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Mechanisms controlling pathogenesis and survival of leukemic stem cells

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Stem cells are an integral component of normal mammalian physiology and have been intensively studied in many systems. Intriguingly, substantial evidence indicates that stem cells also play an important role in the initiation and pathogenesis of at least some cancers. In particular, myeloid leukemias have been extensively characterized with regard to stem and progenitor cell involvement. Thus, as a focal point for both scientific and therapeutic endeavors, leukemic stem cells (LSC) represent a critical area of investigation. LSC appear to retain many characteristics of normal hematopoietic stem cells (HSC) as evidenced by a hierarchical developmental pattern, a mostly quiescent cell cycle profile, and an immunophenotype very similar to HSC. Consequently, defining unique properties of LSC remains a high priority in order to elucidate the molecular mechanisms driving stem cell transformation, and for developing therapeutic strategies that specifically target the LSC population. In this review, we discuss emerging concepts in the field and describe how various molecular and cellular characteristics of leukemia cells might be exploited as a means to preferentially ablate malignant stem cells.
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Introduction

A fundamental characteristic of primary tumors is a marked degree of cellular heterogeneity. Multiple different cell types are commonly found within clonal tumor populations, indicating that specific mechanisms must exist to drive processes of differentiation and/or change from the tumor-initiating cell (Foulds, 1969, 1975). Genomic instability is one feature of many tumors that may be responsible for diversity in the malignant population. A second source of change may arise from intrinsic development processes such as those normally found within stem cell-based hierarchies. This phenomenon is certainly present in the context of hematologic malignancies such as myeloid leukemia, where tumor stem and progenitor cells have been studied in detail (Passegue *et al*., 2003).

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Evidence supporting a stem cell origin for leukemia dates back several decades. Beginning in the mid 1960s, it was first demonstrated that a small subset of murine leukemia cells gave rise to clonally derived colonies both *in vitro* and *in vivo*, results that paralleled similar observations in normal hematopoietic stem and progenitor cells (Bruce and Gaag, 1963; Wodinsky *et al*., 1967; Park *et al*., 1971). Subsequent studies in humans used the X-linked gene glucose-6-phosphate dehydrogenase (G-6-PD) to monitor hematopoietic populations in leukemia patients heterozygous for the A and B isoenzymes (Fialkow *et al*., 1967, 1977). These experiments further established the clonal nature of leukemic stem cells (LSC) by demonstrating single-enzyme phenotypes in multiple hematopoietic lineages. More recently, modern methods of stem cell analysis have been employed to demonstrate that leukemic growth potential resides in a rare and phenotypically distinct subset of malignant populations (Lapidot *et al*., 1994; Bonnet and Dick, 1997; Blair *et al*., 1998; Blair and Sutherland, 2000). Thus, using the same tools employed to characterize stem cell development in normal hematopoiesis, a relatively clear picture of malignant stem cell involvement in myeloid leukemia has also been obtained.

A general schema depicting how malignant stem cells arise is shown in Figure 1. Notably, like their normal counterparts, LSC are central to the growth and perpetuation of downstream daughter cells. LSC undergo processes of self-renewal and at least partial differentiation in a fashion analogous to normal hematopoietic stem cells (HSC). Differentiation from the LSC population gives rise to 'blast' cells, which represent arrested or aberrant stages of myeloid development. Consequently, an important aspect of myeloid leukemia biology is that the tumor population is heterogeneous and that LSC are biologically distinct from the more differentiated blast cells. Hence, elucidating the specific nature of LSC is an essential step towards ultimately curing leukemia, and has indeed been an active area of research. However, a practical consequence of the tumor heterogeneity mentioned above is that strategies for inducing cell death must address the unique survival mechanism(s) of each different cell type within the malignant population. This problem is particularly challenging in stem cell-based malignancies, where the critical target cells are typically rare and possess unique molecular characteristics. A further difficulty is that extrinsic factors may aid intrinsic cell survival mechanisms to protect cells from

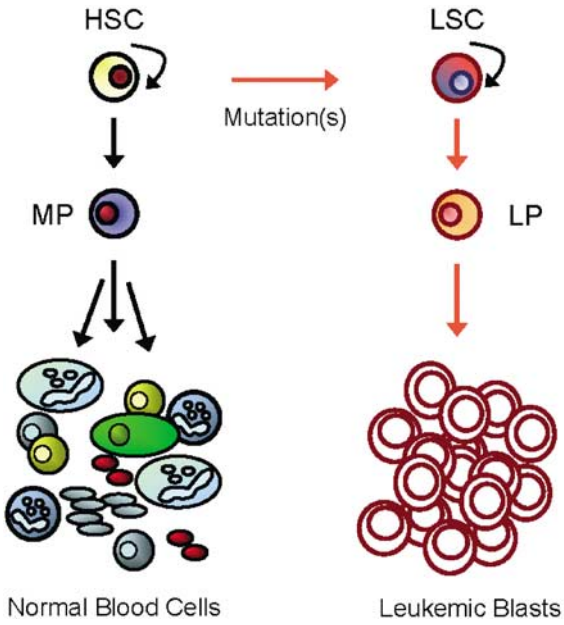

Figure 1 Stem cell basis for myeloid leukemia. Normal hematopoietic stem cells (HSC) typically display a balanced program of self-renewal vs differentiation. HSC first differentiate to myeloid progenitors (MP) and then proceed to various mature lineages. Mutation at the stem cell level leads to a leukemic stem cell (LSC). The LSC retains the hallmark stem cell properties of self-renewal, strong proliferative capacity, and differentiation potential; however, normal developmental pathways are arrested at an intermediate stage of maturation. Leukemic progenitors (LP) can usually be detected, but the majority of cells manifest as a leukemic 'blast' cell population

apoptotic stimuli. Evidence that the local microenvironment is critical for controlling basic mechanisms of self-renewal and differentiation exists for normal stem cells (Schofield, 1983; Lemischka, 1997). Based on these studies, it seems likely that the tumor microenvironment is also critical for self-renewal of LSC. Thus, a major challenge for stem cell targeted therapy is to identify apoptotic stimuli that effectively target the tumor stem cell population while simultaneously sparing normal stem cells; and to do so in the context of a largely uncharacterized *in vivo* microenvironment. To meet this challenge, development and analysis of sophisticated LSC experimental systems is essential. As described below, recent investigations are beginning to explore novel methods of LSC analysis and have provided intriguing insights into the nature of stem cell malignancy.

Experimental systems for LSC analysis

Seminal studies in the past 10 years have formally identified and characterized malignant stem cells in human acute myelogenous leukemia (AML). Using primary human leukemia specimens in conjunction with xenogeneic transplantation models, investigators have described the cell surface phenotype, self-renewal frequency, and developmental characteristics of AML stem cells (Lapidot *et al*., 1994; Blair *et al*., 1997, 1998; Bonnet and Dick, 1997; Blair and Sutherland, 2000; Jordan *et al*., 2000). These studies have subsequently permitted a more detailed molecular analysis of human LSC (Guzman *et al*., 2001a, b) and hold promise for evaluation of stem cell-targeted therapeutic strategies. Similar studies have also reported substantial progress in defining characteristics of chronic myelogenous leukemia (CML) stem cells (Holyoake *et al*., 2002).

Studies of human LSC have been substantially aided by the recent evolution of several murine model systems, which provide a novel means of analysis for stem cell-based pathogenesis. Perhaps most importantly, retroviruses encoding defined human leukemia translocations have been employed to transduce normal murine hematopoietic cells with a variety of oncogenes (Kamps and Baltimore, 1993; Li *et al*., 1999; Lavau *et al*., 2000b; Kroon *et al*., 2001; Thorsteinsdottir *et al*., 2001). Typically, donor hematopoietic cells are infected *ex vivo* with one or two retroviral vectors and then transplanted into appropriate recipient animals. Such an approach can be further enhanced by employing donor hematopoietic cells with various genetic lesions (Li *et al*., 2001; Tomasson *et al*., 2001). Similarly, recent studies have also employed conditional alleles of leukemic oncogenes in transgenic mice as a means to model early stages of myeloid leukemia (Braun *et al*., 2004; Chan *et al*., 2004). Studies using these murine models have shown that the pathology of animals bearing different oncogenic genes varies widely depending upon the specific type of mutation(s) introduced. For example, introduction of activated kinases such as BCR/ABL, Flt3, and TEL/PDGFR, can induce myeloproliferative disease with varying degrees of severity, suggesting that as single lesions these genes are not sufficient to induce acute leukemia (Tomasson *et al*., 2000; Van Etten, 2001; Kelly *et al*., 2002). In contrast, several transcription factor mutations have been shown to induce acute myeloid leukemia (e.g. Nup98/HoxA9, MLL-ELL, MLL-CBP, etc.); however, the latency of disease progression is typically several months, indicating that secondary mutations are required for complete transformation (Lavau *et al*., 2000a, b; Kroon *et al*., 2001). Prior to the onset of acute disease induced by leukemic transcription factors, aberrancies in stem cell behavior have been observed, but typically with little pathology. Further, several recent studies have attempted to model acute leukemia pathogenesis by introducing combinations of two leukemic translocations. Specifically, by expressing both an activated hematopoietic kinase and a mutated leukemic transcription factor, these studies mimic the molecular genetic profile commonly observed in human AML (Dash and Gilliland, 2001). Upon dual expression of such genes, a very rapid evolution of acute leukemic disease is evident, thus strongly indicating that specific pairs of mutations are sufficient to generate *de novo* AML in mice (Cuenco and Ren, 2001; Dash *et al*., 2002; Mayotte *et al*., 2002). This observation is supported by other studies in which animals bearing a single mutation rapidly progress to myeloid leukemia upon exposure to mutagenic agents such as nitrosourea (Yuan *et al*.,

2001). An important feature to note is that a stem or progenitor cell origin is either directly or indirectly evident for almost all experiment systems described to date. Thus, it appears that the aberrant developmental hierarchy that arises from stem cell-based malignancies can be directly studied *in vivo* using murine experimental systems.

An intriguing example of how murine models can be used to study stem cell pathogenesis was recently described by Cozzio *et al*. (2003). These investigators employed a retrovirus to introduce the MLL-ENL translocation (a known leukemic oncogene) into highly purified stem and myeloid progenitor cell populations. Gene-modified cells from each population were then independently analysed *in vivo* for leukemic potential. Virtually identical disease was generated by transduced HSC, common myeloid progenitors (CMP), and granulocytic/monocytic progenitors (GMP), but no disease was produced by megakaryocytic/erythroid progenitors (MEP). These studies suggest that mutation of self-renewing HSC is not always strictly necessary to manifest AML, but rather that mutation of certain myeloid progenitors may also be sufficient. Thus, the data indicate that expression of MLL-ENL either conferred self-renewal properties on progenitor cells that are normally only transient, and/or rapidly led to secondary mutations that conferred enhanced self-renewal and a transformed phenotype. It will be interesting to determine whether other types of mutations are also sufficient to confer self-renewal properties on myeloid progenitors. One preliminary report indicates that expression of the BCR/ABL oncogene in CMP or GMP is not sufficient to induce myeloid disease (G Gilliland, personal communication), suggesting that activated kinases may be less effective mediators of self-renewal than transcription factors. Collectively, the available data indicate that LSC arise not only as a function of the target cell (hematopoietic stem cell (HSC) vs progenitor) but also as a consequence of the specific type of mutation. In the future, analysis of multiple mutations should serve to further define the process of stem cell transformation, as well as other disease parameters such as mechanisms of drug resistance.

Recent studies have also begun to model leukemogenesis using primary human cells. Transduction of normal CD34+ cells with the AML1-ETO translocation inhibits differentiation and increases self-renewal and survival of primitive hematopoietic cells *in vitro* (Mulloy *et al*., 2002). Similarly, expression of BCR/ABL in human CD34+ cells enhances the growth of primitive myeloid cells and inhibits apoptosis (Zhao *et al*., 2001). These studies permit a variety of molecular and cellular analyses that have begun to define how oncogenes function in primitive human hematopoietic cells. Although, successful xenotransplantation of translocation-bearing human cells into NOD/SCID mice has not yet been achieved, as gene transfer methods improve, it should be possible to evaluate *in vivo* characteristics of various leukemic oncogenes. In addition, investigators have successfully transplanted primary leukemic cells into immune-deficient mice, and have demonstrated the utility of this approach for characterizing stem cell biology (Dick, 1996; Bonnet and Dick, 1997; Wang *et al*., 1998). Particularly interesting are recent studies by Hope *et al*. (2004) that have used traditional methods of retroviral marking to monitor the *in vivo* fate of multiple LSC clones. Importantly, these studies demonstrate that the LSC population is heterogeneous, and that growth potential varies considerably among LSC derived from the same patient. These observations reflect the biological similarity of normal vs AML stem cell populations and suggest that early differentiation steps in the LSC pool are essentially intact, despite subsequent downstream aberrancies.

Self-renewal and stem cell pathogenesis

The characteristic of self-renewal is often described as a hallmark of normal stem cells and is perhaps the most important intrinsic cellular property that is subverted during stem cell tumorigenesis. Indeed, inappropriate regulation of self-renewal mechanisms appears to be a key component of stem cell malignancy. In considering the pathogenesis of stem cells, one important issue to consider is whether mechanisms of self-renewal and genomic stability are linked. A potential association between self-renewal and genomic stability arises from intriguing studies of different genes that affect the self-renewal process. Some genes that mediate increased self-renewal appear to be benign with regard to subsequent mutational events. For example, constitutive expression of the AML1-ETO translocation product has been shown to increase the self-renewal frequency of stem cells, but results in no apparent pathogenic consequences (de Guzman *et al*., 2002; Mulloy *et al*., 2002). Presumably, only subsequent random mutations provide a molecular context in which a leukemic role for AML1-ETO becomes apparent. In contrast, other genes with known self-renewal potential, such as HoxA9, have been demonstrated to induce cytogenetic aberrations when expressed in an unregulated fashion (G Sauvageau, personal communication). While not being sufficient to generate overt disease, it is possible that activation of HoxA9 predisposes stem cells to subsequent oncogenic events by virtue of decreasing genomic stability. Interestingly, HoxA9 upregulation is commonly observed in AML (Golub *et al*., 1999; Lawrence *et al*., 1999).

In attempting to understand how genetic instability or mutations might affect the stem cell pool, an important observation to consider is that LSC maintain a largely quiescent cell cycle status (Holyoake *et al*., 1999; Guzman *et al*., 2001a; Guan *et al*., 2003). Therefore, mutations that affect genomic stability will typically only manifest themselves in the small percentage of cycling cells. In a multistep pathogenic process, one might imagine a quiescent 'preleukemic' stem cell pool carrying an initial single oncogenic mutation. As this pool gradually enters cycle, genomic instability caused by the initial mutation could induce secondary

mutations. Alternatively, random secondary mutations could also contribute to disease progression. Once two or more oncogenic lesions occur, such cells would then give rise to acute disease. In some cases it is possible that cancers arising in this fashion would be ablated by standard chemotherapy. However, conventional drugs are unlikely to affect the originating 'preleukemic' stem cell population for at least three reasons. First, the preleukemic cells are primarily in G_0, thus conventional 'cycle-active' chemotherapy drugs will generally not be effective. Second, the preleukemic population may be more developmentally primitive than later stage tumor cells, and thereby possess natural mechanisms of survival such as drug efflux pumps, etc. Third, because the preleukemic population bears fewer oncogenic lesions, it is likely to be more biologically similar to normal cells and thus less susceptible to tumor-specific drugs.

A detailed consideration of early events in stem cell pathogenesis must also take into account the specific cellular targets involved. An initial mutation could occur in a stem cell, which might confer upon the preleukemic population natural self-renewal properties intrinsic to the parental stem cell. Alternatively, the initial mutation could occur at a later developmental stage, such as in a myeloid progenitor (MP) cell. These cells, while possessing substantial proliferative and developmental potential, do not undergo significant self-renewal and are more actively cycling (Akashi *et al*., 2000; Manz *et al*., 2002). In this case, the initial mutation would be expected to confer some degree of self-renewal, or at least sufficient genomic instability to quickly generate subsequent mutations. Importantly, the preleukemic cells are likely to reflect the natural biological properties of their normal parental cell type. For example, preleukemic HSC may retain a largely quiescent cycle status, whereas preleukemic MP might have increased cell cycle activity. Biological properties such as these might directly influence the relative degree of drug responsiveness of primitive leukemia populations.

Figure 2 illustrates three possible scenarios by which LSC could be formed, and how their genesis might influence therapeutic outcome. The first scenario (panel a) depicts LSC that arise directly from normal HSC. The initial mutation occurs in an HSC, leading to the formation of a preleukemic stem cell. Secondary mutation(s) in the pre-LSC then gives rise to LSC. Both the initial and secondary mutation(s) in this scenario are at the stem cell level. The second scenario (panel b) shows an initial mutation at the HSC level, followed by differentiation to a preleukemic MP stage and subsequent secondary mutation(s) leading to the LSC. A third possible scenario (panel c) suggests that HSC first differentiate to normal MP, and then undergo primary and secondary mutations to ultimately generate LSC. In all three scenarios, once LSC are formed, subsequent differentiation generates the leukemia blast population. While the differences in each scenario are subtle and may not be readily evident in the LSC population, the ramifications with regard to therapy are significant. Considering the path by which each type of LSC is generated, the therapeutic outcome of treatment falls into at least three categories. In the first category, the

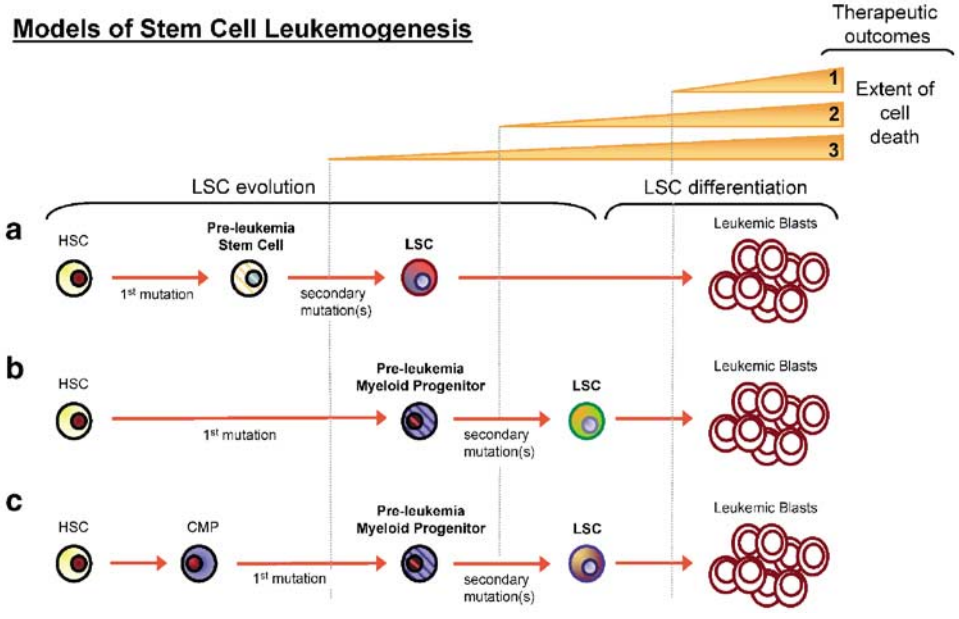

Figure 2 Models of stem cell leukemogenesis. The figure depicts three possible scenarios for the evolution of AML (panels a–c). Hematopoietic stem cells (HSC), myeloid progenitors (MP), or both populations are potential targets for primary and secondary mutations leading to acute disease. Cells bearing a single mutation are termed 'preleukemic' and upon undergoing subsequent mutation give rise to leukemic stem cells (LSC). LSC in turn give rise to the majority of malignant cells found in the leukemia population (blasts). Three possible therapeutic outcomes are illustrated by the arrows above the chart. The length of each arrow denotes the degree to which a particular therapeutic regimen might affect the leukemic blasts, stem/progenitors cells, or preleukemic cell types

therapeutic agent(s) destroys leukemic blasts but the LSC, regardless of its origin, survives (outcome #1, Figure 2). This might be because the LSC retains certain properties of the normal HSC or MP that render them resistant to drug therapy. Therefore, a clinical remission is achieved but the disease relapses relatively fast, driven by surviving LSC. In the second category (outcome #2), the therapeutic agent(s) destroys leukemic blasts and the LSC that originated from preleukemic MP. This gives a relatively stable remission for scenarios b and c since only the residual preleukemic cells survive. However, the LSC originating from HSC (panel a) are spared and could cause relatively fast relapse of the disease. Of course, the presence of preleukemic MP (b and c) can also lead to relapse, but remission may be more durable. In the final category (outcome #3), the therapeutic agent(s) destroys leukemic blasts and the LSC for all three scenarios, as well as the preleukemic MP in panels b and c. In this situation, leukemia deriving from a myeloid progenitor origin might be completely cured but leukemia with an HSC origin is likely to eventually relapse due to the presence of residual pre-LSC. Relapsed disease arising from preleukemic populations is likely to be caused by new secondary mutations, thereby leading to AML cells that may be biologically distinct from the original disease.

As illustrated in Figure 2, the level of success with therapeutic drugs may depend upon the cell type in which mutation(s) initially occur. HSC populations bearing the initial mutation (panel a) are less likely to be targeted, and these preleukemic cells can cause disease relapse. This might be particularly evident if the preleukemic mutations result in genomic instability and/or increased self-renewal (e.g. HoxA9). If the initial mutation is at the MP level (panels b and c), the preleukemic cells might be more sensitive to therapy. Irrespective of whether the initial mutation is at the HSC or MP level, selective pressure from chemotherapy could result in the development of new mutations that render the preleukemic and/or LSC populations increasingly drug resistant. Subsequent relapsed disease would then be expected to respond poorly to further cycles of chemotherapy.

Molecular mechanisms controlling growth and survival of LSC

The various scenarios described in Figure 2 serve to highlight the potential complexity of stem cell-based malignancies and emphasize the need for better molecular characterization of mechanisms specific to the LSC population. To this end, it is instructive to consider the vast number of studies describing numerous mutations that occur in AML (Lowenberg *et al*., 1999; Dash and Gilliland, 2001). Characterized leukemia mutations impact a wide range of cellular pathways and processes including proliferation, cell cycle, apoptosis, cytokine responsiveness, adhesion, morphology, etc. More specifically, aberrant activation of signaling pathways such as Flt3, Ras, PI3 kinase, NF-κB, Stat3/5, and others have been described in detail by many groups (Gilliland and Griffin, 2002; Ravandi *et al*., 2002; Steelman *et al*., 2004). While very little is known as yet about how these anomalies function at the stem cell level, a number of studies have begun to suggest pathways that may influence LSC survival. We note however, that it is important to validate any potential LSC-specific mechanism before drawing conclusions as to its relevance. Recent analysis of the BCR/ABL pathway in CML provides an interesting case in point. The Abl kinase inhibitor imatinib (also known as STI-571 and Gleevec) has a strong cytotoxic effect on the vast majority of CML cells by specifically inhibiting the kinase activity of the BCR/ABL oncogene (O'Dwyer and Druker, 2000). Thus, one might conclude that all CML cells have acquired a critical dependence on BCR/ABL activity for survival. However, recent studies by Graham *et al*. (2002) have suggested that imatinib is not cytotoxic to the CML stem cell population, but rather only cytostatic. A large proportion of CML stem cells are quiescent (Holyoake *et al*., 1999), suggesting that constitutive kinase activity is necessary for survival of actively cycling CML cells, but perhaps not for quiescent or less metabolically active CML stem cells. Further, anecdotal evidence from clinical experience indicates that CML is effectively suppressed as long as patients continue to take imatinib, but that relapse occurs when treatment is discontinued. The experience with imatinib thus demonstrates that the role of specific pathways in mediating drug sensitivity in the LSC population cannot necessarily be inferred by studies of more differentiated leukemic cells.

Although key survival mechanisms in human LSC have not yet been directly identified, several lines of investigation have suggested pathways that may play a central role. For example, analysis of primary AML LSC has shown constitutive activation of the NF-κB transcription factor complex in a large percentage of specimens (Guzman *et al*., 2001a). This important transcription factor has been the focus of numerous studies in the cancer field (Mayo and Baldwin, 2000; Orlowski and Baldwin, 2002). In the vast majority of cases, activation of NF-κB is directly linked to increased growth and survival of tumor cells. Thus, if LSC acquire NF-κB dependence as part of the pathogenic process, then inhibiting this pathway may be an apoptotic stimulus and/or sensitize LSC to a variety of other agents. This concept is supported by studies in other tumor types, where loss of NF-κB is strongly associated with increased apoptosis and sensitivity to chemotherapy (Mayo and Baldwin, 2000; Wang *et al*., 1999). Notably, none of the commonly used AML chemotherapy agents (Ara-C, anthracyclines, etc.) inhibit NF-κB; but rather act to further upregulate NF-κB activity (Brach *et al*., 1992; Laurent and Jaffrezou, 2001; Tergaonkar *et al*., 2002). Hence, toxicity of some drugs may be at least partially 'masked' by increased NF-κB, which is likely to have a prosurvival function. As yet, no clear or consistent mechanism has been described to explain the constitutive NF-κB activity found in primary AML cells. Activating mutations of the Flt3

and Ras genes are commonly observed in AML (Stirewalt *et al*., 2001), and evidence suggests Flt3 can activate Ras (Dosil *et al*., 1993), which in turn may stimulate NF-κB (Baldwin, 1996). However, a recent report using the Flt3 inhibitor AG1296 described little to no inhibition of NF-κB activity, despite clear inhibition of Flt3 (Birkenkamp *et al*., 2004). In the same study, treatment with the farnesyl transferase inhibitor (FTI) L-744832 resulted in some NF-κB inhibition; however, the broad activity FTIs precludes specific analysis of Ras. Thus, the relative contribution of Flt3 and Ras signaling with regard to NF-κB remains uncertain. Moreover, no available data indicate how such pathways directly affect the biology of LSC.

A second mechanism implicated in LSC survival is signaling via the PI3 kinase pathway. Like the other pathways mentioned above, constitutive PI3 kinase activity has been reported for a large percentage of primary AML specimens (Xu *et al*., 2003; Zhao *et al*., 2004). In addition, at least two studies have demonstrated loss of LSC as a result of treatment with drugs that inhibit PI3 kinase activity (Wierenga *et al*., 2003; Xu *et al*., 2003). Interestingly, PI3 kinase is known to activate NF-κB in some circumstances, thereby suggesting a common survival pathway in which both factors are involved. Evidence supporting this theory was recently reported by Birkenkamp *et al*. (2004) in studies where treatment of primary AML cells with the PI3 kinase inhibitor LY294002 resulted in downregulation of NF-κB activity.

In addition to pathways controlling survival, exciting recent studies have also begun to describe genes regulating self-renewal mechanisms in both normal and leukemic stem cells. Signaling via Notch, Sonic Hedgehog, and Wnt pathways are all implicated in controlling HSC self-renewal (Reya *et al*., 2001). Similarly, the polycomb gene Bmi-1 has been shown to directly mediate self-renewal of both normal and leukemic stem cells (Lessard and Sauvageau, 2003; Park *et al*., 2003). Interestingly, the data from Bmi-1 studies supports the concept that basic mechanisms of self-renewal are shared between normal and malignant stem cells. If true, then the regulation of self-renewal pathways becomes a focal point for approaching LSC-specific therapies. Indeed, a key question becomes – will modulation of self-renewal provide therapeutic benefit in the context of AML? Although inhibition of self-renewal might slow expansion of the LSC population, it is not necessarily a cytotoxic signal. Thus, one can imagine that inhibited self-renewal processes might simply force LSC into a dormant condition. In addition, if self-renewal mechanisms are conserved, then inhibition of such pathways is likely to also affect normal HSC. Thus, understanding how self-renewal processes are linked to mechanisms of survival is a critical issue to consider in devising LSC-targeted therapies.

While it is attractive to suggest that inhibition of self-renewal pathways might impair survival of LSC, a direct link between self-renewal and antiapoptosis signals has not been clearly established in stem cells. However, one possible consequence of blocking self-renewal could be a commensurate increase in differentiation pressure, which may in turn deplete the LSC compartment. Indeed, using differentiation as a means to treat hematologic malignancy has been highly successful in the context of acute promyelocytic leukemia (APL), where all trans retinoic acid (ATRA) induces remission for a majority of patients (Tallman *et al*., 2002). The underlying mechanism is clearly related to providing a strong differentiation signal to the APL cells. Similarly, recent studies have shown that ligation of the CD44 antigen is a differentiation signal for primary AML cells *in vitro* (Charrad *et al*., 1999). Further, initial results by Jin *et al*. (2003) showed *in vivo* reduction of LSC activity in an NOD/SCID xenograft model using CD44 antibody treatment. This observation is intriguing in that modulation of CD44 binding might function as a differentiation signal to the LSC, or alternatively, as a means to inhibit cellular interactions with the hematopoietic microenvironment. Hence, treatment with anti-CD44-based drugs may represent an exciting strategy to diminish LSC self-renewal and/or to mediate extrinsic survival signals. Notably, the affect of the marrow microenvironment remains largely unexplored with regard to LSC biology. While several studies have demonstrated the phenomenon of cell adhesion-mediated drug resistance (CAM-DR) for hematologic malignancies (Hazlehurst and Dalton, 2001), details of this phenomenon have not been described at the stem cell level. Also, one report suggests that primary AML blasts can generate conditions that promote their adhesion to endothelial cells (Stucki *et al*., 2001). Thus, a role for integrin-mediated signaling or a similar mechanism of extrinsic control seems possible for primitive leukemic cells. As suggested by the CD44 studies, a potentially interesting strategy to address this issue might be the use of monoclonal antibody therapy to inhibit microenvironment signals from stimulating/supporting primitive leukemic cells. For example, treatment with anti-VEGF-R antibody reduces *in vivo* angiogenic activity and appears to either directly or indirectly inhibit the growth of leukemic cells (Dias *et al*., 2001; Zhu *et al*., 2003). Either alone, or in combination with cytotoxic drugs, this approach may yield interesting results. Similarly, antibody-based inhibition of cytokines or adhesion molecules that modulate hematopoietic growth might also sensitize leukemic cells to various forms of treatment.

Effects of current therapies on the LSC

The mainstay of AML therapy for over 10 years has been remission 'induction' therapy using a combination of Ara-C (cytarabine) and an anthracycline (typically daunorubicin or idarubicin), followed by several months of 'consolidation' therapy consisting of multiple cycles of Ara-C (Perry, 2001). Although, induction therapy often achieves remission, if not followed by consolidation therapy, most patients rapidly relapse. This observation suggests that in the context of a stem cell-based disease such as AML, induction regimens do not

effectively target the leukemic or preleukemic stem/progenitor populations (see Figure 2). Experimental evidence directly supporting this hypothesis has come from two recent studies in which both Ara-C and daunorubicin were shown to be less toxic to primitive AML cells in comparison to more mature leukemic blasts (Costello *et al*., 2000; Guzman *et al*., 2001a). Moreover, given the known characteristics of human LSC, especially the lack of cell cycle activity, there is little reason to believe that current chemotherapy regimens will preferentially target malignant stem cells. Interestingly, despite the lack of a clear mechanism, consolidation therapy with Ara-C improves the length and durability of AML remission. This observation appears somewhat paradoxical considering the fact that Ara-C is preferentially toxic to cells in S phase, yet AML LSC are mostly quiescent. One possible explanation for the utility of Ara-C derives from murine hematopoietic transplant models. It has been commonly observed that insult to the hematopoietic system with cycle-active drugs such as 5-fluorouracil (5-FU) induces a transient increase in the cell cycle activity of quiescent stem cells (Harrison and Lerner, 1991). Presumably, this phenomenon is caused by homeostatic mechanisms that regulate repopulation of the hematopoietic compartments after drug treatment. One might imagine that similar mechanisms exist within the AML population, and that ablation of blast cells might induce increased cell cycle activity in the LSC population. If so, then for at least transient periods after one dose of Ara-C, a relatively large proportion of LSC could be susceptible to subsequent administration of drug. If appropriately timed over multiple cycles, the net effect of this phenomenon could be substantial ablation of the LSC pool, which then might lead to relatively durable remission.

In addition to a mostly quiescent cell cycle status, another complication related to targeting tumor stem cells derives from their potential expression of membrane efflux pumps. Normal HSC are known to express surface membrane proteins such as MDR1 and Bcrp1/ABCG2 that function to efflux certain molecules (Chaudhary and Roninson, 1991; Zhou *et al*., 2001). Chemotherapy agents such as anthracyclines are substrates for these efflux pumps and are removed from stem cells relatively fast. Whether or not efflux mediators are present in LSC has not yet been studied in detail, but given the similarity of LSC to normal HSC, the presence of such molecules is certainly plausible.

To date, the only therapeutic approach that has attempted to directly target leukemic progenitor cells has been the antibody-based drug gemtuzumab ozogamicin (Mylotarg). This anti-CD33 monoclonal antibody is conjugated to the toxic antibiotic calicheamycin and preferentially targets cells expressing the CD33 antigen (Hamann *et al*., 2002). However, the degree of CD33 expression on primitive leukemia stem cells has not been clearly determined and appears to be variable. One possible explanation for varying levels of CD33 expression may derive from the models depicted in Figure 2. LSC deriving from MPs, where CD33 expression is already present, may be more likely to retain the antigen. In contrast, more primitive HSC, which do not normally express CD33, may fail to upregulate the gene upon transformation to a leukemic phenotype. Nonetheless, antibody-based regimens have demonstrated strong promise in oncology and future efforts to target LSC are clearly warranted. Another possible antigenic target is the CD123 molecule, which encodes the interleukin-3 receptor alpha chain. Several studies have indicated increased CD123 expression in myeloid leukemias (Testa *et al*., 2004), and one report describes strong expression of CD123 on the LSC population but not on normal HSC (Jordan *et al*., 2000). The differential expression of CD123 on malignant stem cells makes it a potentially attractive target for therapy.

Strategies to identify LSC-specific apoptotic mechanisms

As yet, the pathways that specifically regulate LSC survival are unclear; however, there are recently described stimuli that trigger robust apoptosis in the LSC population while sparing normal HSC. Thus, such stimuli must be targeting pathways unique to the LSC, and represent potentially powerful tools to identify mechanisms controlling survival in malignant stem cells. For example, work from our laboratory has shown that treatment of normal vs leukemic cells with the combination of a proteasome inhibitor (MG-132) and the anthracycline idarubicin is sufficient to induce preferential apoptosis of LSC (Guzman *et al*., 2002). Moreover, the cell death observed is very rapid, occurring in approximately 12 hours *in vitro*. Subsequent studies have shown similar results using the clinically approved proteasome inhibitor, bortezomib, also known as PS-341 or Velcade™ (MLG and CTJ unpublished). Interestingly, unlike almost all chemotherapy agents in current use, proteasome inhibitors are well known to downregulate NF-κB activity (Sunwoo *et al*., 2001; Hideshima *et al*., 2002), thereby supporting a role for NF-κB in LSC survival. Importantly though, several studies suggest that NF-κB is not the only factor mediating survival of AML cells (Turco *et al*., 2004). Rather, it appears to be one of several pathways that contribute to drug resistance. Indeed, direct inhibition of NF-κB does not induce the same degree of rapid apoptosis seen with MG-132 + idarubicin (Guzman *et al*., 2002). However, a markedly increased sensitivity to chemotherapy agents has been observed in primary AML cells when NF-κB is downregulated using molecular genetic methods (Romano *et al*., 2000; Birkenkamp *et al*., 2004).

A second pathway implicated in LSC-specific cell death is controlled by p53. Treatment of primary AML cells with proteasome inhibitors and idarubicin induced clear activation of p53 and increased levels of the p53 target genes GADD45, p21, and Bax, all of which are strongly implicated in p53-mediated apoptosis (Guzman *et al*., 2002). Interestingly, the p53 gene is wild type in most leukemia specimens (Stirewalt *et al*., 2001), suggesting that strategies involving activation of the

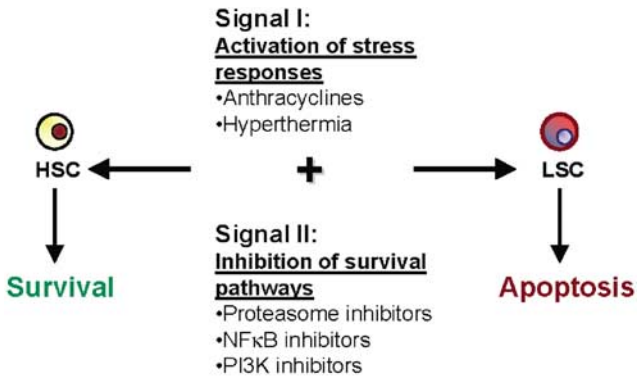

Figure 3 Model for LSC apoptosis. Two types of extrinsic stimuli are proposed to preferentially induce apoptosis in leukemic stem cells (LSC) while sparing normal hematopoietic stem cells (HSC). The combination of certain inducers of cellular stress (signal I), and an inhibitor of specific survival pathways (signal II) is sufficient to mediate LSC-specific cell death

p53 pathway may be applicable to the majority of AML patients.

As described earlier, another pathway recently linked to LSC survival is the PI3 kinase pathway. Studies by Xu *et al*. have demonstrated a reduction in LSC after treatment with the PI3 kinase inhibitor LY294002. Similarly, Wierenga *et al*. showed that the drug ET-18-OCH_3, a known PI3 kinase inhibitor (Ruiter *et al*., 2003), is also preferentially toxic to LSC in comparison to normal HSC. Interestingly, ET-18-OCH_3 was more effective when combined with heat shock (Wierenga *et al*., 2003). This observation, in conjunction with the proteasome inhibitor data described above, may begin to suggest basic rules that dictate survival of LSC. Figure 3 illustrates a proposed model for the preferential induction of apoptosis in the LSC population. Current evidence indicates that when specific types of cellular stress are combined with inhibition of survival signals, LSC are induced to undergo apoptosis while normal HSC are spared. For example, treatment with the anthracycline idarubicin is known to induce genotoxic stress via the generation of oxygen free radicals and induction of DNA strand breaks (Gutteridge and Quinlan, 1985). Used alone, idarubicin does not have a significant tumor-specific effect on LSC (MLG and CTJ unpublished). However, in combination with proteasome inhibitors, which are known to block survival signals (i.e. NF-κB and downstream targets), a robust LSC-specific apoptosis is observed. Similarly, hyperthermia induces the heat-shock response, and in combination with PI3 kinase inhibition (via ET-18-OCH_3 treatment) also fulfills the criteria of the proposed model. Most of the drugs/stimuli listed in Figure 3 demonstrate some degree of LSC toxicity when used as single agents but exhibit substantially enhanced activity when used in the combinations shown. Several of these agents are appropriate for clinical use and represent possible novel therapeutic options for AML patients. Additional testing in animal models will further validate their potential utility *in vivo*.

Summary

Myeloid leukemia is typically a disease of stem or progenitor cell origin. Importantly, the malignant stem/progenitor cell is biologically distinct from more differentiated blast cells and in most cases is unlikely to be effectively targeted by standard chemotherapy agents. Recent studies have described experimental systems for analysis of both human and murine LSC that will greatly improve our understanding of stem cell-based pathogenesis and provide models for testing new therapeutic strategies. These systems are beginning to define the specific cellular targets of transformation, the molecular mechanisms of pathogenesis, and the *in vivo* biology of LSC. Furthermore, combinations of specific agents have been shown to preferentially induce apoptosis in human LSC, despite their predominantly quiescent cell cycle status. Molecular analyses indicate that signal transduction pathways such as those mediated by NF-κB and PI3 kinase are directly implicated in the survival of human LSC and represent interesting targets for intervention. In addition, activation of p53-mediated apoptosis pathways has also been associated with LSC death. Taken together, these findings suggest that LSC-targeted treatment regimens can be achieved using clinically relevant drugs and might be effectively added to traditional regimens as a means to achieve more durable remissions in AML.

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