PATHOGENESIS AND PROGRESSION OF CHRONIC MYELOID LEUKEMIA

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C hronic myeloid leukemia (CML) is a myeloproliferative disorder whose clinical hallmark is the abnormal expansion of clonal hematopoiesis still capable of achieving terminal differentiation. CML exhibits a biphasic clinical course: it originates as an indolent disease, the initial chronic phase, which results from clonal expansion of a pluripotent or multipotent hematopoietic progenitor cell compartment retaining normal phenotype and normal functioning. The real leukemia, termed blast crisis, represents the ineluctable outcome of the chronic phase of the disease. It is marked by the emergence within the clonal hematopoiesis of fully transformed cell clone(s) arrested at an early stage of differentiation, either myeloid or lymphoid.

CML clonal hematopoiesis is identified by a cytogenetic marker, the Philadelphia (Ph1) chromosome.1 The Ph1 chromosome is generated by a reciprocal translocation between the long arms of chromosomes 9 and 22. As a consequence of this translocation, most of the c-abl protooncogene located on chromosome 9 joins to a gene located on chromosome 22, called the breakpoint cluster region (bcr). The resulting rearranged gene, the bcr/abl chimera, contains approximately the first half of the bcr-coding sequences starting from the breakpoint cluster region, followed by the abl sequences downstream to exon 2.2 It transcribes an 8.5 kb mRNA that codes for a 210 kDa hybrid product, termed p210, in which the normal N-terminus sequence of the c-abl polypeptide has been replaced by the bcr-coded sequences. Clinical and experimental evidence supports the crucial role of p210 in determining and sustaining CML.3

Normal and clonal hematopoiesis coexist in CML

A prominent feature of CML is that the mutagenic event leading to bcr/abl rearrangement spares some normal hematopoiesis. The persistence of normal, Ph1-negative and non clonal hematopoiesis was first demonstrated by the Vancouver group in studies evidencing a progressive exhaustion of clonal Ph1+ hematopoietic progenitors maintained under long-term culture,4,5 and was supported by more recent studies that indicate a marked prevalence of normal progenitors within the putative hematopoietic stem cell compartment, which is functionally characterized by its ability to sustain prolonged hematopoiesis under long-term culture system (LTC-IC) and phenotypically identified as CD34+/CD33−/HLA-DR/low rhodamine expressing.6,7

One unanswered question is whether the size of normal hematopoiesis is maintained over the course of the disease. This question is not just speculative, but has clinical implications. In fact, since at the moment autotransplant of CD34+/LTC-IC selected progenitors seems to be a promising approach for curing CML patients who lack a sibling bone marrow donor, the progressive decrease in the size of Ph1- hematopoiesis, which may parallel disease progression, restricts autologous bone marrow transplantation (ABMT) to an early stage of the disease. However, in our experience and that of other groups, some normal hematopoiesis is still present in the terminal phase of CML.8

A second question concerns the transcriptional silence of the bcr/abl chimera: is it associated with cell quiescence and is whether eventually reverted by proliferation and differentiation along the myeloid pathway.9,12 Combined analy-
sis of the level of bcr/abl rearrangement, transcription and product within selected progenitor cell compartments is required for proper identification and quantitation of real normal hematopoiesis, in order to validate any strategy for either CML bone marrow harvest purging or in vitro expansion of early progenitors intended for ABMT.

Regarding the pathogenesis and progression of CML, the basic question is: Why, given the coexistence of normal and clonal hematopoiesis at a very early stage of commitment still possessing self-renewal, does the natural history of the disease see the expansion of the clonal Ph1+ hematopoiesis over the normal one? The most likely answer resides in p210 bcr/abl-mediated release of CML hematopoiesis from regulatory controls on proliferative activity, which lets clonal progenitors take the first step toward leukemic transformation. Since the loss of control of proliferation in CML progenitors is associated with additional events (extension of cell survival, priority of replicative over restraining pathways and/or inactivation of tumor suppressor genes) which themselves harbor genetic instability potential, the final result will be the inevitable onset of more transforming genetic mutation(s) and the emergence of more aggressive clone(s).

In the present review we will briefly summarize the biomolecular events associated with p210 bcr/abl expression in order to define the role it plays in both the pathogenesis and progression of CML.

**P210 bcr/abl has multiple effects on signal transduction**

P210 bcr/abl has intrinsic tyrosine kinase activity, which leads to constitutive phosphorylation on tyrosine of the intracytoplasmic substrate proteins necessary for the transduction of mitogenic signals. Part of the proteins constitutively phosphorylated by p210 are also evoked in response to IL-3. The partial convergence of potential substrates for p210 bcr/abl and IL-3 may contribute to the exaggerated proliferative response of CML progenitors to growth factors in vitro.

The oncogenic potential of the chimera product, which overall results in an upregulation of its tyrosine kinase activity, was first attributed to the abl section because of its stringent similarity in both structure and functional activity with the gag-ABL fusion product of the Abelson murine leukemia virus (AMuLV). More recently, a great deal of interest has been directed towards bcr, how stable interaction with abl might perturb both its own and abl normal functioning and get involved in cell transformation. The bcr contribution to p210 bcr/abl oncogenic potential is due to the kinase activity of the product of its first large exon, the gene section shared by the two alternative products of the chimera.

Besides its ability to autonomously trigger the mitogenic signal, p210 bcr/abl elicits its biological effects by interacting with other signal transduction pathways. Actually, the matter of substrates for p210 kinase activity is quite far from being a clear framework and shows a great deal of redundancy, whose functional relevance is still obscure.

The most prominent result of both bcr and abl kinase activity is the switching on of the Ras-signalling pathway, which in turn provides active GTP-bound forms of Ras proteins access to cortical cytoskeleton components, in particular to actin filaments. As a final consequence, the activation of the ras pathway relieves clonal progenitors of the need for growth factor ligand to cognate receptors and contact-mediated events for transduction of the mitogenic signal.

Constitutive p210 bcr/abl signal transduction is further provided by the serine-threonine kinase activity of the bcr product and by heavy phosphorylation of the p41 (p39) CRKL product, the specific ligand binding for c-ABL. Alternatively, since CRKL can be envisioned as a candidate for differentiative functions and since in normal hematopoietic progenitors it is present only in the non-phosphorylated form, its phosphorylation by p210 could possibly result in a loss of function and account for the accumulation of myeloid progenitors located at an intermediate level of differentiation in CML chronic phase.

Additional targets for p210 bcr/abl kinase activity are the src-related tyrosine kinases...
p53/p56 (the protein tyrosine kinases associated with the cytosolic domains of CD4 and CD8 T antigens), the p145 c-kit receptor tyrosine kinase, the cell-lineage specific tyrosine kinase p93 c-fes, the proteins of the 14-3-3 family, the p95 product of the vav protooncogene, the Jak tyrosine kinase family, the p85 subunit of phosphatidylinositol-3-kinase, and the focal adhesion phosphotyrosine paxillin. Finally, the transactivation of early nuclear events c-fos and c-jun by some p210 activated pathways, including p21 ras, MAP-2 and p70 raf-1, further contributes to the withdrawal of clonal hematopoietic progenitors from proliferation control.

**p210 bcr/abl affects adhesive properties of clonal hematopoiesis**

The second distinctive feature induced in CML progenitors by p210 expression is a change in their adhesion properties. Since adhesive ligands represent a tool for extensive cross talk between cells and keep a large number of cell functions (such as gene expression, cell growth and differentiation, cytoskeletal structure, cell-mediated immunity, etc) under control, altered adhesion of CML hematopoiesis has multiple consequences.

Part of the adhesive defect of CML hematopoiesis is mediated through abnormalities of phosphatidylinositol (PI)-linked surface receptors, which are involved in both reduced adhesion of CML progenitors to the bone marrow stromal compartment, the hematopoietic microenvironment, and decreased expression of ectoenzyme leukocyte alkaline phosphatase. In particular, deficient expression of one PI-linked cell surface cytoadhesion molecule, lymphocyte-associated antigen three (LFA 3), has been associated with abrogation of the immune-mediated control on the size of the Ph1+ cell clone (for details see the section dedicated to the immunologic surveillance of CML hematopoiesis).

The most relevant consequences of changes in the adhesion properties of CML hematopoiesis, however, arise from muddled interactions of Ph1+ progenitors with the hematopoietic microenvironment. Adhesion to specific cellular and extracellular components of the bone marrow microenvironment is the main way for ordered progression of hematopoiesis; it provides stem cell protection for the most primitive progenitors, allows migration of already committed progenitors to specific sites of differentiation and, as a final step, it regulates the traffic through the endothelial walls and the release into the bloodstream of mature elements. The whole process requires multiple and discrete recognition events and is properly regulated in a cell type- and shape-specific fashion.

Adhesion of the putative hematopoietic stem cell, phenotypically recognized as CD34+/CD33+/HLA-DR+, to special niches within the bone marrow microenvironment is functional to restriction of its proliferative activity. The adhesive interaction required at such a very early stage of hematopoiesis results from the ligand of β1 integrins VLA-4 and VLA-5, the homing lymphocyte receptor (CD44) and the cell surface proteoglycan receptor with distinct functional domains of fibronectin (one component of the hematopoietic microenvironment extracellular matrix): 75 Kd RGDS-containing fragment and the 33/66 Kd heparin-binding C-terminal. Hematopoietic inhibitory factors MIP-1α and TGF-β have been proposed as soluble messengers involved in contact-mediated inhibitory effects on cell proliferation.

The intrinsic defect in adhesion to the bone marrow microenvironment of CML hematopoietic progenitors, first described by Gordon et al., is indeed due to their reduced ability to adhere to both intact fibronectin and its 75 Kd or 33/66 Kd fragments. As a first consequence, impaired adhesion to the stromal microenvironment allows CML progenitors to cycle continuously, independently of physiological stimuli that induce cell cycle arrest on the normal counterpart. The biochemical events underlying the adhesive defect of Ph1+ progenitors have not been identified. They are not sustained by defective expression of cell surface adhesive receptors and have been tentatively ascribed to a non functional state of cell receptors or, alternatively, to lack of additional cell surface receptors whose cooperation is required for activation of integrin-mediated adhesion.
The other distinctive change in the adhesion properties of Ph₁⁺ progenitors results from increased expression of the \( \alpha_2 \) - and \( \alpha_6-\beta_1 \) integrin receptors VLA-2 and VLA-6, the ligands for collagen type IV and laminin. Since both proteins are almost exclusively distributed in the basement membranes, this adhesive defect might account for CML progenitor ability to penetrate the subendothelial basement membrane and egress from bone marrow before completion of the maturation process, as well as for illegitimate colonization of non-hematopoietic tissues, in particular the spleen and liver.

**p210 bcr/abl expands life expectancy of CML progenitors**

The expression of p210 bcr/abl prolongs cell survival by inhibiting apoptotic cell death. Since the rate of cell accumulation, a critical step for control of the proper size of any tissue, results from a balance between the rate of cell proliferation and the rate of cell death, the p210 bcr/abl-induced loss of control on cell life expectancy is a further cause of the abnormal expansion of clonal Ph₁⁺ over normal hematopoiesis. In that sense, p210 bcr/abl shares similarities with the \( bcl-2 \) deregulated activation that results from a t(14;18) chromosomal translocation associated with low-grade follicular non-Hodgkin’s lymphoma.

Actually, abnormal prolongation of cell survival is a rather common pathway of neoplastic progression since it contributes to increasing additional genetic alterations and favors the emergence of more aggressive molecular clones.

**Is there a role for hematopoietic microenvironment in the pathogenesis and progression of CML?**

The bone marrow microenvironment is a complex system. It consists of a variety of cell types, including cells of mesenchymal and hematopoietic origin, and of extracellular matrix (ECM) components. Cellular and extracellular components together provide a definite architecture, intended to deliver the proper messages (growth or inhibitory factors, mostly bound to the ECM) to the right cells.

Whether and how the hematopoietic microenvironment contributes to the deregulation of CML hematopoiesis has been, and still is, a controversial issue. To date, cells of mesenchymal origin (i.e. stromal fibroblasts and adipocytes) do not seem leukemic in nature since they lack both the Ph₁ chromosome and bcr/abl rearrangement, whereas cells of hematopoietic origin (i.e. stromal macrophages) are malignant. Moreover, the bone marrow stromal compartment produces normal levels of soluble messages and adhesive molecules, which overall seem to function properly (and Santucci, unpublished data); however, some evidence supports a failure in this functioning, possibly involved in the selective growth advantage for the malignant clone. First, the CML hematopoietic microenvironment has a reduced ability to support the growth of early normal hematopoietic progenitors since the soluble isoform of stem cell factor is probably involved in favoring clonal versus normal progenitors. In addition, IFN-\( \alpha \), the only agent capable of controlling the expansion of clonal hematopoiesis, does not affect the intrinsic adhesion properties of CML progenitors but rather interacts with the bone marrow microenvironment, by restoring stromal-mediated adhesion of early hematopoietic progenitors. Adhesive interactions restored by IFN-\( \alpha \) have been identified by Dowding et al. in neuraminic acid (sialic acid), a negatively charged non-reducing salt, by Verfaille et al. in the upregulation of \( \alpha_4- \) and \( \alpha_5-\beta_1 \) integrin receptors, associated with the reinduction of TGF-\( \beta \) and MIP1-\( \alpha \) activity, and by Santucci et al. in reinduced expression of \( \alpha_1- \) and \( \alpha_3-\beta_1 \) integrin receptors on the bone marrow stromal fibroblast surface. Identification of the hematopoietic microenvironment as the alternative target for IFN-\( \alpha \) activity in CML further supports some role for this compartment in the pathogenesis of CML.

Increased production of IL-1\( \beta \) by mononuclear Ph₁⁺ cells associated with IFN-\( \alpha \) resistance (likely an advanced stage of the disease), IL-1\( \beta \)-induced expression of IL-1\( \beta \), IL-6 and GM-CSF gene expression, IL-1\( \beta \)-induced increase of LIF gene expression, as well as IFN-\( \alpha \) ability to reduce IL-1\( \beta \) synthesis are all elements sup-
porting the view that the induction of a para-crine loop is a critical step for progression of the disease.

**Immunologic tolerance of p210 bcr/abl rearranged progenitors favors the expansion of clonal hematopoiesis**

Clinical outcomes of T-depleted bone marrow transplantation (BMT) and recent evidence of reinduction by donor T cells of durable complete remission after relapse following BMT suggest that an immune response to CML may occur and offer a hope of developing therapeutic vaccine strategies.

Like many other proteins expressed by altered cancer related genes, the p210 product of the bcr/abl rearranged gene is a potential T cell target. In general, T cells do not recognize intact proteins, but rather short peptide fragments (8-12 amino acids in length) from intact proteins processed intracellulary and presented at the cleft of major histocompatibility complex (MHC)-encoded molecules. A 12 amino acid residue from the p210 bcr/abl joining region, composed of 6 bcr, one fusion and 5 abl amino acids of the a2b3 rearrangement product, can be processed by the antigen-presenting cells (APCs). As a consequence, the segment of the joining region, which has a proper molecular configuration, binds the cleft of class II MHC molecules and reaches sufficient concentration to elicit T cell stimulation. Given this experimental basis in CML, the adoptive transfer of immuno-tumor antigens specific for CD4+ (capable of evoking APC expression of class II MHC molecules) or CD8+ (capable of evoking class I MHC-restricted catalytic responses) T cells seems feasible.

Another mechanism for immune-mediated control of Ph1+ clonal hematopoiesis is possibly related to the HLA class I phenotype. The fusion region-processed peptide presented at the T-cell receptor by HLA class I molecules might be potentially recognized as a non-self sequence.

Efforts to correlate the degree of immune-mediated susceptibility of CML hematopoiesis in association with either fusion sequence (a2/b2 or a2/b3), with any HLA class I allele (HLA-A or HLA-B) have been unsuccessful. However, since the enhancement of the immune response related to increased HLA class I expression is among the beneficial effects of IFN-α treatment in CML, the relationship between clinical outcome of IFN-α therapy and HLA phenotype still represents a matter of investigation and could possibly provide a key for understanding the mechanisms involved in immune mediated control of the disease.

The immunotolerance of the Ph1+ clone also arises from deficient LFA3-mediated adhesion of CML progenitors to immunocompetent cells. LFA3 (identified by the monoclonal antibody CD58) is a widely expressed cell surface protein whose only known function is as the binding ligand for the T-cell surface protein CD2. CD2/ LFA3 adhesive interaction in a subset of human T cells and early (CD34+) hematopoietic progenitors plays a role in controlling the size of the actively cycling stem cell pool. LFA3-deficient expression is thus an additional way for CML hematopoiesis to escape growth regulation and to enlarge illegitimately.

**Conclusions**

We have seen that multiple biomolecular events are associated with p210 bcr/abl expression. Taken together, they are more than sufficient to induce and sustain expansion of clonal Ph1+ hematopoiesis. Yet, the chronic phase of CML is not a leukemia: in fact, it does possess the basic traits of complete neoplastic transformation, i.e. fully transformed phenotype and serial transplantability in animal recipients, two features achieved by terminal phase hematopoiesis of the disease (blast crisis).

The theoretical model of the multistep pathogenesis of cancer postulates that multiple genetic alterations are required to attain complete malignant transformation, but the first essential step is the initiation of deregulated proliferation. Accordingly, p210 bcr/abl expression, the molecular marker of chronic phase of CML, is associated with illegitimate expansion of myelopoiesis, and additional cytogenetic and/or molecular aberrations become apparent as the dis-
ease progresses to blast crisis, where it is associated with a fully transformed phenotype.

The transition from chronic phase to blast crisis would require that the additional mutagenic event(s) occurs(s) not just in any clonal hematopoietic progenitor, but rather in a very early progenitor endowed with sufficient self-renewal to sustain the expansion of a fully transformed clone over the chronic phase one. The long interval elapsing from p210 bcr/abl-induced deregulation of proliferation to the appearance of fully transformed clone(s) might result from successive waves of molecular aberrations (as in colorectal tumors), or could rather depend on the relative scarcity of preleukemic/initiated hematopoietic stem cells. Actually, there is evidence in favor of both hypotheses. The clinical outcome of IFN-α therapy, which correlates IFN-α unresponsiveness with more aggressive disease, supports the importance of the cumulative number of additional genetic alterations for progression of the disease. On the other hand, the significant reduction in the size of clonal rearranged hematopoiesis present at the onset of blast crisis following a complete or major IFN-α response, as well as the observation that minimal persistence of clonal rearranged progenitors following allogeneic BMT does not predict disease relapse would seem to be consistent with the theory that the fewer the number of rearranged hematopoietic progenitor cells, the lower the probability of accumulating additional genetic mutations at an early stage.

One final consideration concerns the ineluctability of the transition of CML from the indolent chronic phase to a real leukemia. Whatever the sequence of biomolecular events, there is most likely a least common denominator between the p210 bcr/abl-mediated pathways that sustain the pathogenesis and the progression of CML. Deregulated proliferation takes place at expense of the cell cycle quiescence phase (G0-G1) and probably involves abrogation of control over the progression from G1 to S phase (G1/S checkpoint). The relevance of the G1/S checkpoint recently came into the limelight as being critical in both the pathogenesis and the progression of cancer. In functional terms, it represents an overlapping section of pathways controlling cell proliferation and DNA repair. Accordingly, in CML p210-associated abrogation of control over cell cycle progression has two major consequences. First, a loss of control over the size of clonal hematopoiesis, which expands illegitimately over the residual normal one; second, a loss of control over the quality of replicated DNA. The former plays a role in the pathogenesis of CML and the latter is critical for progression to blast crisis by favoring propagation of additional genetic errors.

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PATHOGENESIS AND PROGRESSION OF CHRONIC MYELOID LEUKEMIA

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MOLECULAR EVENTS IN CML PROGRESSION

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Progression toward an acute leukemic phenotype is almost the rule in Ph+ CML, whereas it is present in about 15-20% of PV, MF and ET patients. No common molecular mechanism responsible for this acute transformation has been detected so far in either Ph-positive or Ph-negative chronic myeloproliferative disorders (CMD). In the past we had looked for structural alteration of p53 and Ras genes in a large series of Ph-positive and Ph-negative CMDs analyzed in different phases of their disease, the results showed that p53 and Ras gene mutations significantly correlate with the progression of the disease only in Ph-negative myeloproliferative disorders, but not in CML. Since homozygous deletion of the cyclin-dependent kinase 4 inhibitor gene (CDK4i), a putative tumor suppressor gene located on chromosome 9p21, represents a very common genetic event in human cancer, we decided to investigate whether the occurrence of similar deletions could possibly be one of the mechanisms underlying the disease progression in Ph-positive CML. Whereas none of the 22 chronic phases examined presented alterations, we found that 3 of 17 total blast crises (18%) showed homozygous deletion of the CDK4i gene. The deletions were restricted to cases of lymphoid blast crisis; they were present in 3 of 8 (40%) of the lymphoid and in none of the 9 myeloid cases examined. The fact that the chronic phase DNA obtained at diagnosis in one of these cases lacks the homozygous deletion observed in blast crisis suggests that the final deletion event took place in concomitance with the progression of the disease. Finally, analysis of polymorphic regions on chromosome 9p21 flanking the CDK4i gene on both sides showed that the deletions at 9p21 differ from case to case and are characterized by a wide range of extensions. A concomitant search for possible involvement of the p53 tumor suppressor gene in the same series of patients revealed mutations of the gene and loss of heterozygosity at 17p only in myeloid blast crisis, suggesting the presence of distinct molecular pathways in the pathogenesis of lymphoid and myeloid blast crisis.

CLONAL AND NON CLONAL HEMATOPOIESIS IN CHRONIC MYELOGENOUS LEUKEMIA

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Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder arising at the level of the pluripotent hematopoietic stem cell. The hallmark of the disease is the Philadelphia (Ph) chromosome, which results from the rearrangement of the bcr and abl genes. Clinical and experimental evidence suggests that normal hematopoietic stem cells may be present within CML marrow. The availability of culture techniques for assaying progenitor cells at different ontogenetic stages, and cytogenetic as well as molecular techniques for detecting the Ph chromosome allow quantitative evaluation of the relationships between clonal and non clonal progenitors in CML marrow. It was that aim of the present study to investigate the cytogenetic status of the progenitor cells generated by the mononuclear (MNC) and CD34+ cell fractions. Both MNC- and CD34+ -derived progenitors were further fractionated according to their sensitivity to mafosfamide (Mafo) and their capacity for stromal adher-
A significant increase of Ph-neg clones could be obtained by combining CD34 selection with either stromal-adherence or mafosfamide incubation. Enrichment of Ph-neg progenitors by CD34 selection plus mafosfamide incubation was not significantly improved by stromal-adherence. Based on these data, it is concluded that currently available cell culture techniques associated with chromosomal or molecular analysis allow quantitative investigation of progenitor cell compartments in CML patients. These studies may have physiopathological and therapeutic relevance.

### EVALUATION OF PH-NEGATIVE HEMATOPOIESIS IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA. ANALYSIS OF LTC-LCS AND CLONOGENIC CELLS REGENERATING AFTER CHEMOTHERAPY AND COLLECTED IN PERIPHERAL BLOOD. INDICATIONS FOR AN APPROACH AT DIAGNOSIS AND IMPROVED PERSPECTIVES FOR AUTOGRFTING

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Interactions between normal and leukemic cells and, in particular, the suppression of normal hematopoiesis apparently associated with expansion of the leukemic cells is an unresolved issue in leukemia. Moreover, it is unclear whether the persistence of suppression leads to the exhaustion of the suppressed population. We show here that chronic myeloid leukemia (CML) patients at diagnosis have a substantial hematopoietic reservoir that seems to undergo progressive exhaustion in relation to the duration of the disease. We analyzed 42 patients with CML: 9 at diagnosis and 33 more than 12 months after diagnosis. All patients were treated with IC: idarubicin+cytarabine+etoposide. G-CSF (3-10 ug/kg) was given from day +8 after ICE. We evaluated the parameters reported in the table and compared them with collections obtained from peripheral blood from normal donors (NPBC) after 5 days of G-CSF (3-10 ug/kg). The results show: 1) significant differences between collections at diagnosis versus >1 yr; 2) collections of Ph-negative cells at diagnosis can almost reach the same range as that of normal donors; 3) normal Ph-negative hematopoiesis is progressively reduced during the course of the disease. In conclusion, mobilization at diagnosis seems the most profitable for collection and manipulation of Ph-negative cells in view of autografting.

Evaluation of normal hematopoiesis is measured by the ability of normal cells to regenerate faster than leukemic cells after chemotherapy. This, in our opinion, is a more profitable method for collecting Ph-ve cells than sorting them from CML steady state bone marrow.

In fact, patients at diagnosis can be divided in two groups: 1) those who show persistence of normal hematopoiesis in their marrow, and 2) those in whom normal hematopoiesis is not detected. In the same of the latter, but not in all, an overshoot of normal hematopoiesis following chemotherapy can be obtained and exploited.

### PROLIFERATIVE AND INHIBITORY FACTORS IN CHRONIC MYELOGENOUS LEUKEMIA

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Normal and clonal hematopoietic progenitor cells were demonstrated to coexist in CML and recent studies have shown that expansion of the leukemic clone occurs at the level of intermediate-late precursors, whereas residual normal cells reside within the CD34+DR- lin- cell compartment. In this study, CD34+ cells and more immature CD34+DR- lin- cells were highly purified from the bone marrow (BM) of CML patients at diagnosis. Primitive hematopoietic progenitor cells were tested for their colony forming ability in response to early and intermediate-late colony stimulating factors (CSFs), and the presence of the BCR-ABL transcript in individually plucked colonies was detected by nested RT-PCR. Molecular analysis revealed that 64.5±16% of CD34+ cells and 46.5±9% of earlier precursors did not express the BCR-ABL transcript (p<0.05). Clonogenic assays demonstrated a remarkable proliferation of CML cells in the presence of SCF, IL-11, IL-3, GM-CSF and EPO. Specifically, SCF and EPO stimulated 135±31% of CFU-C derived from CD34+DR- lin- cells generated by PHA-LCM. Conversely, optimal stimulation of normal primitive precursors required co-incubation with 3 or more CSFs. Moreover, SCF and IL-3 induced selective survival and expansion of residual normal hematopoietic cells in long-term cultures of CML marrow. Parallel in vitro studies were performed to evaluate the inhibitory activity of transforming growth factor-β3 (TGF-β3) on normal and leukemic CD34+ cells and CD34+DR- lin- cells.

Our results showed a higher degree of colony-stimulating activity inhibition of leukemic cells than that exerted by TGF-β3 on their normal counterparts. In contrast to benign progenitor cells, the inhibitory activity of TGF-β3 on CML cells was not counteracted by the early-acting CSFs IL-11 and SCF. Experiments carried out using murine (32D) and human (M07e) CSF-dependent cell lines and their subclones made CSF-independent by transfection of the BCR-ABL sequence indicated that the differences in responsiveness to TGF-β3 are directly related to BCR ABL expression. Further investigations on cell cycle distribution of CML cells and mRNA expression of selected CSF receptors are currently underway to elucidate the role of TGF-β3 in the inhibition of leukemic development.
Normal and clonal hematopoietic progenitor cells have been demonstrated to coexist in chronic phase chronic myelogenous leukemia (CML), but few data are available on the presence of non neoplastic hematopoiesis during the blast transformation phase. We used reverse transcription-polymerase chain reaction (RT-PCR) to investigate the expression of the bcr-abl transcript of individual hematopoietic progenitors in a CML patient in blastic phase. We showed that non clonal hematopoiesis is induced to re-emerge by conventional chemotherapy that includes fludarabine. In addition, we confirmed that some pluriotent CD34+/CD33-/DR- cells circulating in the peripheral blood are non clonal.

Our data provide an encouraging basis for further studies addressing the issue of in vitro purification of normal hematopoietic stem cells in advanced-stage CML and their use in the setting of autologous bone marrow transplantation.

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THE EXPRESSION OF LEUKEMIA INHIBITORY FACTOR GENE mRNA IN CHRONIC MYELOID LEUKEMIA

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Human interleukin for DA1a (HILDA)-leukemia inhibitory factor (LIF) (LIF-HILDA) is a cytokine able to induce differentiation along the macrophage pathway in the murine M1 cell line by suppressing proliferation. In addition, it promotes growth of the murine DA1a cell line and inhibits spontaneous in vitro differentiation of embryonal mouse stem cells (ES).

We examined the structure of the gene that encodes the LIF/HILDA cytokine in 55 cases of chronic myeloid leukemia by Southern blotting and demonstrated the characteristic germline configuration in both Philadelphia chromosome negative and positive cases.

Examination of mRNA expression of LIF/HILDA in the 55 cases (19 in chronic phase, 36 in blast crisis) revealed that the percentage of positive cases rose from 31% in the chronic phase patients to 92% of those in blast crisis. Polymerase chain reaction (PCR) analysis of the Northern blot negative cases, both chronic phase and blast crisis, mainly confirmed the Northern blotting results and increased the positive cases only slightly.

Detailed immunological phenotyping of the blast crisis patients failed to reveal any differences in LIF/HILDA mRNA expression between the myeloid and the lymphoid phenotype cases. Since this cytokine blocks differentiation in some cell systems and stimulates proliferation in others, it could favor the evolution of chronic myeloid leukemia from chronic phase to blast crisis by blocking differentiation of pluripotent stem cells, while, at the same time, stimulating their proliferation.

CONSTITUTIVE EXPRESSION OF IL-1β, M-CSF AND c-fms DURING THE MYELOID BLASTIC PHASE OF CHRONIC MYELOGENOUS LEUKEMIA

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Non random additional chromosome abnormalities occur in over 80% of patients during the myeloid blast crisis (BC) of chronic myelogenous leukemia (CML). However, these cytogenetic changes have been reported to precede the clinical signs of CML-BC by several months to years, suggesting that other biological events may participate in the multistep process of acute CML transformation. Autocrine production of growth factors has recently been shown to occur in several hematological malignancies and, in particular, in acute myeloblastic leukemia (AML).

We evaluated 13 adult patients with CML in myeloid blast crisis. At the time of the study all patients had >50% blast cells in both the peripheral blood and bone marrow with morphological and immunological characteristics of myeloid blasts.

We demonstrated that the IL-1 gene is expressed in almost all cases of CML in myeloid blast crisis. The secretion of IL-1 from CML blasts in culture supernatants was confirmed in all patients. A high proportion of cases showed constitutive expression of the M-CSF gene, and many of the same patients often had simultaneous coexpression of the proto-oncogene c-fms, which encodes for the M-CSF receptor. After exposure of leukemic cells to phorbol myristate acetate (PMA), release of M-CSF protein was documented in three of five patients studied. No significant interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) or granulocyte colony-stimulating factors (G-CSF) were detected in these patients, indicating that different growth factor secretion patterns exist in AML and CML, where distinct molecular events are probably involved in the control of leukemic proliferation. The simultaneous production of active cytokines such as M-CSF and IL-1 is a common finding during the terminal phase of CML, even though an actual role for them in the differentiation and progression of CML remains to be proven. Nonetheless, it is likely that these pleiotropic molecules can determine several aspects of the biology and clinical behavior of this disease.

BCR-ABL ANTISENSE OLIGONUCLEOTIDES: ADVANCES IN THE TREATMENT OF CML CELLS

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The evidence that the Philadelphia chromosome product p210 plays a critical role in the pathogenesis of chronic myeloid leukemia (CML), and the absence of the bcr-abl gene transcript in non-malignant cells makes this messenger RNA (m-RNA) an ideal target for antisense strategy. The most effective antileukemic activity has been reported using bcr-abl antisense oligodeoxynucleotides (As ODNs) on CML cell lines and on primary cells from patients in blastic phase. We studied the effect of 26- and 16-mer phosphorothioate As ODNs complementary to the bcr-abl junction on the colony-forming ability of mononuclear and CD34+ enriched cells from patients with CML in chronic phase, comparing the sensitivity of mononuclear cells to that of CD34+ enriched cells. In the 27 cases tested,
an overall recovery of 41.7% clonogenic cells was found when 26-mer junction-specific As ODNs were tested on mononuclear cells, confirming the therapeutic effect of the 26-mer junction-specific ODNs on the leukemic cell mass in the majority of newly diagnosed chronic myeloid leukemia (CML) patients. However, when the 16-mer As ODNs were used, 6/16 and 5/6 patients tested on mononuclear and CD34+ cells, respectively, showed specific sensitivity to As ODNs. Down-regulation of p210 was observed in 3/6 cases tested for p210 expression, with a good correlation between the As ODN effect on leukemic colony formation and protein levels. Five patients in advanced phase of the disease, suitable for in vitro treatment with As ODNs, were selected for a phase-1 autograft trial using bone marrow purged in vitro. A median of 3.5 (range 1.8-6.7) × 10^6 mononuclear cells were recovered after Ficoll separation containing a median of 3.3% (0.7-13) CD34+ cells and 1.0 × 10^5 (3.8 × 10^5-2.6 × 10^6) clonogenic cells. Incubation with junction-specific As ODNs was prolonged for 24 hours using a concentration of 150 μg/mL of As ODN in a medium containing 4% autologous serum, IL-3 and GM-CSF. After incubation, a median of 50% (47-82) mononuclear cells, 59% (23-85) CD34+ cells and 38% (21-66) clonogenic cells were recovered, respectively. Patients received busulfan (16 mg/kg) and VP-16 (40 mg/kg) and were autografted with the bcr-abl As ODN treated bone marrow cells. Bone marrow engraftment was observed in all the cases. Platelets > 50 × 10^9/L were reached in 4 evaluable cases after a median of 77 days (22-180); neutrophils > 0.5 × 10^9/L were reached in 5 patients after a median of 26 days (20-30). The patient autografted in second chronic phase died at day +210 in blastic transformation; the other 4 patients are in chronic phase after 7-18 months. These results indicate that incubation with bcr-abl As ODNs does not affect short- or long-term bone marrow engraftment and that autograft with ODN-purged BM cells may prolong the duration of chronic phase in this high-risk group of patients.

MOLECULAR MECHANISM OF INTERFERON THERAPY: THE INDUCTION OF EXPRESSION OF IFN-α RESPONSE GENES


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Interferons (IFN) belong to a large family of naturally occurring cellular polypeptides that possess antiviral activity, exert antiproliferative effects on rapidly dividing cells, and are involved in the modulation of immune responses. Treatment with α-interferon (α-IFN) adequately controls the leukemic cell mass in the majority of newly diagnosed chronic myeloid leukemia (CML) patients. However, the degree of response ranges from no hematological response to complete suppression of the leukemic clone. The molecular mechanism(s) by which α-IFN elicits these responses is presently unknown, but in vitro studies have indicated that α-IFN might function by a) selective toxicity against the leukemic clone, or b) enhancement of immune regulation and modulation of bone marrow microenvironmental regulation of hematopoiesis. As for selective suppression of the Ph+ clone, the exact mechanism for α-IFN-induced selective suppression of the Ph clone and re-emergence of Ph-negative hematopoiesis is that cells expressing P210 bcr/abl are more sensitive to growth inhibition by α-IFN that their normal counterparts. Molecular events and genetic heterogeneity result from the different positions of the breakpoint in M-BCR that lead to two types of transcripts, b2-a2 or b3-a2. We analyzed 244 patients affected by CML: the transcript type was b2-a2 in 44% and b3-a2 in 56% of cases. We found no differences between the two groups regarding as a prognostic factors, time to progression to accelerated or blastic phase, overall survival, or karyotype response after one year of IFN therapy. The molecular mechanism of this responsiveness is still unknown, but an immunological effect has been postulated. Depending on the type of fusion, two different series of non potentially self-immunogenic peptides may be produced. If they are detected on leukemic cells by HLA class I molecules induced by α-IFN (such as Tyk2 ISGF3), they may be recognized as cytotoxic CD8 lymphocytes. One theory is that the leukemic cell clones may be protected from host defenses by a decrease in the expression of those HLA class I alleles able to bind specific fusion region peptides. A state of anergy of specific CML clones can also be hypothesized, which in turn could depend on the low density of the peptides on the surface of the leukemic cells. If this is the case, one of the beneficial effects of α-IFN treatment in these patients may be due to enhancement of the immune response related to increased HLA class I expression. To test this point, the frequencies of HLA-A and B alleles were compared between b2-a2 and b3-a2 transcripts in 135 Italian CML patients. These with b2-a2 junctions numbered 58, while 77 showed the b3-a2 junction; they were compared to 1092 normal controls. Out of the 135 CML patients 51 had an HLA-B35-containing haplotype (37.8%). This proportion was significantly higher than that observed in controls (315 out of 1092; 28.8%; p< 0.02). Twenty-five of the 58 b2-a2 pts carried the B35-containing haplotype (43%) vs. 26 of the 77 b3-a2 pts (33.8%). The higher frequency of Italian CML patients with an HLA B35 antigen could support the purported inability of the immune system to give rise to a T-cell mediated response and, possibly, to a bcr-abl restricted GVL effect.

References

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IMMUNE-MEDIATED AND UNUSUAL COMPLICATIONS DURING α-INTERFERON THERAPY IN CHRONIC MYELOGENOUS LEUKEMIA

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The occurrence of immune-mediated and unusual complications was evaluated in 581 patients with Philadelphia chromosome (Ph)-positive chronic myelogenous leukemia (CML) treated with α-interferon (IFN-α) containing regimens at M.D. Anderson Cancer Center. Well-documented and clinically evident complications developed in 35 patients (6%) after a median of 14 months of IFN-α treatment. Hypothyroidism developed in 11 patients (2%) immune mediated hemolysis in seven (1%) and connective tissue disease in 11 (2%). Other unusual side effects included congestive heart failure (4 patients), porphyria cutanea tarda (3 patients), membranous glomerulonephritis (1 patient), and vitiligo (1 patient). Interferon treatment was discontinued in 19 patients, and the dose was reduced in five. Ten of 11 patients (91%) with immune-mediated hypothyroidism and eight of 11 patients (73%) with connective tissue diseases had some degree of cytogenic response at the time of the event suggesting a possible relationship between the way in which IFN suppresses the Ph-positive clone and induces some of the immune-mediated complications (IMC). Although the frequency of IMC is low, patients treated with IFN should be monitored for signs and symptoms of autoimmune. On the basis of these results, we analyzed 49 CML patients in chronic phase who had shown different responses to IFN-α treatment, in an attempt to identify different lymphocyte sub-population patterns related to different response levels to IFN-α treatment. We found that the absolute number of lymphocytes was substantially the same in the different groups of patients. We observed a remarkable rebound of CD3, CD4, CD8, CD19 absolute number in complete cytogenetic response (CCGR) patients after discontinuation of IFN treatment. Patients in CCGR showed a higher number of CD56-positive cells than other groups of patients, but the differences were not statistically significant. Patients with resistant disease as well as those in partial or complete hematological remission showed a lower number of CD19-positive cells than patients in complete, partial or minor cytogenetic response. It is difficult to understand what these small differences mean and we are not able to interpret these data completely. In conclusion, we failed to identify a clear pattern of positivity that was specific for patients who had obtained different levels of response to IFN treatment. Although we observed no important changes in the absolute number of cells expressing CD3, CD4, CD8, CD56 or CD19, we think that changes might be observed according to function.

**EFFECTS OF HOMOHARRINGTONINE ALONE AND IN COMBINATION WITH α-INTERFERON AND CYTOSINE ARABINOSIDE ON IN VITRO GROWTH AND INDUCTION OF APOPTOSIS IN CHRONIC MYELOID LEUKEMIA AND NORMAL HEMATOPOIETIC PROGENITORS**

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Homoharringtonine (HHT) is a cephalotoxine alkaloid that showed clinical efficacy in the chronic phase of chronic myeloid leukemia (Ph'CML); as a single agent it proved to be effective in controlling leukocytosis and producing a sporadic karyotypic conversion. Its clinical use in combination with IFN-α for the treatment of CML could be considered. In fact, although IFN-α as a single agent induces a hematologic response in 60%-80% of patients, less than 30-40% of responding patients obtain a karyotypic conversion, varying from minor to complete (Ph neg. metaphases > 33% to 100%), or a significant increase in the duration of the chronic phase. In this study we evaluated the growth inhibition and the induction of apoptosis due to HHT alone and in combination with IFN-α and ara-C on normal and Ph'CML cells (both in the chronic and the blastic phase) hematopoietic progenitors. We studied bone marrow from 10 normal subjects, 10 CML chronic phase patients and 10 CML acute phase patients. Cells (at a final concentration of 3×10^5/mL) were plated in 24-well plates with HHT (10-50-200 ng/mL) alone or in combination (50 ng/mL with ara-C (100 ng/mL) and/or IFN-α (1000 U/mL). After 72 hours of incubation cells were resuspended and counted. CFU-GM colonies were cultured with HHT (0.1-1-10 ng/mL) alone or in combination (1 ng/mL) with ara-C (1 ng/mL) and/or IFN-α (100 U/mL). Apoptosis was quantitated by flow cytometry. The IC50 of HHT, evaluated after 72 hours of culture, was 135 ng/mL in CML-CP; in CML-AP and in normal cells, respectively, it was 240 ng/mL and 250 ng/mL, highly superior to that observed in CML-CP. Two different combinations of HHT with IFN-α or/and Ara-C determined a comparable increase of cytotoxicity in CML-CP (37% vs 43% vs 40%), with HHT as standard. On the contrary, in CML-AP and normal progenitors the drug combinations determined only a slight increase of cytotoxicity. Comparable results were obtained in semisolid cultures. The induction of apoptosis proved to be dose-dependent in CML-CP and normal controls; no changes were
observed in CML-AP. In conclusion, HHT was able to inhibit cell growth in CML chronic phase at doses significantly lower than in CML acute phase and in normal cells. The association IFN-α+HHT was again significantly more active in CML chronic phase than in CML acute phase and in controls. In addition, the data are consistent with statistically superior effect of the association IFN-α plus ara-C and HHT on chronic phase, if compared to IFN-α used as a single drug. Apoptosis data were in line with inhibition experiments since induction of apoptosis proved to be dose dependent in CML chronic phase, whereas no effect was seen in CML acute phase. These data are concordant with recent evidence showing that HHT belongs to the category of MDR-related drugs whose antileukemic effect is modulated by P 170 glycoprotein expression. In fact, P 170 expression is increased in CML acute phase with respect to CML chronic phase; the significantly different cytotoxicity and apoptosis inducible observed by us provide experimental evidence for the differences in clinical results between chronic phase (responsive to HHT) and acute phase (where the drug is ineffective).

RESISTANCE TO HUMAN RECOMBINANT INTERFERON α2a (IFNα2a) IN Ph+ CHRONIC MYELOID LEUKEMIA PATIENTS: THE ROLE OF NEUTRALIZING ANTI-IFNα2A ANTIBODIES (nIFN α2a Abs) AND THE USE OF LYMPHOBLASTOID IFNα (IFNα-Ly)

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In this study we evaluated: a) the frequency and the relevance of nIFNα2a Abs in a cohort of 118 Ph+ CML pts; b) the hematologic and karyotypic response to IFNα-Ly in 17 Ph+ CML pts resistant to nIFNα2a.

Using an IFNα antiviral neutralization bioassay the frequency of nIFNα2a Abs was evaluated in 118 Ph+ CML patients (pts) before (22 cases), during (67 cases), and after (29 cases) discontinuation of IFNα2a therapy (average dose from 6 to 9 MU/day). The results are reported in the Table. Out of 67 pts studied during IFNα2a treatment, 15 (22%) developed nIFNα2a Abs (titer ranging from 1:40 to 1:20,480) and 11/15 were hematologically and/or karyotypically (H/K) unresponsive to therapy. Out of 52 nIFNα2a Ab negative patients only 11 were (H/K) unresponsive. The negative relationship between the positivity of nIFNα2a Abs and the hematologic and karyotypic response was highly significant (p<0.0001).

IFNα-Ly was given at escalating doses of 3,6,9 MU/daily in 17 patients (pts) with Ph+ CML in chronic phase who had discontinued IFNα2a between the 5th and 60th month (mean=24, median=13) because they were hematologically (12/17) and/or karyotypically (17/17) unresponsive, and/or nIFNα2a Ab positive (9/17).

<table>
<thead>
<tr>
<th>nIFNα2a Abs</th>
<th>pts studied</th>
<th>positive</th>
<th>negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior to treatment</td>
<td>22</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>During IFNα2a treatment</td>
<td>67</td>
<td>15 (22%)</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12/15</td>
<td>11/52</td>
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<tr>
<td>p&lt;0.0001</td>
<td></td>
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<td></td>
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<tr>
<td>After IFNα2a discontinuation</td>
<td>29</td>
<td>9 (31%)</td>
<td>20</td>
</tr>
<tr>
<td>Total cases studied</td>
<td>118</td>
<td>24 (20%)</td>
<td>94</td>
</tr>
</tbody>
</table>

After a 12-month IFNα Ly treatment, a hematologic response was obtained in 8/12 hematologically and karyotypically unresponsive patients, and was maintained in 2 out of the remaining 5 pts who were hematologically responsive but karyotypically unresponsive (4 cases) or nIFNα2a Ab positive (1 case). Out of 10 hematologically responsive pts who completed 12 months of treatment with IFNα-Ly, 9 pts did not achieve any karyotypic conversion (Ph neg 100%) and 1 obtained a minimal karyotypic response (Ph neg 21%). No difference was observed in response between the group of nIFNα2a Ab positive patients and the group who were negative. In conclusion, these results show that: a) a significant proportion of Ph+ CML patients receiving chronic treatment with IFNα2a develop nIFNα2a Abs, which are associated with a loss of IFNα2a efficacy; b) a change in therapy to a non cross-reactive type of IFNα (IFNα-Ly) can induce a hematologic response in most patients unresponsive to IFNα2a, whether nIFNα2a Ab positive or negative, but it seems to be incapable of producing a karyotypic conversion.