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Autostimulatory Mechanisms in Myeloid Leukemogenesis

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WEHI-274 is a monocytic leukemia that arose in a BALB/c mouse infected with Abelson murine leukemia virus. A series of subclones were derived from early passages of this tumor. Three subsets of these leukemogenic subclones were identified. Two subsets demonstrated autostimulatory patterns of growth. This was due to the ectopic production of the T-cell lymphokine the panspecific hemopoietin IL-3 in one case and of the T-cell lymphokine granulocyte-macrophage colony-stimulating factor (GM-CSF) in the other. The third type of subclone did not secrete any autostimulatory growth factor. In the subclone producing IL-3, one copy of IL-3 gene was rearranged and abnormal IL-3 RNA transcripts were present in the nucleus. Subclones producing GM-CSF also contained abnormal GM-CSF RNA transcripts, although no rearrangement of the GM-CSF gene was detected. All three sets of subclones shared a common rearrangement of one c-*myb* oncogene, suggesting that they share a common ancestor. These results suggest that initiation or progression of leukemogenic behavior in this abnormal clone occurred in three different ways, two of which involved autostimulation by the ectopic activation of T-cell lymphokine genes.

Key words: c-*myb* rearrangement, granulocyte-macrophage colony-stimulating factor, panspecific hemopoietin, interleukin-3, autostimulation, myeloid leukemia

There are now many examples of soluble polypeptides that stimulate the growth of specific types of cell. It is also well-established that the growth of tumor cells is usually much less dependent on such soluble growth factors than is that of their normal counterparts. In 1961 Hsu [1] proposed that one way in which a cell could become independent of exogenous sources of a growth factor would involve infection by a virus that carried the gene coding for that growth factor. Based on observations that tumor cells released "transforming growth factors" that caused nontransformed cells to assume some of the growth characteristics of transformed cells, Sporn and Todaro [2] elaborated upon the autocrine hypothesis and pointed to its implications

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for new therapeutic strategies. Evidence that the initiation of the production of an autostimulatory factor could actually cause a change to an oncogenic growth pattern came from experiments showing that the initiation of the ectopic production of a hemopoietic growth factor normally released from activated T cells, coincided with leukemogenesis [3]. Shortly thereafter two groups noted that the *v-sis* oncogene of the simian sarcoma virus encoded a product that closely resembled platelet-derived growth factor [4,5]. These experiments validated Hsu's earlier speculation and strongly supported the link between pathological production of an autostimulatory growth factor and oncogenesis.

Because the physiological source of several of the hemopoietic growth factors is well established, the hemopoietic system has provided several clear instances of tumors exhibiting ectopic production of hemopoietic growth factors [3,6,7]. In the case of the myelomonocytic leukemia WEHI-3B it is now clear that the ectopic production of a T-cell lymphokine panspecific hemopoietin (IL-3) or interleukin-3 (IL-3) results from pathological disruption of the growth factor gene [8]. Another monocytic leukemia, WEHI-274.14, also constitutively produces IL-3 and moreover appears to require this factor for its growth [7].

The myelomonocytic leukemia WEHI-3B provides an instructive example of how the behavior of tumor cells can change with continued passage. When initially isolated, WEHI-3B required an exogenous source of hemopoietic growth factors for optimal growth in agar. However, with one possible exception [9], the clones of WEHI-3B that are in use today no longer respond to IL-3. This loss of responsiveness to IL-3 probably reflects strong selective pressure during its long passage history against cells that depended on an autostimulatory mechanism and responded to IL-3 and selection for variants that no longer depended on IL-3 and thus had a growth advantage over the autostimulatory parental cell, especially at low cell densities.

The monocytic leukemia WEHI-274 arose in a BALB/c mouse that had been infected with the Abelson murine leukemia virus. Based on the history of WEHI-3B, we reasoned that cell lines that had been selected for the capacity to grow from single cells in vitro in the absence of exogenous sources of growth factors could differ substantially from the major population of leukemic cells in vivo. Therefore, we thawed an early frozen sample of WEHI-274 and plated out the cells in vitro in the presence or absence of a source of IL-3, namely, medium that had been conditioned by WEHI-3B. In parallel, cells were also cultured in vitro at high cell density in medium alone. Cells from both populations were then plated in agar, either in the presence or absence of WEHI-3–conditioned medium. Much higher numbers of colonies grew in the cultures in which WEHI-3–conditioned medium was present, suggesting that the bulk of cells in the original tumor responded to IL-3 in vitro. A number of these clones were plucked from the agar cultures and studied further. Subsequent experiments with fractionated WEHI-3B–conditioned medium and with purified or synthetic IL-3 demonstrated that the active factor in the WEHI-3B–conditioned medium was IL-3.

Previously we had shown that one such clone, WEHI-274.14, showed an autostimulatory pattern of growth when plated in agar at varying cell densities. Thus, whereas in the presence of WEHI-3–conditioned medium, WEHI-274.14 formed colonies in agar at cell densities ranging from 10^2 to 10^4/ml, in the absence of WEHI-3B–conditioned medium, colony growth occurred only at the highest cell density of 10^4/ml [7]. This pattern of dependence on an exogenous source of growth factor at

low cell densities but not higher cell densities is a characteristic sign of an autostimulatory mechanism. The presence of this mechanism was confirmed when conditioned medium was harvested from high density cultures of WEHI-274 (10^5/ml) grown in the absence of exogenous sources of IL-3 and was shown to enhance in a dose-dependent fashion the growth of WEHI-274.14 cells at low cell densities [7].

The nature of this autostimulatory factor was further investigated by biochemical techniques and shown to be indistinguishable from T cell-derived IL-3 in terms of its apparent molecular weight on gel filtration and behavior on reverse-phase HPLC [7]. Here we describe further the molecular nature of the factor produced by WEHI-274 and present evidence that one IL-3 gene in WEHI-274.14 has undergone a pathological rearrangement. We report that other clones of WEHI-274 isolated in parallel with WEHI-274.14 also show evidence of an autostimulatory mechanism but one that involves not IL-3 but a second T-cell lymphokine, GM-CSF. These subclones of WEHI-274 together with a third class of clone, which appears to produce neither IL-3 nor GM-CSF, share a common rearrangement of a c-*myb* gene, indicating that they arose from a common progenitor.

MATERIALS AND METHODS

Frozen stocks of WEHI-274 cells that had been passaged several times from the animal to high density in vitro cultures and back were obtained through the kindness of Dr. A. Harris at the Hall Institute. Conditioned medium was produced from WEHI-3B cells as described elsewhere [6]. The medium used was RPMI 1640 supplemented with 10% fetal calf serum, 2-mercaptoethanol 5×10^{-5} M, and glutamine 2.8×10^{-3} M supplemented with WEHI-3B–conditioned medium (3% of 10-fold concentrate) as indicated. In some experiments the medium was gelled by the inclusion of 0.3% Bacto-agar (Difco Laboratories, Detroit, MI).

Total cellular RNA was extracted by standard methods, and polyadenylated RNA was isolated using an oligo-dT cellulose column. Southern and Northern blot analyses were peformed by standard methods. The IL-3 probe was the 400 bp HindIII-XbaI cDNA fragment containing most of the coding region of the mature protein. The GM-CSF probe was a PstI-EcoRI cDNA fragment containing the entire coding region of the mature protein. The c-*myb* probe consisted of the 1.3 kb HindIII-EcoRI genomic DNA fragment, which comprises the second exon of the murine c-*myb* gene. All probes were labeled by random priming with random hexamers after the method of Feinberg [10].

Antipeptide antibodies were raised against synthetic peptides corresponding to defined regions of the IL-3 sequence and were affinity-purified and coupled to Sepharose beads as detailed elsewhere [11].

IL-3 was assayed as described elsewhere using a PSH-dependent line, R6X. GM-CSF was assayed using a colony assay as described elsewhere [6].

RESULTS

Evidence of Pathology of IL-3 Gene in WEHI-274.14

Southern blot analysis of DNA from the WEHI-274.14 subclone using a IL-3-specific probe indicated that one copy of the IL-3 gene was rearranged (see Table I). Digestion of normal mouse liver DNA with EcoRI resulted in hybridization with a

TABLE I. Organization of the IL-3 and c-*myb* Genes by Southern Blot Analysis of an EcoRI Genomic Digest

	IL-3 cDNA probe		c-myb genomic probe	
DNA source	No. of bands	Fragment size (kb)	No. of bands	Fragment size (kb)
Germline	1	8.5	1	6.5
274.14	2	8.5 and 9.1	2	6.5 and 2.8
274.28	1	8.5	2	6.5 and 2.8
274.3	1	8.5	2	6.5 and 2.8

single 8.5 kb fragment corresponding to that reported for the germline IL-3 gene [8]. However, in the case of DNA from WEHI-274.14, the IL-3 probe hybridized with two bands, one corresponding to a single germline copy of the IL-3 gene and the other to a larger, 9.1 kb fragment (Table I). Digestion of DNA from WEHI-274.14 with Bam-HI and hybridization with a IL-3 probe revealed a band of 3.1 kb in addition to the germline bands of 2.8 kb and 8.5 kb. Information from restriction maps of a IL-3 gene and data from other experiments in which DNA from WEHI-274.14 was digested with various restriction enzymes localized the rearrangement of the IL-3 gene in WEHI-274.14 to a region betwen 0.5 and 1.5 kb 5′ to the transcription-initiation site of the normal IL-3 gene.

Abnormal IL-3 Transcripts in WEHI-274.14

Northern blot analysis of whole-cell, polyadenylated RNA extracted from WEHI-274.14 cells revealed a surprising result (Fig.1). The IL-3-specific probe hybridized with three major transcripts of 8.0 kb, 4.5 kb, and 1.5 kb, contrasting with the 1.3 kb primary transcript seen in activated T cells. As shown in Table II, the 8.0 kb species was the major transcript and was present at high levels, which were markedly disproportionate to the relatively small amounts of biologically active IL-3 released by this cell line. Fractionation experiments indicated that the large, 8.0 kb and 4.5 kb transcripts occur predominantly in the nucleus and that, in accord with the low levels of IL-3 activity produced, only low levels of the smaller 1.5 kb cytoplasmic species could be detected. It is interesting to contrast this with the situation in the myelomonocytic leukemia WEHI-3B where the IL-3 RNA transcripts are indistinguishable from those in activated T cells.

Nature of the IL-3 Molecule Released by WEHI-274.14

Data showing that the IL-3 gene was rearranged in WEHI-274.14 (Table I) and that there were abnormal IL-3 transcripts present (Table II) strongly supported biochemical and biological evidence suggesting that WEHI-274.14 released a molecule related to IL-3 [7]. Experiments using antibodies specific for defined peptides corresponding to regions of the IL-3 amino acid sequence have further clarified the nature of the molecules with IL-3 bioactivity secreted by WEHI-274.14 cells. Thus antibodies specific for epitopes determined by the six N-terminal amino acids of IL-3 bound up to 70% of bioactivity released by WEHI-274.14 [12]. This observation indicated that these six amino acids, which are present on the primary products secreted by activated T cells but which had been cleaved from the subpopulation of molecules analyzed as IL-3 by Ihle et al [13], are also present on the bulk of IL-3 secreted by WEHI-274.14.

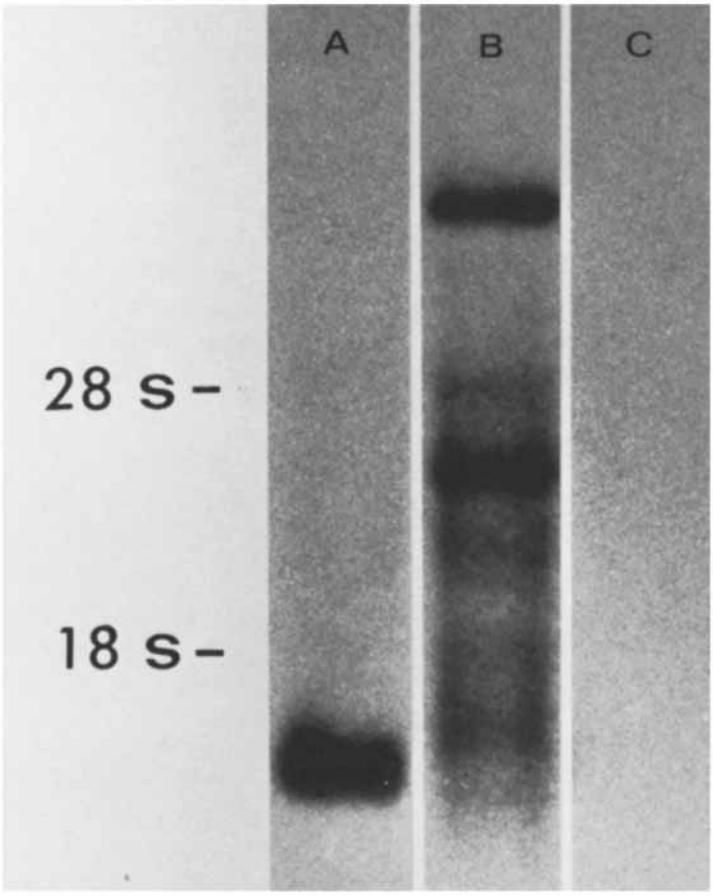

Fig. 1. Northern blot analysis using a IL-3-cDNA probe of whole cell polyadenylated RNA from A) WEHI-3B, showing a normal-sized 1.3 kb transcript; B) WEHI-274.14, showing the abnormal transcripts; and C) a control line, the IL-3-dependent R6-X, showing no IL-3 transcripts.

TABLE II. Detection of Specific IL-3 and GM-CSF Gene Transcripts by Northern Blot Analysis of Whole Cell Polyadenylated RNA

	IL-3 cDNA probe		GM-CSF cDNA probe	
Source of RNA	Presence of transcript	Dominant transcript (kb)	Presence of transcript	Dominant transcript (kb)
Activated T cell	+	1.3	+	1.1
274.14	+	8	–	—
274.28	–	—	+	10
274.3	–	—	–	—

Similar experiments showed that antibodies to peptides corresponding to the N-terminal 29 amino acids predicted by the nucleotide sequence of the IL-3 cDNA clones and also to peptides corresponding to the middle of the molecule (residues 91–112) also bound IL-3 bioactivity from WEHI-274.14 (Ziltener et al, in preparation). Together with data on the apparent Mr. and hydrophobicity of the IL-3 from WEHI-274.14 [7], these experiments indicate that the IL-3 from this leukemia is very similar, if not identical, to the T-cell product.

IL-3 and GM-CSF Genes in Other Subclones of WEHI-274

When other subclones of WEHI-274 that were derived at the same time as WEHI-274.14 were examined for abnormalities of IL-3 genes, different results were obtained. Although in two cases, ie WEHI-274.28 and WEHI-274.25, the cells released an autostimulatory factor, in neither case could IL-3 bioactivity be detected in supernatants of high density cultures. Moreover, there was no evidence for rearrangement of the IL-3 gene (Table I). Analysis of the factor in conditioned medium form WEHI-274.25 or WEHI-274.28 suggested that these lines produced a second T-cell lymphokine, GM-CSF. Southern blot analysis revealed a rearrangement of the GM-CSF gene in these subclones (Leslie et al, in preparation).

GM-CSF RNA in Subclones of WEHI-274

Northern blot analysis showed no evidence of RNA transcripts that hybridized with a GM-CSF probe in either WEHI-274.14 or WEHI-274.3 (or WEHI-3b). However, in WEHI-274.28 and WEHI-274.25 the GM-CSF probe hybridized with a strikingly abnormal pattern of transcripts, somewhat reminiscent of that seen in WEHI-274.14 with IL-3 probes. Thus there was a large (10 kb), abnormal transcript, together with one smaller transcript (about 1 kb) present at lower levels. In contrast, in activated T cells the GM-CSF transcript was about 1 kb in size. Fractionation experiments indicated that the dominant 10 kb transcript was present in the nucleus and was not detectable in the cytoplasm. None of the transcripts in leukemic cells hybridized with a probe specific for the LTR of Moloney murine leukemia virus.

Rearrangement of the c-*myb* Gene

The results detailed above suggested that there were at least three types of subclones in the mouse carrying the original WEHI-274 leukemia, those producing IL-3 transcripts, eg, WEHI-274.14, those producing abnormal GM-CSF transcripts, eg, WEHI-274.28 or WEHI-274.25, and those producing neither, eg, WEHI-274.3. The relationship between these clones was clarified by experiments that examined the c-*myb* gene in these clones (Table I), and indicated that in all three classes of subclones there was a common abnormality of one c-*myb* gene. This observation strongly suggested that all three subclones were derived from a common progenitor cell.

DISCUSSION

Our experiments show that autostimulatory mechanisms are operating in WEHI-274, a myeloid leukemia that arose in vivo. In this instance there is good biological evidence that factors that are secreted by leukemic cells, ie, IL-3 in the case of WEHI-274.14 and GM-CSF in the cases of WEHI-274.25 and WEHI-274.28, act as autostimulatory factors. At present it is not possible to say whether the production of

these factors is critical for the leukemogenic behavior of these clones or merely enhances their growth; experiments attempting to stop the growth of the leukemic cells with antibodies specific for IL-3 or GM-CSF should shed light on this point. Certainly it is striking that in this one tumor, progression of different subclones has occurred through autostimulatory mechanisms that involve two distinct T-cell lymphokines. If the activation of the IL-3 and GM-CSF genes represent completely independent events, and the rearrangements of the respective genes strongly suggest that this is the case, their occurrence in subclones of a single tumor suggests that the aberrant activation of lymphokine genes might be a relatively common mechanism of oncogenic progression in the appropriate target cells.

The common origin of the three classes of subclones, which was demonstrated by their sharing of a common c-*myb* rearrangement, provides an intriguing insight into the history of this tumor. It seems reasonable to postulate that the initiating event was aberrant activation of one c-*myb* gene in a macrophage precursor. Activation of this c-*myb* gene probably resulted in immortalization of this clone.

Although there is no direct evidence, it may well be that this immortalized clone remained dependent on the growth factors to which monocytic progenitors respond, ie, IL-3, GM-CSF, or CSF-1. If this were the case this clone, although immortal, would not have behaved as a transplantable leukemia. Progression to the leukemic state (or enhanced leukemogenicity) appears to have involved at least three discrete mechanisms. Thus leukemogenic subclones represented by WEHI-274.14 resulted from aberrant activation of the IL-3 gene, whereas leukemogenic subclones represented by WEHI-274.28 or WEHI-274.25 resulted from an activation of a second T-cell lymphokine gene, GM-CSF. In the third group of leukemic clones exemplified by WEHI-274.3, the leukemogenic mechanism has yet to be identified. It should be noted that v-abl sequences were not detected in any of these clones. These experiments point to the power of recombinant DNA techniques in establishing the aberrant production of autostimulatory growth fractor by tumors cells and set the scene for experiments investigating the therapeutic potential of antibodies and other measures designed to interrupt autostimulation.

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