**Cancer Genes and Hematopoiesis**

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**BLOOD CELLS** and their progenitors have been the proving ground for many ideas about development and differentiation, growth factors and receptors, transcriptional control, and normal and cancerous growth.1 Because leukemias, both human and experimental, are especially accessible cancers, they have provided many of the cancer genes and oncogenic mechanisms that we know the most about.2

An abiding hope in the pursuit of leukemogenic genes has been the prospect of understanding hematopoiesis at a molecular level. This notion has its origins in the idea that cancer is a distorted version of normal growth and development.3,4 Cancers arise through mutations affecting a subset of genes, here called cancer genes. Many cancer genes are mutant proto-oncogenes, genes designed to promote normal growth; when made hyperactive by mutations, such as base substitutions, translocations, or amplification, they drive inappropriate growth in a dominant fashion. Conversely, tumor suppressor genes normally restrain growth, but inactivation by base changes, deletions, and chromosomal loss also contributes to oncogenic change. As these principles came into view over the past two decades, it has become an article of faith that genes identified through the study of cancer would ultimately illuminate our picture of normal developmental events, as well as normal growth.

To what extent has this conviction been sustained through the study of blood cells? Our objectives here are to review the criteria by which we implicate cancer genes in the formation or function of blood cells, to describe the evidence that supports or (in some cases) undermines such suspicions, and then to re-evaluate the premise that cancer genes are useful guides to understanding normal cell function. We will emphasize genetic manipulation of the mouse as a means to test ideas about gene function and to show how the existence of gene families complicates the analysis.

We now have an impressive accounting of the proteins that determine the major events in a cell’s history—its growth and division, differentiation, performance of specialized functions, and programmed cell death. Many of these proteins are encoded by known proto-oncogenes, some by known tumor suppressor genes. They can be classified by their locations—outside the cell, on the cell surface, or in the cytosol or nucleus (Fig 1). Or they may be classified according to their biochemical properties, such as enzymatic activities.

Some of the best understood components are secretory factors and receptors, including those that mediate growth and differentiation of hematopoietic cells. These factors and receptors show a high degree of specificity for each other and for the kinds of effects they produce. Thus, the influence of a secretory factor requires that a cell with the right receptors be at the right place at the right time. This kind of specificity is relatively easy to understand. In contrast, it is difficult to fathom how the intracellular components of signaling pathways produce specific cellular phenotypes in response to extracellular signals. Several signaling mechanisms, enclosed in the boxed area in Fig 1, are known to operate in many different cell types and the hierarchy of the signaling relay has been partially deciphered. Ultimately, the relay must interface with cell-cycle components and transcription factors to determine cell fate. But it is not known how a specific response occurs to a specific message received at the cell surface. In asking whether cancer genes are informative about hematopoiesis, we will confine the discussion to this murky boxed territory of intracellular signaling devices.

**APPROACHES TO HEMATOPOIESIS THROUGH CANCER GENES**

Why might we suspect a cancer gene to be involved in hematopoiesis? The first such genes to be suspects were those frequently mutated in leukemias or lymphomas, either in humans or experimental animals. Some familiar examples include the myb gene, to which we shall return; abl, which will not be further discussed here (see ref 5); and erb B, which encodes the epidermal growth factor receptor 6 and is thus unlikely to be involved in hematopoiesis. The other cause for suspicion comes from gene expression—the finding that a gene is expressed exclusively, effusively, or in a highly regulated fashion in blood cells. Among the genes that meet at least one of these criteria are, again, myb, and several members of the src gene family, discussed below.

At least three general strategies are available for evaluating these suspicions. (1) Relevant genes can be manipulated in cultured blood cells through the use of genetic vectors for ectopic expression or through the use of antisense methods to interfere with endogenous gene expression. These approaches are subject to many qualifications and rarely definitive. (2) In rare instances, receptors encoded by proto-oncogenes (such as Kit) or ligands for such receptors (such as colony-stimulating factor-1 [CSF-1], the Fms ligand, or Steel factor, the Kit ligand) have been directly implicated in hematopoiesis by fortuitous inherited mutations affecting blood cells. The affected cells include hematopoietic stem cells in the case of Kit and Steel, and the result is anemia and pancytopenia.7 The CSF-1 mutation affects the macrophage lineage, causing osteopetrosis due to a block in osteoclast development.8 (3) Methods developed over the past decade now permit any gene suspected of a role in hematopoiesis (or any other function) to be evaluated directly by making a designed germ line mutation.

Central to this third approach is the growth of totipotent...
Fig 1. Classes of proteins involved hematopoietic cell growth, differentiation, programmed death, and specialized functions. Extracellular factors (such as CSF-1 or Steel factor) bind their receptors (the c-Fms protein or the Kit gene product), which initiate a cascade of intracellular signaling responses including activation of tyrosine kinases and phosphatases, enhancement of phosphoinositide (PIs Ins) metabolism, activation of GTP binding proteins and their cognate GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GNEFs), and activation of serine/threonine (Ser/Thr) kinases. This leads to modification (phosphorylation, protein complex formation) of nuclear transcription factors and cell cycle components, producing the altered gene expression and growth regulation required for the appropriate phenotypic response.

Embryonic stem cells (ES cells), derived from the inner cell mass of early mouse embryos. Upon introduction into ES cells of a so-called “targeting vector,” containing a disrupted version of the gene of interest, homologous recombination occurs occasionally with one of the two normal copies of the gene, rendering that copy inactive. Once the rare cell with the disrupted gene has been identified and isolated, it is injected into blastocysts of an appropriate mouse strain. The affected embryos were much paler than their heterozygous litter mates, and peripheral red blood cells (RBCs) were virtually all nucleated. This indicates that they are able to make RBCs efficiently in the yolk sac, but not in the fetal liver, where adult-type erythropoiesis usually commences about day E13 in the mouse. In addition, other aspects of hematopoiesis, including myelopoiesis, were also defective in the homozygous animals.

Similar results have recently been reported by three groups that have made inactivating mutations in the retinoblastoma (Rh) tumor suppressor gene. This was surprising because, unlike myb, the Rh gene has been implicated in many kinds of cancers, but rarely hematopoietic tumors; furthermore, the gene is very widely expressed, and biochemical studies link it to transcriptional control during the cell cycle, a general rather than a lineage-specific phenomenon. Nevertheless, homozygous embryos die at day E13; shortly before death they become profoundly anemic. The hypocellular fetal liver and an excess of nucleated over anucleated RBCs indicate an inability to produce mature RBCs by adult-type erythropoiesis. This unexpected phenotype has yet to be explained.

These two examples show that targeted gene ablation can clearly implicate cancer genes in hematopoiesis, whether or not they were suspected earlier of having such functions. But the still incomplete evidence implicating members of the src gene family in hematopoiesis presents a more vexing set of findings.

There is a multiplicity of protein-tyrosine kinases in cells: Many transmembrane receptors, including some for hematopoietic factors, and several classes of cytoplasmic proteins, including multiple members of families known for their founders (such as src, fgs, abl, csk, and tyk). Members of the several families of cytoplasmic tyrosine kinases are recognized by similarities in amino acid sequence, by the arrangement of functional domains, and by other properties, such as chemical modifications and intracellular locations.

Of the nine genes in the src gene family, four (src, yes, fgr, and lck) were first isolated as active oncogenes, and five others (fyn, lyn, hck, blk, and yck) were later encountered as close relatives (Fig 2). All of the genes are similarly arranged into exons and introns, despite their placement on several different chromosomes. All encode proteins about 500 amino acids in length with similar design: myristic acid at the amino-terminus assists membrane association; an amino-terminal segment varies greatly among family members; two noncatalytic, regulatory regions, known as Src homology 3 and 2 follow the variable domain; and the kinase domain with an autophosphorylation site is next to a short carboxy-terminal region that negatively affects the kinase domain when tyrosine phosphorylated. Thus, Src proteins are themselves regulated by tyrosine kinases and phosphatases. Although many defined proteins are tyrosine phosphorylated in response to activation of Src-like kinases, the physiologically relevant substrates of these kinases remain elusive.

Many, perhaps all, of the Src family kinases are also associated with and affected by different cell-surface proteins, including components of the T-cell receptor, cytokine and Fc receptors, and others. The Src kinases participate in signaling pathways whose downstream components include Ras proteins, serine-threonine kinases, and specific transcription factors like AP-1. Some Src family proteins, like Src itself, are expressed in many cell types. Others, like Lck, Itk, and Fgr, are produced in very few cell types, and these cells are generally hematopoietic (Fig 3).

The first member of the src gene family to be disrupted by gene targeting was src itself, an event reported by Soriano et al in 1991. Homozygous src-deficient animals were born in nearly expected numbers, with apparently normal neurologic function, although they were somewhat smaller than...
their heterozygous littersmates. The viability of these animals was surprising. After all, src is highly conserved in evolution, expressed in virtually all cells in vertebrate animals, and especially active in the brain, where alternative splicing generates special forms of Src protein in higher vertebrates. In addition, the Src kinase is regulated during the cell cycle, suggesting an essential function at the cellular level. Nevertheless, the small src-deficient animals seem to have a single problem: osteopetrosis. The disorder is evident in the long bones, with some displacement of sites for hematopoiesis (but not enough to produce anemia), and in the head, blocking tooth eruption and hence impairing the ability to eat. In subsequent work, Lowe et al.20 have attributed the disease to functionally impaired osteoclasts, cells (like many others) that normally contain high levels of Src protein.

The viability and the relatively limited pathology of src-deficient mice were presumed to depend on the competence of other src family genes to fulfill similar functions in the many cells in which src is believed to act. This presumption is now being tested. Stein et al.21 have mutated two other widely expressed members of the src family—fyn and yes—and have begun to cross mutant mice to generate multiply homozygous mice. Like the src-deficient mice, fyn knockouts have high viability and restricted, albeit very interesting, disorders: subtle impairment of T-cell proliferation21 and defects in higher neural functions, including long term potentiation and learning.22 The yes-deficient mice seem to be entirely normal at current levels of inspection (P. Stein and P. Soriano, personal communication).

However, when bred to form double homozygotes the viability of the mice falls precipitously, and those that are born are especially frail. In addition, the fyn/yes double homozygotes have an unexpected immune complex disease that affects renal glomeruli. The so-called “synthetic lethality” of the src, fyn, and yes mutations has yet to be explained physiologically or biochemically, but it is presumed to reflect inadequate levels of src-like kinases in multiple cell types.

Some of these complexities may be avoided by examining the src family members expressed exclusively in hematopoietic cells (Fig 3). For example, Lck protein is found principally in T cells, and Hck and Fgr proteins are mainly in monocytes, macrophages, and granulocytes. Moreover, in some of these cells no more than two members of the src gene family appear to be active.

A stunning validation of this approach was recently reported by Molina et al.23 The lck gene has been occasionally involved in experimental T-cell lymphoma and is a partner in the 1:7 translocation observed in childhood ALL.24,25 Moreover, expression is largely confined to T cells at several stages of development, including mature T cells in which Lck protein associates with multiple transmembrane proteins, such as CD4, CD8, and the interleukin-2 (IL-2) receptor.18 Elimination of a competent lck gene by homologous recombination produces homozygous mice with an atrophic thymus and a nearly complete arrest of T-cell ontogeny before the CD4/CD8 double-positive stage.23 Thus, lck is required for efficient progression from the double-negative to the double-positive stage of T-cell ontogeny.

Our concurrent experiments with the hck and fgr genes, in conjunction with Phil Soriano, now at the Fred Hutchinson Cancer Research Center (Seattle, WA), have produced more subtle results (Lowell CA, Soriano P, Varmus HE, submitted). Even in the mono-myelocytic series, where both genes are highly expressed, sometimes without other src family members, hematopoiesis appears to occur normally, despite inactivation of both genes.

The strategy used to inactivate these two genes was unexceptional. A bacterial gene conferring antibiotic resistance was inserted in a 5' coding exon of the genes, thus blocking translational initiation. Germ line chimeras were readily obtained with multiple clones of targeted cells, and heterozygous and homozygous animals were generated with the expected Mendelian frequency. As predicted, the Hck and Fgr proteins were reduced in the heterozygous and absent in the homozygous animals. Nevertheless, the hck- and fgr-deficient animals appeared completely healthy, with no overt anatomical or histologic defects and no morphologic abnormalities in peripheral blood or blood forming organs. When hck and fgr mutants were interbred to generate double homozygotes, these findings did not change: the expected number of double homozygotes was born, the animals appeared healthy, and the blood counts were normal. Bone marrow preparations from these hck/fgr double homozygotes were highly cellular, with abundant granulocytes, megakaryocytes, and other elements. In addition, the white pulp of spleens from these animals showed normal extra-medullary hematopoiesis, with basophiles, eosinophiles, and megakaryocytes.

src family members might have important effects on specialized functions of blood cells, even though they are not
required for formation of blood cells. So, having found no evidence for defective hematopoiesis in *hck* and *fgr* mutants, we have begun to examine blood cells from these mice for impaired blood cell functions, including phagocytosis, bactericidal activity, cytokine responsiveness, and cytokine release. At least one of these assays has provided interesting results. Thioglycollate-induced, peritoneal macrophages from *hck*-deficient mice ingest latex beads less efficiently than similar cells from normal animals, suggesting that this kinase plays an essential role in receptor-independent phagocytosis. *fgr*-deficient animals do not show this trait, and the double homozygotes are no more affected that the *hck* homozygotes, even though *fgr* and *hck* proteins are normally found at similar levels in these cells.

We have also tested doubly homozygous *hck*/*fgr*-deficient animals for defects in natural immunity. Unexpectedly, the doubly mutant mice show increased susceptibility to infection by the intracellular bacterium *Listeria monocytogenes*, whereas mice deficient in either gene alone are resistant to *Listeria*. The mechanism of the reduced immunity in the double homozygotes is not clear, but the results show that members of the *src*-gene family share functions in the cells in which they are coexpressed, requiring generation of doubly deficient mutant mice before phenotypic consequences appear.

This principle is further illustrated when *hck*-deficient mice are bred to *src*-deficient animals. In contrast to either of the singly mutant mice, the *hck/src*-deficient mice have hematopoietic deficiencies characterized by anemia, leukopenia, and an accumulation of abnormal, immature, cells in the spleen (our unpublished observation). Again, the mechanisms are not apparent, but the phenotype reveals that Src kinases must cooperate to provide the intracellular signals required for normal hematopoietic development.
CONCLUSIONS

The gene-targeting strategies central to the results discussed here are not yet trivial to perform, but use of them is rapidly transforming the study of mammalian development and disease, with over 200 genes already mutated. The experiments are sometimes complicated by genetic redundancy, and crucial phenotypes may be difficult to perceive. But in several cases, some of which are reviewed here, genes have been unequivocally implicated in major developmental events, like hematopoiesis.

What do these experiments now say about earlier speculations that cancer genes associated with blood cells by tumor genotypes or by expression patterns might have important functions in hematopoiesis? Despite paradigm-filling examples like myb, other genes (like erb-B) that contribute to leukemogenesis, are probably not involved in hematopoiesis. Conversely, cancer genes implicated in hematopoiesis, such as Rb, may not be leukemogenic. Expression patterns appear to be, at best, weakly predictive about the roles of cancer genes in hematopoiesis. The phenotypes of myb- and lck-deficient mice do conform to expectations based on sites of expression. But deficiencies of src, hck, or fgr have not produced the profound developmental abnormalities that might have been expected, whereas the ubiquitously expressed Rb gene is specifically required for erythropoiesis in the fetal liver.

Recent findings suggest that the src family genes may prove to be more important in the regulation of differentiated functions than in growth and development as originally proposed. But the genetic analysis must also take into account the possibility of shared functions; so the jury will be out on src and its cousins until all family members have been inactivated and the appropriate combinations of deficiencies assembled. Finally, we should recall that these new genetic approaches, however powerful, have not, and will not, by themselves answer a central question facing students of any cell lineage: how do cells that receive specific signals at the cell surface then use general signaling devices to make an appropriate phenotypic change?

REFERENCES

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