Bernasconi *et al*., 2001). Nevertheless, low expression levels of $P230^{BCR\text{-}ABL}$ may permit an indolent syndrome (Verstovsek *et al*., 2002). A fourth abl fusion protein is the product of a t(9;12) translocation and has been identified in three patients with acute leukemias of myeloid or lymphoid origin (Papadopoulos *et al*., 1995; Golub *et al*., 1996; van Limbergen *et al*., 2001) as well as two patients with typical or atypical CML (Andreasson *et al*., 1997; van Limbergen *et al*., 2001). This translocation fuses the amino terminus of the ets-like protein, tel, with c-abl (Golub *et al*., 1997). The TEL-ABL fusion shares several important functional characteristics with BCR-ABL fusion proteins. Both bcr and tel contribute oligomerization domains important for transformation (Golub *et al*., 1996), and similar signal transduction pathways are activated by both fusions in factor dependent hematopoietic cell lines (Okuda *et al*., 1996).

C-abl is a 145 kd nonreceptor tyrosine kinase that is predominantly nuclear, however, there is a cytoplasmic fraction bound to F-actin. The tyrosine kinase activity of c-abl is tightly controlled and is activated in S phase or by ATM in response to ionizing radiation (reviewed in van Etten, 1999). The fraction remaining in the cytoplasm is transported to the cell membrane through an N-terminal myristolation sequence. $Abl^{-/-}$ knock-out mice have abnormal spermatogenesis, shortened life span, and are born runted (Tybulewicz *et al*., 1991). An *abl*-related gene (*arg, abl-2*) has been identified on 1q24-25 with structural similarities to abl (Kruh *et al*., 1986). Arg contains SH2, SH3, and tyrosine kinase domains and autokinase activity reminiscent of abl, suggesting possible overlapping functions (Perego *et al*., 1991). The predominant defect in $Arg^{-/-}$ mice are behavioral abnormalities; however, double homozygous $Abl^{-/-}$, $Arg^{-/-}$ mice are nonviable and embryos show defects in neurulation, indicating that abl and arg may complement each other to a limited extent (Koleske *et al*., 1998). Bcr is less well characterized, but neutrophils from *bcr* knock-out mice are diminished in superoxide formation and response to endotoxin stimulation (Voncken *et al*., 1995b).

The tyrosine kinase activity of the BCR-ABL fusion protein is much higher than that of the c-abl protein (Konopka and Witte, 1985). Fusion of *bcr* onto c-*abl* constitutively activates the c-abl tyrosine kinase through protein–protein oligomerization at the N-terminal coiled-coil domain of bcr encoded by the first 63 amino acids (McWhirter and Wang, 1991). This region encodes two α-helices that associate by forming an anti-parallel coiled-coil through the two C-terminal helices (Zhao *et al*., 2002). Oligomerization enhances F-actin binding through the C-terminal actin-binding domain of abl (McWhirter and Wang, 1991). Bcr also contributes a grb2 binding site at Tyr177 that is hypothesized to link BCR-ABL to the Ras-MAP Kinase pathway through the guanine exchange factor sos. Mutation of tyrosine to phenylalenine (Y177F) abrogates grb2 binding and attenuates Ras activation to impair fibroblast transformation (Pendergast *et al*., 1993). One effect of cytoplasmic localization and increased tyrosine kinase activity of BCR-ABL is to induce the formation of multimeric protein complexes that may affect cellular proliferation, adhesion, and apoptosis, eventually leading to transformation (Melo, 1996). These complexes include the adapter proteins CrkL, the focal adhesion proteins paxillin and talin, and the Ras activating proteins Shc and p62 Dok (Tauchi *et al*., 1994; Salgia *et al*., 1995; Carpino *et al*., 1997; Heaney *et al*., 1997; Yamanashi and Baltimore, 1997).

Important c-abl protein domains that are retained in the $P210^{BCR\text{-}ABL}$ fusion protein include an SH3 domain, which may negatively regulate transformation,

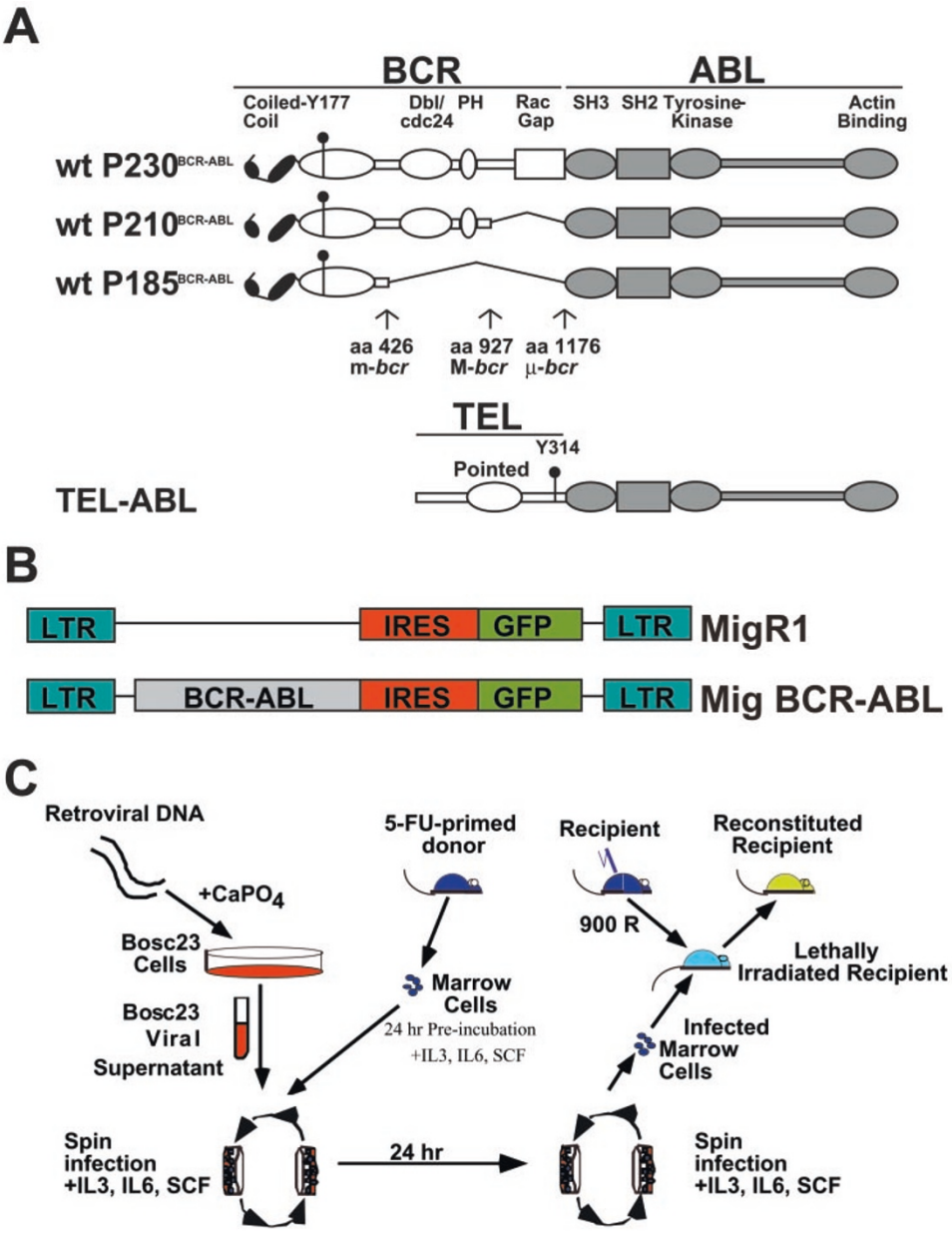

Figure 1 (**a**) Structures of the four abl translocation proteins associated with human malignancies. The coiled-coil domain of BCR-ABL mediates oligomerization, grb2 binds to Tyr177 (Y177). The central portion of bcr, preserved in $P230^{BCR-ABL}$ and $P210^{BCR-ABL}$, contains a region that shares homology with Rho guanidine nucleotide exchange factors, dbl and cdc24 (Dbl/cdc24) and pleckstrin homology (PH) domains. The abl region, conserved in all four fusion proteins, contains src homology domains (SH3 and SH2), a tyrosine kinase domain, and a C-terminal actin binding domain. The Tel gene from chromosome 12 contributes a Pointed domain to TEL-ABL that mediates self association. (**b**) Retroviral vectors used in bone marrow transplant assays. MigR1 is an MSCV-based retroviral vector that expresses GFP. BCR-ABL variants are cloned upstream of the internal ribosomal entry site (IRES). The IRES allows for transcription of a bicistronic message that is independently translated. This strategy increases the likelihood that both proteins will be expressed independently in transduced cells and will not be vulnerable to promoter competition. (**c**) Protocol for retroviral transduction of 5-FU treated BM and subsequent reconstitution of BM into a lethally irradiated recipient. After prestimulation, BM cells are transduced by two rounds of spin infection (viral infection under centrifugation) in the presence of the retroviral supernatant

an SH2 domain which binds phosphotyrosine, a tyrosine kinase domain whose presence is absolutely essential for transformation, sequences for nuclear localization, F- and G-actin binding, and DNA binding (Figure 1a) (Jackson and Baltimore, 1989; Kipreos and Wang, 1992; McWhirter *et al*., 1993; van Etten *et al*., 1994; Melo, 1996b). Until recently, the contribution of these functional domains to the overall transforming capacity of BCR-ABL was tested in cell lines for anchorage independent growth or for cell survival and proliferation in the absence of growth factors. However, at times these assays have been

contradictory, and the next step was to evaluate the *in vivo* role of BCR-ABL.

One potentially relevant approach for investigating CML is to use primary human tissue for study. Primary tissue has fewer phosphorylated proteins than cell lines and have narrowed the range of proteins to include those that are highly phosphorylated in patients with CML such as, $p62^{dok}$, and crkL, that may cooperate with $P210^{BCR\text{-}ABL}$ in the development of leukemia (Oda *et al*., 1994; Carpino *et al*., 1997). However, relevant functional assays do not exist for the evaluation of patient tissue, which is a major deficiency in using human tissue to study the pathogenesis of CML. One promising methodology for identifying the CML reservoir cell is transferring human cells into NOD/SCID mice. This approach has successfully identified the cell type causing AML (Lapidot *et al*., 1994). Although a report shows that CML cells engraft at low frequency in NOD/SCID mice (Lewis *et al*., 1998), the utility of this assay for studying the pathogenesis of CML has not yet been shown.

The wealth of evidence showing that the Ph chromosome is associated with CML in conjunction with *in vitro* studies demonstrating that $P210^{BCR\text{-}ABL}$ transforms hematopoietic and fibroblast cells provides strong evidence that the t(9;22) translocation initiates CML. The direct link between genotype and phenotype has greatly aided in the development of a mouse model. From this data, several investigators have hypothesized that mice could be made to succumb to a CML-like disease if $P210^{BCR\text{-}ABL}$ could be introduced into the appropriate HSC at the correct expression level. This has been the challenge of the last 10 to 12 years, during which time several models have been developed utilizing differing approaches.

BCR-ABL transgenic models were one of the first approaches used to express BCR-ABL. To overcome the embryonic lethality when BCR-ABL is driven by the bcr promoter in transgenic mice, $P190^{BCR\text{-}ABL}$ (Heisterkamp *et al*., 1990) and $P210^{BCR\text{-}ABL}$ (Honda *et al*., 1995; Voncken *et al*., 1995a) have been expressed under the control of the metallothionine promoter. These mice survive through gestation and birth until moribund with disease several weeks later. Through this approach, $P190^{BCR\text{-}ABL}$ precipitated a B-cell leukemia with a latency of 4 to 28 weeks, whereas $P210^{BCR\text{-}ABL}$ transgenic mice developed either a B- or T-cell leukemia with a longer latency of 8 to 44 weeks. $P190^{BCR\text{-}ABL}$ was also expressed by homologous recombination into the bcr gene in embryonic stem cells, giving rise to a B-cell acute lymphoblastic leukemia after 4 months (Castellanos *et al*., 1996). These methods have not been able to reproduce the myeloid expansion of CML; however, the acute lymphoid leukemia commonly precipitated by both the transgenic and knock-in $P190^{BCR\text{-}ABL}$ mice is closer to the human ALL disease.

One potential explanation for the failure to achieve a CML-like disease in the aforementioned murine models is that the BCR-ABL fusion may not have been expressed at the optimal level in the cell type that gives rise to the disease. In studies in which $P210^{BCR\text{-}ABL}$ was expressed under the control of the *tec* promoter, which is largely restricted to the hematopoietic compartment, the founder line of the *tec*:BCR-ABL transgenic mice preferentially developed lymphoblastic leukemia; however, the F1 line showed marked myeloid expansion with 60–90% granulocytes in the PB at 8–10 months of age (Honda *et al*., 1998). Breeding of these mice with mice heterozygous for P53 resulted in the rapid development of clonal T-cell neoplasms that had lost both P53 alleles (Honda *et al*., 2000). These findings suggest that the tec:BCR-ABL transgenic mice may be a useful murine CML model.

An alternative approach to obtain sufficient $P210^{BCR\text{-}ABL}$ expression in the progenitor cell that causes the CML-like disease is to transplant lethally irradiated syngenetic mice with genetically modified BM that stably expresses $P210^{BCR\text{-}ABL}$ in HSCs following retroviral transduction. The efforts in our laboratory are focused on this latter approach, and we have developed a murine model of CML that is characterized by short disease latency and increased penetrance that has proven useful for investigating the role of BCR-ABL in the pathogenesis of CML.

Establishment of an efficient and reproducible mouse model of CML

Early murine bone marrow transplant (BMT) models using $P210^{BCR\text{-}ABL}$ were successful in generating mice that developed a myeloproliferative disease (MPD), yet only after a long latency, low penetrance, and a high incidence of acute leukemias and macrophage tumors (Daley *et al*., 1990; Kelliher *et al*., 1990). $P210^{BCR\text{-}ABL}$ expression was driven from the promoter of the myeloproliferative sarcoma virus (MPSV) and was stably expressed in BM from donor mice that had been injected with 5-Fluorouracil (5-FU) to enrich for cycling HSCs. Lethally irradiated BALB/c mice were reconstituted with BM heterogeneous for expression of $P210^{BCR\text{-}ABL}$. Eventually 20–30% of mice developed a CML-like disease with a latency of 63 days. These models clearly showed that $P210^{BCR\text{-}ABL}$ expression in a early hematopoietic progenitor led to a MPD closely resembling human CML.

The disadvantage of these early CML murine models was the low incidence of CML disease, which made it difficult to utilize the model for investigating issues relating to pathogenesis. The murine disease was difficult to induce due to difficulties in preparing high titer retroviral supernatants capable of infecting rare HSCs (Daley *et al*., 1991; Gishizky *et al*., 1993). We took several approaches to overcome this deficiency. First, we developed a methodology to produce high-titer, helper free retrovirus by transient transfection. Highly transfectable 293T cells were transfected with plasmids that encode Moloney packaging functions containing the gag, pol, and ecotropic envelope genes. The resulting cell line, termed BOSC 23, produces

$>10^6$ infectious units/ml of supernatant (Pear *et al*., 1993). Second, to maximize expression of the transgene in hematopoietic progenitors, we utilized retroviral vectors containing the murine stem cell leukemia virus (MSCV) long terminal repeats (LTR) (Figure 1b). The MSCV LTR is capable of high and sustained expression in immature hematopoietic cells, embryonic stem cells, and carcinoma cells (Hawley *et al*., 1993). Also with the hope of increasing the pool of hematopoietic stem cells (HSC) that could be transduced, we included stem cell factor along with IL-3 and IL-6 in the cytokine cocktail (Figure 1c).

An additional drawback of the early $P210^{BCR\text{-}ABL}$ BMT models was the inability to easily track $P210^{BCR\text{-}ABL}$ progenitor cells and their progeny after the BM was infused into the recipient mouse. Only a fraction of stem cells are successfully transduced, creating two BM compartments, one that expressed BCR-ABL and the remainder that was not transduced. To facilitate identification of the transduced cells, we subcloned BCR-ABL into the MCSV 2.2 vector (Hawley *et al*., 1994) along with an ECMV internal ribosomal entry site (IRES) that creates a bicistronic message that permits expression of green fluorescent protein (GFP) (Figure 1b). This strategy should alleviate the effects of promoter competition that frequently causes down-regulation of protein expression in internal promoter vectors (Schott *et al*., 1996). GFP expression is detectable within 48 h after transduction by its autofluorescence properties using either a fluorescent microscope or flow cytometry. This allows for rapid quantitation of retroviral titer and obviates the need to determine titer by drug selection, Southern blotting, or PCR. The MigR1 vectors express both *in vitro* and *in vivo*, and most importantly, GFP does not appear to affect hematopoietic development. The fluorescence intensity of GFP correlates with BCR-ABL expression (JA Wertheim, unpublished results). In addition to mice given BM expressing a GFP-only vector control, the non-transduced (GFP^-) compartment serves as an internal, negative control.

The combination of these alterations in the transduction and culture of BM progenitors had a dramatic effect on the disease induced by transplanted $P210^{BCR\text{-}ABL}$-expressing BM progenitors. All recipient mice receiving $P210^{BCR\text{-}ABL}$-expressing marrow died within four weeks of BMT due to a lethal MPD (Figure 2) (Pear *et al*., 1998). As early as 15 days post BMT, mice developed cachexia, lethargy and poor grooming. White blood cell counts were greater than $35\,000/\mu l$ and consisted of mature granulocytes, primarily polymorphonuclar neutrophils and metamyelocytes. Upon sacrifice, $P210^{BCR\text{-}ABL}$ recipient mice had marked splenomegaly, and contained a predominance of $Gr\text{-}1^+/Mac\text{-}1^+$ cells in the BM, PB, and spleen (Figure 3). In addition, they had pulmonary hemorrhage that appeared to be due to expansion of the myeloid cells into the pulmonary vessels (Figure 4). The pulmonary hemorrhage appeared to be the cause of death. In our initial experiments, the murine CML-like disease was frequently monoclonal; however our

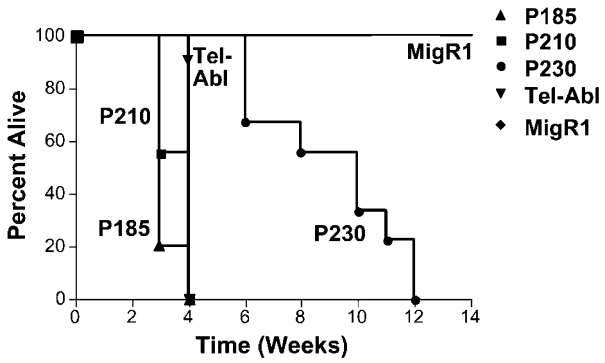

Figure 2 Survival of mice following BM transfer. $P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$, and TEL-ABL mice died or developed severe disease by 4 weeks post BM transplant. Both MigR1 (GFP control) and $P230^{BCR\text{-}ABL}$ mice were healthy at this time. Subsequently, the $P230^{BCR\text{-}ABL}$ mice succumbed to murine CML

subsequent results and those of others (Zhang and Ren, 1998; Li *et al*., 1999) show that $P210^{BCR\text{-}ABL}$ induces a polyclonal disease, suggesting that the fusion protein is both necessary and sufficient to induce the murine CML-like disease. The murine CML-like disease is capable of serial passage, although this is an inefficient process in our hands. Although the MPD can be passaged to several different recipient mice, eventually all mice develop a fatal T-cell leukemia/lymphoma (Pear *et al*., 1998). The T-cell leukemia is derived from the same clone as the MPD, suggesting that it has undergone blast transformation. This model has been adapted by several other laboratories and the ability of $P210^{BCR\text{-}ABL}$ to induce a rapid and fatal MPD has been observed by multiple investigators (Zhang and Ren, 1998; Li *et al*., 1999). The BCR-ABL induced MPD characterized by a fulminant and lethal expansion of non-blastic myeloid cells will be termed 'murine CML'.

Comparison of the murine myeloproliferative disease with human CML

With the development of a murine CML BMT model that efficiently results in a MPD, the question arises as to how relevant this model is to human CML. Murine CML shares many features with human CML. The murine MPD is characterized by a marked leukocytosis comprised primarily of mature neutrophils and myelocytes expressing the BCR-ABL fusion protein (Figure 4). Basophils and eosinophils are present in some mice. Blast forms are rare and lymphadenopathy is absent. The BM is hypercellular and there is splenomegaly; both due to a marked increase in maturing myeloid cells (Figure 4). There is also extensive extramedullary hematopoiesis (EMH) in the spleen and liver. Both the murine and human disease undergo blast transformation, and the blasts express the BCR-ABL protein and are derived from the same clone as the MPD.

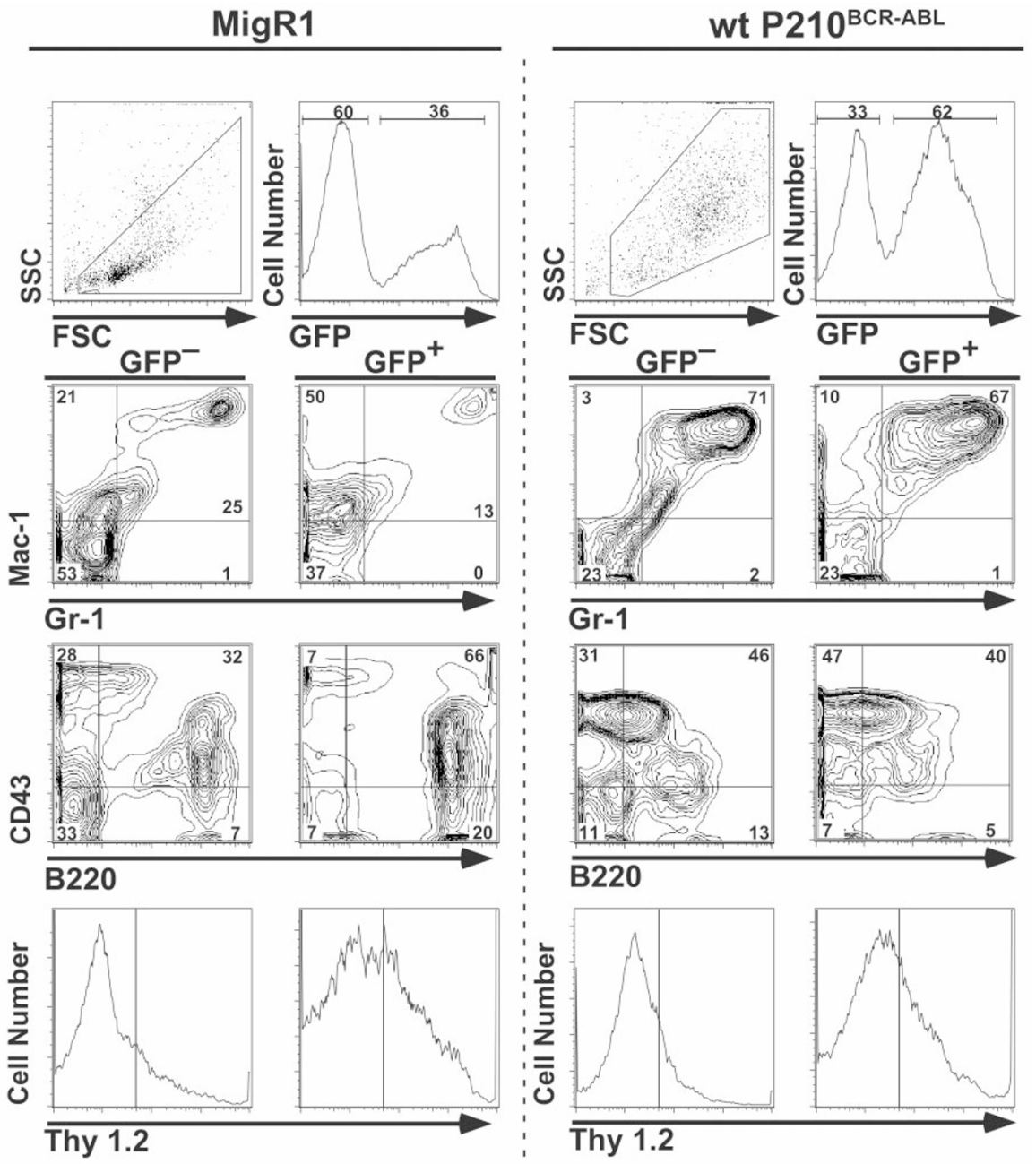

Figure 3 BM, PB and spleens from diseased BCR-ABL mice or healthy control mice (MigR1) were analysed to determine the immunophenotype of transplanted cells present in these organs. A flow cytometry profile of BM cells from a representative diseased wild type $P210^{BCR\text{-}ABL}$ mouse and a MigR1 mouse is shown. Live cells are gated for GFP expression that correlates with expression of the BCR-ABL insert. The majority of GFP^+ positive cells in wild type $P210^{BCR\text{-}ABL}$ mice stain Mac-1^+/Gr-1^+ indicating that the tumor is of granulocytic origin. GFP^- cells also stain Mac-1^+/Gr-1^+. This is most likely due to growth factors that are released by GFP^+ cells that cause bystander cells to proliferate

Despite these marked similarities, there are several features of murine CML that are dissimilar to human CML. Murine CML is a much more aggressive MPD than chronic phase CML. Murine CML occurs more rapidly, and leads to death. This rapid and fulminant disease course is likely to be a manifestation of the induction regimen. In patients, the disease arises from a single (or few) cell(s) in a previously immunocompetent individual. In contrast, murine CML is induced by transferring a population of BM progenitor cells expressing BCR-ABL to a severely immunocompromised, lethally irradiated host. The possibility that the host immune status is an important determinant of disease progression is suggested by the results of our single attempt to induce CML in non-irradiated recipients (a total of eight mice). These mice did not develop a fatal MPD during the 12 month observation period, as compared to their lethally irradiated

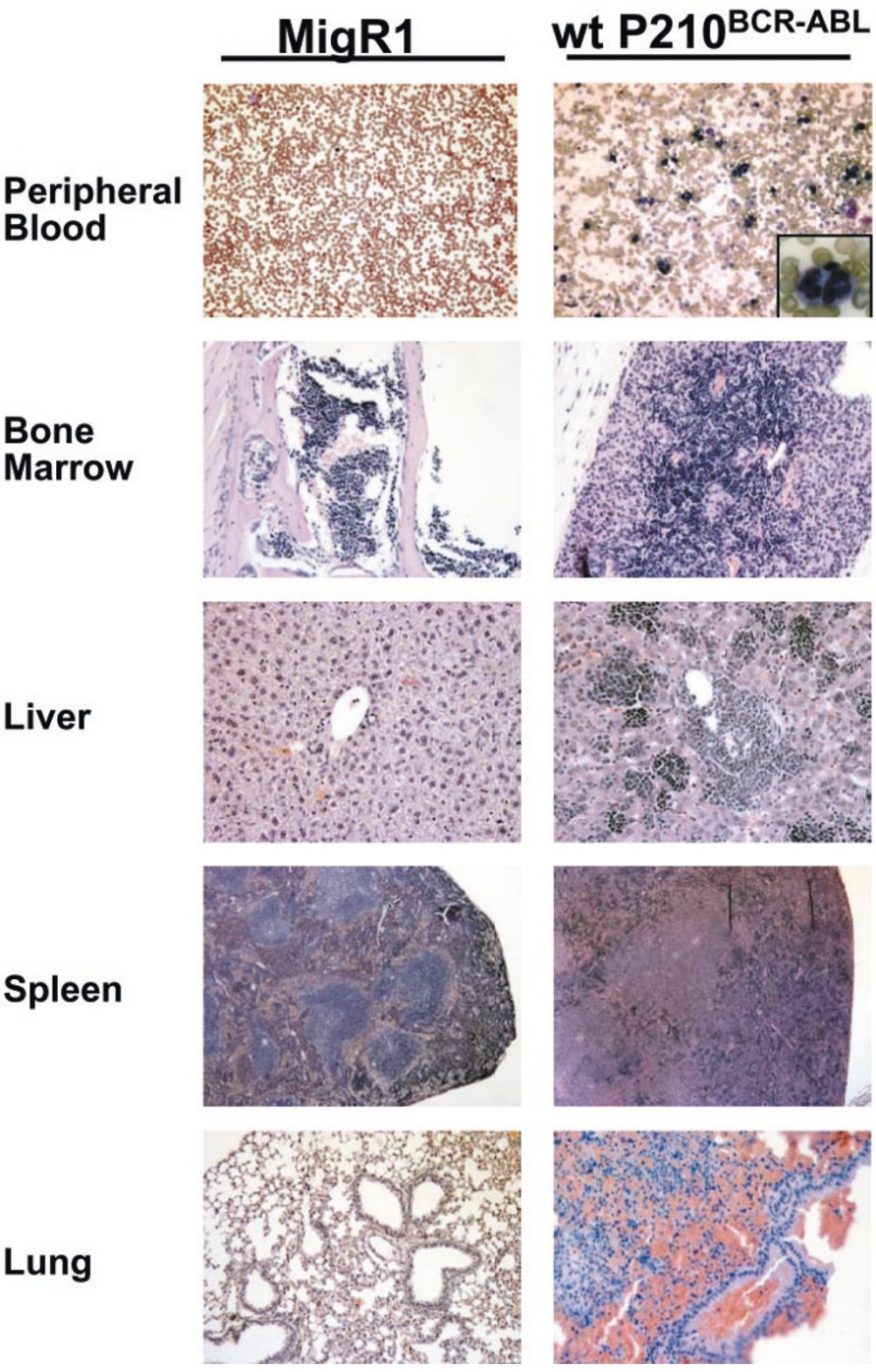

Figure 4 PB, BM, liver, spleen and lungs are shown from healthy MigR1 mice or diseased $P210^{BCR\text{-}ABL}$ mice at time of sacrifice. The PB of $P210^{BCR\text{-}ABL}$ mice contains mature neutrophils and the marrow is packed with granulocytes. The architecture of the liver and spleen are replaced by infiltrative neutrophils and the lung parenchyma is congested with erythrocytes indicating pulmonary hemorrhage

counterparts that had died by 4 weeks after receiving $P210^{BCR\text{-}ABL}$-transduced BM cells. This result suggests that altering the number of transduced input cells and the conditioning regimen of the recipient mice is likely to alter the disease course.

Unlike human CML, in which pulmonary manifestations are rare, murine CML is characterized by pulmonary hemorrhage secondary to myeloid proliferation and EMH in the lungs. This feature of the disease may also be due, in part, to our transplantation regimen of intravenous injection of 400 000 cells which is likely to cause some cells to lodge in the pulmonary vasculature due to the 'first pass effect'. It should be noted that the cellular proliferation in the lungs is not

entirely due to intravenous injection because neither the Y177F nor the $P230^{BCR\text{-}ABL}$ mice develop pulmonary hemorrhage (see below). Blast transformation in our murine model is usually T-lymphoid (rare in human disease) and myeloid blast crisis is not observed. This difference may be a manifestation of serial transplantation that is required to observe blast transformation. Spangrude *et al*. (1995) have shown that enriched hematopoietic progenitors lose their ability for multi-lineage reconstitution after three rounds of serial transplantation and show an increasing bias towards T-cell repopulation. Furthermore, engraftment of human CML cells into NOD/SCID mice resulted in a preponderance of T cells engrafting in the spleen (Lewis *et al*., 1998). Thus, serial transplantation itself may predispose towards T-cell blast crisis, and the current model may be insufficient for studying myeloid blast crisis. With the use of tyrosine kinase inhibitors and other methods to prolong the disease course in mice (Wolff and Ilaria, 2001), it may be possible to observe myeloid and/or lymphoid blast crisis in individual mice without reliance on serial transplantation.

In summary, murine CML is not identical to human CML. Murine CML does, however, exhibit the cardinal features of human CML. Murine CML is a biphasic disease that initially presents as a MPD and subsequently undergoes blast transformation. Both the myeloproliferative and blast phases of the disease are associated with expression of the introduced BCR-ABL fusion protein. The disease originates in a pluripotential hematopoietic cell that gives rise to a clonal malignancy involving both myeloid and lymphoid lineages. As such, murine CML is a unique and valuable model for investigating: (i) the role of BCR-ABL in transformation; (ii) the changes that occur in progenitor cells as the result of BCR-ABL expression; and (iii) the emergence of blast transformation.

$P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$ and TEL-ABL induce a similar myeloproliferative disease; whereas $P230^{BCR\text{-}ABL}$ induces a less severe myeloproliferative process

Four different abl fusion proteins are associated with human malignancy: $P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$, $P230^{BCR\text{-}ABL}$, and TEL-ABL (reviewed in Melo, 1996a). Each of these proteins is primarily associated with a different malignancy: $P210^{BCR\text{-}ABL}$ is found in nearly all patients with CML and 40% of patients with Ph+ ALL, $P185^{BCR\text{-}ABL}$ is rare in CML, but occurs in 60% of patients with Ph+ ALL, $P230^{BCR\text{-}ABL}$ is most commonly associated with N-CML, and TEL-ABL is a rare fusion in childhood ALL and CML. It is unclear whether the different abl transforming proteins are associated with specific disease spectrums as a result of the amount of bcr (or tel) fused to abl, or if the disease specificity is determined by the cell type in which the chromosomal translocation occurs.

We have utilized the murine assay to compare disease induction between $P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$, TEL-ABL, and $P230^{BCR\text{-}ABL}$. The cDNAs for the four abl fusion proteins were cloned into the MigR1 control vector allowing us to normalize the viral titer and ensure that normalized viruses gave equivalent levels of BCR-ABL expression following transduction of NIH3T3 cells. Mice receiving $P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$, and TEL-ABL developed the rapid onset of a fulminant CML-like disease that caused all recipient mice to become severely ill by 3–4 weeks following BMT (Figure 2, Table 1). The disease precipitated by $P185^{BCR\text{-}ABL}$ and TEL-ABL is similar to murine CML of $P210^{BCR\text{-}ABL}$ and is characterized by a high WBC (greater than 70 000 per μl) composed of >80% granulocytes, splenomegaly, and hepatomegaly. The only apparent difference in the diseases caused by the three different abl transforming proteins is the extent of pulmonary hemorrhage. All of the $P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$ and 50% of the TEL-ABL mice had extensive pulmonary hemorrhage, whereas the other half of the TEL-ABL mice had pulmonary infiltrates comprised of myeloid cells and EMH, but did not exhibit hemorrhage. The disease in the TEL-ABL mice differs in some respects from the recent results of van Etten and colleagues where they analysed a similar fusion (Million *et al*., 2002). These investigators observed a slightly longer latency (~1 week) and 50% of the mice succumbed to a $P210^{BCR\text{-}ABL}$-like disease whereas the other mice developed a small bowel syndrome characterized by neutrophilic infiltrates into the small intestine and endotoxemia. The difference between those mice that developed the typical CML-like disease and the small bowel syndrome is not clear. Possibilities include retroviral titer and/or transduction conditions, and this may also explain the differences between our results and those of van Etten's group.

Expression of GFP as a surrogate marker has allowed us to characterize the cell lineages involved in the leukemias caused by the three different transforming proteins. Compared to MigR1 control mice, there is a significant increase in the percentage of cells that express the GFP surrogate marker in the BM, PB, and spleens of BCR-ABL mice (Figure 3). The increased percentage of GFP positive cells in the diseased mice suggests that expression of the abl fusion proteins gives the cells a growth and/or survival advantage.

Consistent with our hypothesis that $P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$, and TEL-ABL cause a similar disease in our assay, the phenotypes of the cells populating the

Table 1 Frequency of murine CML induction by four different Ab1 transforming proteins

cDNA	*# of mice*	*# with CML*	*% with CML*	*Latency*
P210+GFP	16	16	100	3–4 weeks
P185+GFP	10	10	100	3–4 weeks
TEL-ABL+GFP	10	10	100	3–4 weeks
P230+GFP	16	16	100	6–14 weeks
GFP	9	0	0	No disease

The results comprise two independent experiments

spleen (as well as BM and PB) of the diseased mice are similar between the three different abl fusion proteins. The great majority of cells in the spleen (as well as PB and BM) express both Gr-1 and Mac-1 suggesting that they are granulocytes of varying differentiation stages (as indicated by the wide range of Gr-1 staining intensity) (Figure 3). There are also a significant percentage of nucleated red blood cells ($Ter119^+$ cells). There are few lymphocytes ($B220^+$, $Thy1.2^+$) present in the diseased mice. In contrast, the majority of cells in the control mice are lymphocytes. These results show that $P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$, and TEL-ABL have the capacity to cause a similar CML-like disease (Table 1). This suggests that the different spectra of human disease associated with these proteins is likely to result from the cell type in which the fusion protein is expressed and/or level of expression of the fusion protein, rather than an intrinsic property of the abl fusion protein.

In contrast to $P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$, and TEL-ABL, all of the $P230^{BCR\text{-}ABL}$ mice were healthy, without evidence of cachexia, lethargy, or labored breathing, at the time that all of the $P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$, and TEL-ABL mice succumbed to murine CML (Figure 2, Table 1). Although the $P230^{BCR\text{-}ABL}$ mice remained clinically healthy, their WBC began to rise precipitously and the differential counts suggested murine CML (not shown). The rising WBC alarmed us, since $P210^{BCR\text{-}ABL}$ mice developed severe symptoms after precipitous rises in their WBC. In order to investigate the etiology of the rising WBC, four mice were sacrificed at 5 weeks post BMT. The BM and spleens were similar to other CML mice, but there was minimal liver involvement and no evidence of pulmonary involvement, either grossly or microscopically. Over the next 6 weeks, four out of five mice remained outwardly healthy; however, their WBC continued to rise and they were sacrificed at various time points between 7.5 and 11 weeks post BMT. These mice were similar to the other $P230^{BCR\text{-}ABL}$ mice in that they had extensive spleen and BM involvement by the myeloproliferative process, but minimal hepatic and no pulmonary manifestations. Immunophenotyping of the involved tissues showed that the spleen, BM, and PB contained significant numbers of myeloid ($Gr\text{-}1^+$, $Mac\text{-}1^+$) cells and the pattern of involvement resembled that found in the $P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$, and TEL-ABL mice. One of the nine mice developed cachexia and died at 10 weeks post BMT.

These findings suggest that $P230^{BCR\text{-}ABL}$ induces a less severe MPD than $P210^{BCR\text{-}ABL}$, $P185^{BCR\text{-}ABL}$ and TEL-ABL which is consistent with the milder myeloproliferative disease (termed N-CML) associated with $P230^{BCR\text{-}ABL}$ expression in humans (Melo, 1996a). Patients with N-CML frequently have a more benign disease course characterized by lower absolute WBC, less splenomegaly, and they rarely develop blast crisis (Melo, 1996a). It should be noted that there may be overlap between the $P210^{BCR\text{-}ABL}$ and $P230^{BCR\text{-}ABL}$ diseases, and there is a report of one patient expressing the $P230^{BCR\text{-}ABL}$ fusion protein whose disease underwent blast transformation (Wada *et al*., 1995; Mittre *et al*., 1997). Van Etten and colleagues also investigated the $P230^{BCR\text{-}ABL}$ fusion in a murine BMT model and found that $P230^{BCR\text{-}ABL}$ induced a similar disease as $P185^{BCR\text{-}ABL}$ and $P210^{BCR\text{-}ABL}$ (Li *et al*., 1999). As discussed above, the differences with our results may result from differences in retroviral titers and/or transduction conditions. The differences between the results of the van Etten group and our group suggests that experimental conditions can influence the disease phenotype. Nevertheless, this may have physiological relevance as BCR-ABL fusion proteins are associated with a wide variety of human leukemias. Factors such as the cell type in which they are expressed and the level of expression may influence the phenotype of the disease. The murine model offers an opportunity to discriminate between these possibilities.

Use of the CML murine model to determine the role of BCR-ABL domains in causing CML

We and others have used the murine CML model to evaluate the contribution of various signaling domains within BCR-ABL to the development of murine CML. The ability to rapidly and reproducibly induce the murine MPD provides the ability to assay the requirement for different regions of BCR-ABL to induce murine CML. A number of investigators have examined $P210^{BCR\text{-}ABL}$ mutants for their ability to transform fibroblasts or hematopoietic cells *in vitro;* however, these studies have yielded equivocal and sometimes conflicting results regarding the ability of some BCR-ABL mutants to transform fibroblasts or hematopoietic cells (McWhirter *et al*., 1993; McWhirter and Wang, 1993; Pendergast *et al*., 1993). Because murine CML approximates many aspects of human CML, including having its origin in a HSC, the BMT assay is likely to provide a more relevant assay for studying the role of BCR-ABL in the pathogenesis of CML than assays based on immortalized cultured cell lines.

$P210^{BCR\text{-}ABL}$ Tyr177 binding site for grb2 is necessary for CML development

Grb2 is an adapter protein that mediates Ras activation (Lowenstein *et al*., 1992). It has the capacity to interact directly with both tyrosine 177 (Y177) in bcr through its SH2 domain and with proline rich sequences in c-abl by its SH3 domains (Pendergast *et al*., 1993; Ren *et al*., 1994); however, interaction with Y177 appears to be the physiologically relevant interaction (Pendergast *et al*., 1993). Mutation of Y177 to phenylalanine (Y177F) causes a marked decrease in fibroblast transformation by Y177F BCR-ABL, whereas this mutation does not affect the ability of Y177F BCR-ABL to confer factor independent growth on hematopoietic cell lines (Pendergast *et al*., 1993; Goga *et al*., 1995) Because of these discrepant results, it was unclear if the direct interaction between

BCR-ABL and grb2 played a role in the pathogenesis of CML.

To understand the importance of grb2 binding tyrosine Y177, and the ability of Y177F BCR-ABL to induce murine CML, we cloned Y177F BCR-ABL into the MigR1 vector. Y177F did not interact with grb2; however, its *in vitro* tyrosine kinase activity was similar to wild type BCR-ABL (He *et al*., 2002). Similar to previous results, Y177F failed to transform NIH3T3 fibroblasts, but efficiently caused 32D cells to become IL-3 independent. In the BMT assay, Y177F transduced cells failed to induce a MPD in recipient mice (Table 2). Instead, 11 out of 12 mice receiving Y177F transduced BM cells developed an immature clonal $CD4^+/CD8^+/GFP^+$ T-cell lymphoma with a latency of 9–20 weeks. The inability of Y177F to induce a fatal MPD was also reported by Ren's and van Etten's groups (Million and van Etten, 2000; Zhang *et al*., 2001a). Thus, the interaction between Y177 from bcr and grb2 appears to be essential for murine CML development; however, it is not required for other types of leukemias. The precise pathways activated through Y177 signaling are not known. One possibility is Ras, as grb2 mediates Ras activation through Sos, and Y177F fibroblasts fail to show Ras activation (Cortez *et al*., 1995; Goga *et al*., 1995). One potential problem with this hypothesis was that very little BCR-ABL protein localized to regions where Ras activation occurs (Van Etten *et al*., 1994; Skourides *et al*., 1999). A very recent study provides a new perspective on the role of grb2 binding by BCR-ABL. In work from Sattler *et al*. (2002), the BCR-ABL:grb2 interaction recruits the scaffolding adapter Gab2 to the SH3 domain of grb2. This association enhances both PI3K/Akt and Ras/Erk activation. Of particular interest, BM cells from Gab knockout mice are highly resistant to myeloid transformation and partially resistant to lymphoid transformation (Sattler *et al*., 2002).

The N-terminal coiled-coil domain is necessary for $P210^{BCR\text{-}ABL}$ induced CML

The coiled-coil domain likely mediates oligomerization of BCR-ABL, which in turn is proposed to activate tyrosine kinase activity and F-actin binding (McWhirter and Wang, 1991). The presence of the coiled-coil domain has been shown to be critical for transformation of fibroblasts *in vitro* (McWhirter *et al*., 1993). To investigate the function of the coiled-coil domain in the pathogenesis of murine CML, we utilized two different mutants: (1) in one construct (Δ1–63) BCR-ABL, the first 63 amino acids from BCR-ABL were deleted, and (2) in the second construct (1–63) BCR-ABL, the first 63 amino acids of bcr were fused to the c-abl common first exon (Table 2, Figure 5a). The latter construct deletes amino acids 64 to 927 of BCR-ABL. Deletion of the first 63 amino acids of BCR-ABL $P210^{BCR\text{-}ABL}$ is dispensable for growth factor independence of 32D cells (Table 2) although these cells proliferate slower than wild type $P210^{BCR\text{-}ABL}$ (He *et al*., 2002) (JA Wertheim, in preparation). Deletion of the coiled-coil domain also leads to a slight reduction in autokinase activity in whole cell lysates, with a minimal effect on *in vitro* phosphorylation of the crk substrate (He *et al*., 2002). The 1–63 BCR-ABL mutant had no effect on tyrosine kinase activity or on the transformation or growth of 32D cells (He *et al*., 2002; JA Wertheim, in preparation).

In the BMT assay, neither the 1–63 BCR-ABL nor the Δ1–63 BCR-ABL mutants were able to induce a MPD. 1–63 BCR-ABL induced a T-cell leukemia in six out of nine mice with a latency of 14–25 weeks (Table 2). Restoration of a portion of the SH2 binding domain including Y177 by fusing the first 210 amino acids of bcr onto c-abl (1–210) BCR-ABL (Figure 5a) restored the CML disease leading to death in 15 out of 15 mice within 5 weeks (Table 2). All of these animals

Table 2 Analysis of BCR-ABL mutants

Construct	*Functional deletion*	*In vitro tyrosine kinase activity*	*Fibroblast transformation*	*32D cell transformation*	*Disease in murine model*	*Adhesion to fibronectin*
Wild Type P210 BCR-ABL	None	Increased	Yes	Yes	CML	Increased
Y177F BCR-ABL	Grb2 binding	Increased	No	Yes	T-ALL	Increased
1-210 BCR-ABL	Part of SH2-binding, DBL, PH	Increased	NT	Yes	CML CML + B-ALL	Increased
Δ1-63 BCR-ABL	Coiled-Coil/ Oligomerization	Increased	No	Yes	T-ALL	Baseline
1-63 BCR-ABL	Y177, SH2-binding, Ser/Thr kinase, DBL. PH	Increased	No	Yes	T-ALL	Baseline
BCR-ABL ΔActin	F-, G-actin binding NES	Increased	No	Yes	CML	Baseline
BCR-ABL K1176R	Tyrosine kinase inactive	Baseline	No	No	No	Increased
Empty vector	No insert	Baseline	No	No	No	Baseline

Comparison between the effect of several of the mutants on *in vitro* transformation of fibroblasts and hematopoietic 32D cells as well as the ability to induce the murine CML-like disease and modulate cell adhesion of 32D cells to fibronectin. *In vitro* tyrosine kinase activity was determined by the ability to phosphorylate GST-crk. Fibroblast transformation studies are from McWhirter *et al*. (1993); McWhirter and Wang (1993); Pendergast *et al*. (1993). The *in vivo* analysis of P210K1176R is from Zhang *et al*. (1998). Adhesion data is from Wertheim *et al*. (2002); JA Wertheim, in preparation). NT = not tested

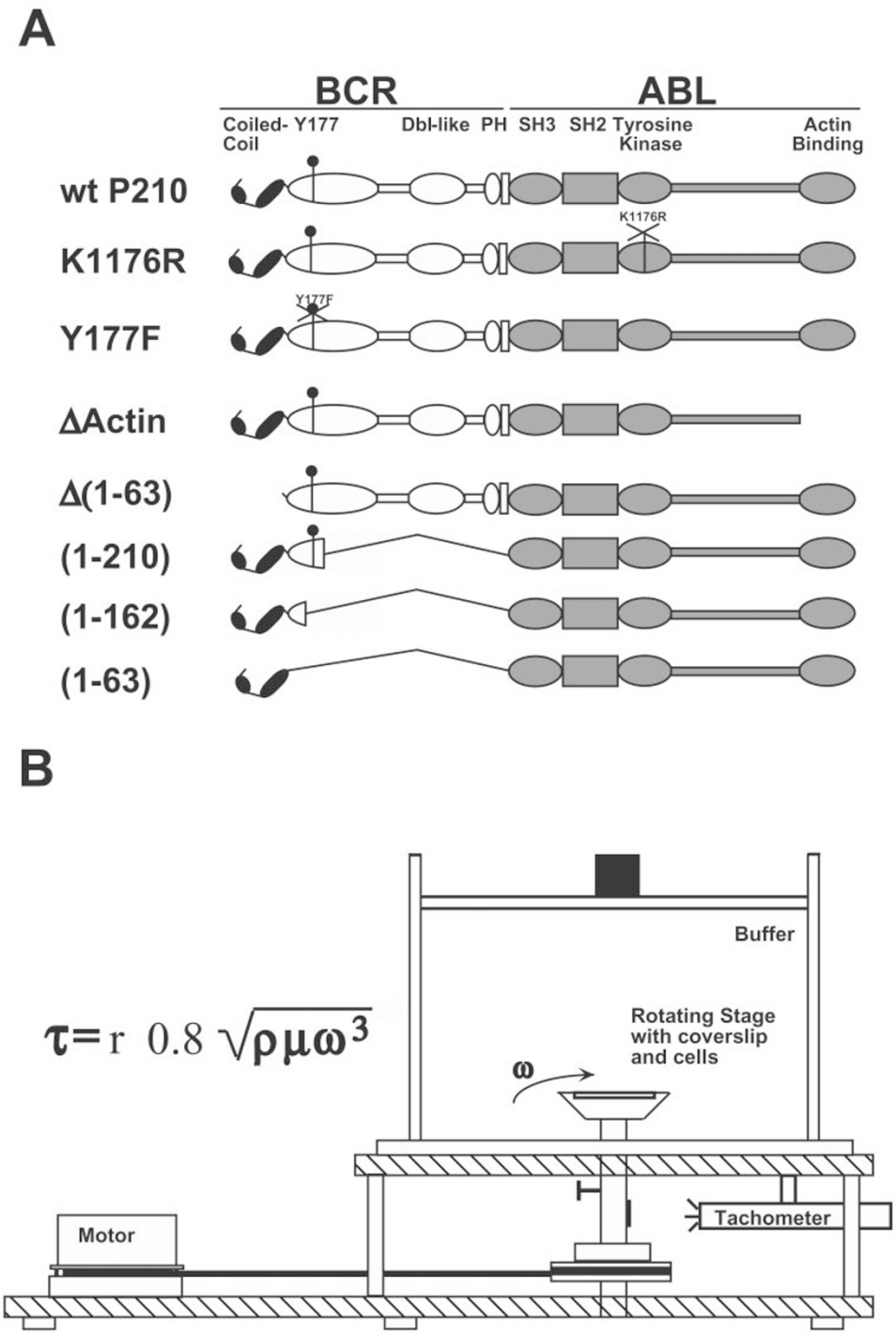

Figure 5 BCR-ABL mutants and schematic representation of the spinning disk cell detachment device. (**a**) BCR-ABL mutants were cloned into the MigR1 vector for mouse BM transplantation studies or the pK1 vector containing the puromycin resistance gene in place of GFP for adhesion experiments. K1176R is a lysine to arginine point mutation in the kinase domain that abrogates tyrosine kinase activity. Y177 is the phosphorylated tyrosine that binds grb2, PH stands for the plextrin homology domain, and the actin binding domain contains a nuclear export signal between the G- and F-actin binding sites. (**b**) 32D cells adhere under static conditions to a monolayer of fibronectin before the stage is rotated. Movement of the stage creates laminar flow across the cover slip that forms a shear stress gradient that dislodges bound cells. The shear stress varies linearly with the radius, thus cells at the center of the cover slip experience little to no detachment force whereas cells near the periphery experience the highest force. For this system, shear stress is calculated from the equation shown in the figure where τ is the shear stress, r is the distance from the center of the cover slip, ρ is the density, μ is the dynamic viscosity and ω is the angular velocity

developed features of murine CML. Of interest, a subset of these mice developed a concomitant B cell leukemia that was transplantable (He *et al*., 2002).

Three out of 10 mice given BM expressing Δ1–63 BCR-ABL developed T-cell acute lymphocytic leukemia, one mouse was dispatched at day 97 without evidence of disease, and the remaining mice were healthy 167–210 days after BMT. One possibility that could account for the absence of a MPD in Δ1–63 BCR-ABL mice is that the coiled-coil region may decrease tyrosine kinase activity below a threshold level needed to cause CML. Alternatively, deletion of the coiled-coil domain, which abrogates the colocalization of BCR-ABL with F-actin, may be less oncogenic in our model because $P210^{BCR\text{-}ABL}$ is dissociated from F-actin. To test this hypothesis, we gave mice BM expressing BCR-ABL ΔActin that lacks the G- and F-actin binding domain (Figure 5a, Table 2). We found that five out of five mice developed murine CML. Further supporting the hypothesis that the coiled-coil domain regulates tyrosine kinase activity, Ren and colleagues found that BM cells expressing a mutant lacking both the coiled-coil domain and the abl SH3 domain which negatively regulates tyrosine kinase activity, resulted in murine CML (Zhang *et al*., 2001a). The results of several groups (Million and Van Etten, 2000; Zhang *et al*., 2001a) show that both elevated tyrosine kinase activity, which can be facilitated by oligomerization, and signaling through grb2 are required for efficient murine CML induction. As such, both the oligomerization domain and grb2 binding site in bcr are potential therapeutic targets in combination with tyrosine kinase inhibitors.

A quantitative cell detachment device to measure adhesion of $P210^{BCR\text{-}ABL}$ expressing cells to fibronectin

Several laboratories have investigated the effect of $P210^{BCR\text{-}ABL}$ on cell adhesion to stromal cells and fibronectin. All of these groups have used various forms of plate and wash assays with the readout step being a direct count of remaining adherent cells (Bazzoni *et al*., 1996; Kramer *et al*., 1999) or enumeration of colonies after incubation of adherent cells in long-term BM cultures for several weeks (Verfaillie *et al*., 1992). The shortcoming of adhesion studies of $P210^{BCR\text{-}ABL}$-expressing cells is the inconsistent results that may be due to assays that use nonstandard washing techniques to determine cell adhesion. There is also variability between cells used to express $P210^{BCR\text{-}ABL}$. Adhesion experiments like many other studies using primary patient tissue are complicated by the inability to identify the critical hematopoietic cell that is responsible for initiating CML. To partially overcome this, the immature hematopoietic $CD34^+$ population is commonly isolated; however, this population remains heterogeneous. For instance, studies by Verfaillie *et al*. (1992) using $CD34^+$ BM show that the HLA DR^+ fraction adhered less efficiently to irradiated stroma compared to HLA DR^- cells from CML patients, suggesting there may be heterogeneity within the CD34 compartment with regard to adhesion. Additionally, variability among primary human cells harvested from the BM, PB, or umbilical cord blood from CML patients or healthy donors may differ slightly depending upon the place of origin, and the use of CML patient donors in different stages of CML treatment further complicate a complete analysis of the role of $P210^{BCR\text{-}ABL}$ in cell adhesion to fibronectin.

Verfaillie and colleagues have performed the most extensive series of studies analysing adhesion in normal and CML BM by employing a plate and wash assay with fibronectin or stromal cells as an adhesive surface (Verfaillie, 1992; Verfaillie *et al*., 1994). After washing, adherent and non-adherent cell fractions were expanded in colony forming assays over a 5–6 week period, and colonies were enumerated, each representing an adherent or non-adherent cell capable of populating a colony under defined culture conditions. Using this assay, Verfaillie's group found that normal hematopoietic cell proliferation is negatively regulated by adhesion to fibronectin. Moreover $CD34^+$ HLA DR^- cells from CML patients exhibit decreased adhesion to stroma or fibronectin compared to cells from healthy donors, suggesting that contact inhibition of cell proliferation is lost by the expression of Ph^+ (Verfaillie *et al*., 1992, 1997a,b; Hurley *et al*., 1995). Alternatively, Kramer *et al*. (1999) and Bazzoni *et al*. (1996) reported that $P210^{BCR\text{-}ABL}$ and IL-3 lead to increased adhesion between BaF3, Mo7, and 32D cells expressing $P210^{BCR\text{-}ABL}$ and fibronectin. Both studies determined the total number of cells remaining attached to fibronectin after an initial washing period. In contrast to primary cells, expression of $P210^{BCR\text{-}ABL}$ in cell lines resulted in a defined and homogeneous population of cells. The discrepancy between the decreased binding to fibronectin in primary cells from CML patients, and the increase in adhesion found by Bazzoni and Kramer, is unclear, but is likely due to cell specificity.

One drawback of panning-style adhesion assays is that they do not use a standardized detachment force to dislodge adherent cells. This may lead to different results between laboratories due to inconsistent washing techniques. To overcome this problem, we have utilized a quantitative cell detachment device that uses a controllable and measurable detachment force to remove cells from a monolayer of fibronectin (Wertheim *et al*., 2002) (Figure 5b). Additionally, this device allows for a quantitative determination of cell adhesion that can discern small changes in cell adhesion.

Spinning disks have been used to study adhesion between K562 erythroleukemia cells and fibronectin (Garcia *et al*., 1998), between rat osteoblast-like cells and bioactive glass (Garcia and Boettiger, 1999), and between K562 cells and vitronectin (Boettiger *et al*., 2001). Cells are first incubated on a fibronectin-coated cover slip mounted on the stationary pedestal of the spinning disk adhesion device (Figure 5b). Cells bind

fibronectin within 15 min, at which time the pedestal is rotated at a predetermined speed that causes the buffer within the chamber to circulate over the cover slip and detach bound cells. The velocity of the buffer and the force (shear stress) used to detach bound cells is readily calculated (Sparrow and Gregg, 1960) and the detachment force is proportional to the distance of the cells from the center of the cover slip (Figure 5b). After a spin, the remaining cells and their position from the center of the cover slip are determined using optical recognition software. Cells near the center experience little or no detachment force, whereas cells at the periphery are nearly all dislodged after a 10-minute spin. The increasing detachment force creates a distribution profile along the cover slip in which the number of remaining cells decreases as a function of radial distance from the center of the cover slip. A parameter that indicates the degree of cell adhesion is called the critical shear stress, and is the detachment force (shear stress) at which half of the cells are dislodged by the circulating buffer (Cozens-Roberts *et al*., 1990a,b). The critical shear stress of a population of cells incubated under different conditions that either increase or decrease adhesion, such as with integrin blocking antibodies or expression of $P210^{BCR\text{-}ABL}$, can be compared and the effect on adhesion can be quantified.

To assay the effect of $P210^{BCR\text{-}ABL}$ expression on cell adhesion, we expressed $P210^{BCR\text{-}ABL}$ in IL-3 dependent 32D myeloblastic cells. $P210^{BCR\text{-}ABL}$ increased the binding strength of a population of transformed 32D cells to fibronectin by 1.7-fold compared to control cells (Wertheim *et al*., 2002). This finding is in agreement with those of Kramer *et al*. (1999) and Bazzoni *et al*. (1996) showing that $P210^{BCR\text{-}ABL}$ leads to increased adhesion in hematopoietic cell lines.

One mechanism to explain the $P210^{BCR\text{-}ABL}$ increased adhesion is that the tyrosine kinase of $P210^{BCR\text{-}ABL}$ may initiate inside-out signaling causing integrin activation. In this scenario, the $P210^{BCR\text{-}ABL}$-induced increase in fibronectin binding would be dependent on the $P210^{BCR\text{-}ABL}$ tyrosine kinase activity. To evaluate this possibility, we expressed a tyrosine kinase defective form of $P210^{BCR\text{-}ABL}$ (BCR-ABL K1176R) in 32D cells to determine the effect on adhesion. We found that expression of BCR-ABL K1176R or treatment of $P210^{BCR\text{-}ABL}$ cells with STI571 led to attenuation of the wild type tyrosine kinase activity and failed to transform 32D cells, but did not reduce the elevated adhesion seen with wild type $P210^{BCR\text{-}ABL}$. This observation suggests that the adhesion effect of $P210^{BCR\text{-}ABL}$ is independent of its tyrosine kinase activity. We further confirmed this result using the cell line, Meg-01, from a patient with CML and found that STI571 had no effect on cell adhesion; however treatment of these cells with STI571 for 3 h led to a striking decrease in tyrosine kinase activity (Wertheim *et al*., 2002). From this finding, it is unlikely that the tyrosine kinase activity of $P210^{BCR\text{-}ABL}$ enhances cell adhesion through inside-out signaling that would originate from the tyrosine kinase domain of $P210^{BCR\text{-}ABL}$. The tyrosine kinase activity is necessary for *in vitro* cell transformation and for the development of murine CML (Zhang and Ren, 1998). Our observation that defective adhesion persists in the absence of tyrosine kinase activity suggests that abnormal binding to fibronectin is unlikely to be a primary contributor to CML development.

Our structure-function studies using the murine CML assay identified regions other than the tyrosine kinase domain of $P210^{BCR\text{-}ABL}$ that are needed for murine CML. It is possible that these regions, specifically Y177 and the coiled-coil domain, may be involved in regulating cell adhesion. We asked whether cell adhesion is related to the inability of mutations in $P210^{BCR\text{-}ABL}$ to induce the wild type murine CML disease. To achieve this, we evaluated the same mutants (Figure 5a) that we used in our murine CML model by expressing these mutants in 32D cells and evaluating their ability to bind fibronectin in our spinning disk cell detachment device. We found that the effect of these mutants on cell adhesion to fibronectin did not show a clear connection with their ability to induce or attenuate the murine CML disease (Table 2). Thus adhesion to fibronectin cannot be used as a prediction of mutations that will abrogate murine CML.

In contrast to the inability to connect adhesion to disease, we observed a direct effect of $P210^{BCR\text{-}ABL}$ expression on cell adhesion. In particular, we found that deletion of the C-terminal actin-binding domain (ΔActin BCR-ABL) reduced cell adhesion compared to wild type $P210^{BCR\text{-}ABL}$; however, this mutant generates a murine CML disease that is nearly undistinguishable from the wild type disease (JA Wertheim, manuscript in preparation). This suggests that localization of $P210^{BCR\text{-}ABL}$ to F-actin filaments may regulate $P210^{BCR\text{-}ABL}$'s effect on adhesion to fibronectin. Two related lines of evidence support the cytoskeletal involvement of $P210^{BCR\text{-}ABL}$ with its effects on integrin mediated cell adhesion. First, we found that both localization of $P210^{BCR\text{-}ABL}$ to F-actin and cell adhesion are independent of the $P210^{BCR\text{-}ABL}$ tyrosine kinase activity (Wertheim *et al*., 2002). Second, $P210^{BCR\text{-}ABL}$ alters the ability of β_1 integrins to aggregate, or cap, on the surface of cells by modifying cytskeletal-integrin connections (Bhatia *et al*., 1999). Altering the distribution of integrins on the surface of cells by preventing integrin aggregation may lead to the changes in adhesion in $P210^{BCR\text{-}ABL}$ expressing cells.

The coiled-coil domain activates both the c-abl tyrosine kinase activity and the c-terminal actin binding domain. The Δ1–63 BCR-ABL mutant fails to induce the murine CML disease, instead causing a T-cell leukemia with an extended latency. This effect may result from the moderately depressed tyrosine kinase activity caused by the inability of this mutant to oligomerize. However, we find that this mutant fails to elevate cell adhesion (Table 2). This most likely results from the inability of this mutant to co-localize with F-actin filaments, leading to decreased adhesion compared to wild type $P210^{BCR\text{-}ABL}$, in a similar

manner to ΔActin BCR-ABL (JA Wertheim, manuscript in preparation).

It is likely that oligomerization of $P210^{BCR-ABL}$ has a critical role in several functions of $P210^{BCR-ABL}$. Clinical trials showing that the $P210^{BCR-ABL}$ tyrosine kinase inhibitor STI571 can lead to hematologic and cytogenetic remission in a high proportion of CML patients suggests that the tyrosine kinase domain of $P210^{BCR-ABL}$ is central to its ability to cause and maintain the CML disease (Druker *et al*., 2001). Oligomerization enhances tyrosine kinase activity indicating that the coiled-coil domain likely plays a role in CML through its effects on tyrosine kinase activity. Additionally, oligomerization permits co-localization of $P210^{BCR-ABL}$ with F-actin, a process that is unaffected by the tyrosine kinase activity (Wertheim *et al*., 2002). It is likely that co-localization of $P210^{BCR-ABL}$ with F-actin is linked to the effect of $P210^{BCR-ABL}$ effect on cell adhesion since a decrease in tyrosine kinase activity, such as that seen with $\Delta 1-63$ BCR-ABL, is not contributory to the effect $P210^{BCR-ABL}$ on cell adhesion (Wertheim *et al*., 2002). This suggests that that two results of oligomerization, elevation of tyrosine kinase activity and co-localization with F-actin, are primary determinants of murine CML and cell adhesion, respectively.

Together, our cell adhesion results have shown that elevated binding between $P210^{BCR-ABL}$ cells and fibronectin is independent of the tyrosine kinase activity and the IL-3 transformation status of 32D cells. Modulation of adhesion by $P210^{BCR-ABL}$ is a specific effect mediated by localization of $P210^{BCR-ABL}$ with F-actin filaments through either direct modification of F-actin or through recruitment of additional proteins to the cell cytoskeleton.

Conclusions

The mouse model of CML has become one of the standard assays used to understand the role of $P210^{BCR-ABL}$ in causing cancer. We and others have generated protocols to transduce murine BM with high titer retroviral vectors allowing for elevated expression of $P210^{BCR-ABL}$ in HSCs. Transplantation of transduced BM into lethally irradiated recipients causes a rapid MPD in 100% of mice. In general, the phenotypes of murine CML are similar between different laboratories and provide strong evidence that BCR-ABL is both necessary and sufficient for CML induction. The ability to easily reproduce this model should provide the opportunity to utilize it to investigate the events that lead to blast transformation and to use it as a tool for drug development. Several recent reports suggest that the model may be adapted for both of these purposes (Cuenco and Ren, 2001; Wolff and Ilaria, 2001; Dash *et al*., 2002).

Our mutational analysis has identified several domains in $P210^{BCR-ABL}$ that are critical for the development of a murine CML-like disease and/or have an effect on cell adhesion. We and others (Million and van Etten, 2000; Zhang *et al*., 2001a) have shown that oligomerization of $P210^{BCR-ABL}$ and binding of grb2 to Y177 of $P210^{BCR-ABL}$ is required for efficient induction of murine CML. The demonstration that mutation of the grb2 binding site had a profound effect on murine CML illustrates the importance of using relevant assays to model human leukemias as the Y177F mutation blocked fibroblast transformation, but had little effect on *in vitro* tyrosine kinase activity or growth of factor-dependent hematopoietic cell lines (Pendergast *et al*., 1991).

Although the murine models are sensitive to discern effects on the coiled-coil domain and Y177F, they were unable to distinguish effects between $P185^{BCR-ABL}$ and $P210^{BCR-ABL}$, when expressed in HSCs. This was surprising, as $P185^{BCR-ABL}$ is rarely associated with CML and frequently associated with ALL. One explanation is that the expression level of these particular fusions may not mimic their expression in HSCs in patients, as the retroviral LTR leads to relatively high levels of expression. An alternative explanation is that these proteins may not be expressed in the same cell types in human disease. This latter hypothesis runs counter to results that demonstrate $P185^{BCR-ABL}$ expression in SCID repopulating cells and myeloid colony forming cells from patients with ALL suggesting that a multipotent stem cell may initiate disease (Tachibana *et al*., 1987; Cobaleda *et al*., 2000). Nevertheless, van Etten and colleagues found a significant difference in the ability of $P185^{BCR-ABL}$ and $P210^{BCR-ABL}$ to cause leukemia in non-FU (i.e. non HSC) treated cells, with $P185^{BCR-ABL}$ causing a more aggressive disease. This suggests that the cell type in which the fusion proteins are expressed may have an important impact on disease phenotype.

Increased adhesion in $P210^{BCR-ABL}$-expressing 32D cells is independent of tyrosine kinase activity. This finding together with other investigators' experiences with the kinase dead K1176R BCR-ABL mutant that is inert in mice (Zhang and Ren, 1998), suggest that abnormal adhesion to fibronectin alone is not sufficient to induce leukemia. The coiled-coil domain is critical to both disease initiation and $P210^{BCR-ABL}$-mediated elevated cell adhesion in our assay, yet the precise role of the $P210^{BCR-ABL}$ oligomerization domain in contributing to CML and adhesion remains unclear. F-actin localization is also independent of the $P210^{BCR-ABL}$ tyrosine kinase activity. Localization of $P210^{BCR-ABL}$ to the cell cytoskeleton may permit $P210^{BCR-ABL}$ to directly modify F-actin or recruit proteins that may influence cell adhesion. However, localization to the cell cytoskeleton is unlikely to be important in causing murine CML, since the wild type MPD persists in our recipient mice given BM expressing a BCR-ABL mutant that lacks the entire actin binding domain (ΔActin BCR-ABL). Our results showing that the ΔActin mutant fails to attenuate CML contrasts the finding by Heisterkamp *et al*. (2000) that nearly all $P190^{BCR-ABL}$ transgenic mice fail to develop the wild type B-ALL disease when the F-actin binding domain is removed. It is likely that intrinsic differences between

the two murine models or additional bcr sequences in our mutant derived from $P210^{BCR\text{-}ABL}$ permit the CML disease in the absence of F-actin localization.

Our results indicate that the effect of oligomerization on tyrosine kinase activity and actin binding have separate outcomes. Localization of $P210^{BCR\text{-}ABL}$ to F-actin appears to mediate its effect on adhesion. Alternatively, evidence from several laboratories suggests that its effect on tyrosine kinase activity is vital for CML development (Zhang *et al*., 2001a). However, the decline in tyrosine kinase activity is small in the absence of the coiled-coil domain as shown by a minimal effect on *in vitro* crk phosphorylation (He *et al*., 2002). Interestingly, deletion of the SH2 domain of $P210^{BCR\text{-}ABL}$ reduces the BCR-ABL tyrosine kinase activity to half wild type levels, a much larger reduction than what we observe with deletion of the coiled-coil domain, yet removal of the c-abl SH2 domain is compatible with CML development in mice with a longer latency and underlying B-ALL (Zhang *et al*., 2001b). Alternatively, the coiled-coil region may mediate the formation of protein–protein complexes that are necessary for adhesion and/or CML development (Tauchi *et al*., 1997).

Lastly, it should be noted that different groups have identified different phenotypes when expressing the same mutants. For example, the Ren and van Etten laboratories found that the Y177F mutant caused the transient appearance of a MPD prior to lymphoid leukemia/lymphoma, whereas we did not observe evidence of a MPD. These discrepant results may be due to the expression level of the introduced fusion protein, the cell types transduced by the viruses, and/or the number of cells in which the fusion proteins are expressed. Although this slight variability in the murine disease between laboratories provides a challenge in interpreting the results, it may also shed additional insights into human CML where there is variability from patient to patient. Identifying the parameters that cause variability in the murine disease may identify factors that influence the phenotype and disease course in patients. Finally, our experience in developing an efficient mouse model of CML and using it to investigate the role of BCR-ABL in the pathogenesis of CML has taught us that *in vitro* tests to determine oncogenicity, including cell transformation and cell binding assays, cannot always be substituted for experiments that use conditions that closely approximate the natural disease environment.

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