Multistage Mastocytoma Model Characterized by Autocrine IL-3 Production

C. Moroni, I. D. Diamantis, A. Wodnar-Filipowicz, and A. P. K. Nair

A. Introduction

Leukaemogenesis, as tumor formation in general, is a multistage process where alterations of proto-oncogenes play some yet unknown role. To understand this process, it is necessary to know how activated proto-oncogenes (i.e., oncogenes) affect and perturb cellular growth control mechanisms from extracellular growth factors down the signal transduction pathway to the nucleus. We and others have previously reported [1, 2] that one of the frequent proto-oncogene alterations in human leukemias and lymphomas are point mutations of the *ras* genes (predominantly N-*ras*), findings which have led us to ask how an activated *ras* gene might affect responsiveness to hemopoietic growth factors.

We have approached this problem by introducing the viral H-*ras* oncogene into an IL-3-dependent, nontumorigenic mouse mastocyte line (PB-3c) [3] and observed the generation of IL-3-secreting, autocrine mastocytomas following a long latency period [4]. In this report, we summarize our data on this multistage tumor system. In addition, we show that IL-3 gene expression can be induced in normal cells in vitro, and autocrine IL-3 production by the mastocytomas can be downregulated by cell fusion.

B. Results and Discussion

Introducing the v-H-*ras* gene via a retroviral *neo*-selectable retroviral vector into IL-3-dependent mouse mastocytes produced short-term and long-term effects. The immediate effect was a reduction of the IL-3 requirement by about 10- to 20-fold in all cell clones tested. The long-range effect was observed in vivo, where infected cells progressed to autocrine, IL-3-secreting mastocytomas. The salient features of this tumor system are summarized in Table 1, and we wish to emphasize the following points. While v-H-*ras*-expressing cells were able to grow at reduced IL-3 levels, this oncogene did not abrogate the requirement for IL-3, in contrast to effects of the *abl* and *myc* genes observed by other workers when analyzing IL-3-dependent cells [5, 6].

Table 1. Features of the v-H-*ras*-induced mastocytoma model

1. *Parental cell:* immortalized, IL-3-dependent and non-tumorigenic mast cell line (PB-3c). Diploid to near diploid [3]
2. *v-H-ras effect:* reduction, but not abrogation, of IL-3 dependence. Observed in all clones tested
3. Two clonable subpopulations within PB-3c: *"transformation-competent"* cells form mastocytomas following v-H-*ras* infection; *"transformation-non-competent"* cells do not
4. Long-latency *mastocytomas* derived in vivo from transformation of competent subclones by the v-H-*ras* gene. Long letancy suggests a progression step taking place in the animal
5. Tumors secrete IL-3, which forms part of an *autocrine loop* as shown by antibody inhibition of growth during cloning in methylcellulose

Institut für Medizinische Mikrobiologie, Petersplatz 10, CH-4003 Basel, Switzerland

In vivo, v-H-*ras*-expressing cells underwent an unknown progression step yielding autocrine, IL-3-secreting mastocytomas. It was of interest to find that only a subpopulation of PB-3 cells (3/20 clones) was transformable by v-H-*ras;* we call them "transformation competent." Non-competent cells produced no tumors following v-H-*ras*-expression, but they still displayed the altered IL-3 requirement described above. While we have no direct evidence, we assume that transformation-competent cells arose from transformation-non-competent ones, as the responsiveness to transforming steps is usually a function of the doubling times undergone by a cell, in other words, transformability increases with time.

The evidence that IL-3 forms part of an autocrine loop and that IL-3 production is a feature relevant to tumor formation is severalfold. All of over 50 mastocytomas produced IL-3, suggesting this trait was selected for. Furthermore, tumor cells were able to grow in culture in the absence of added IL-3; they had acquired growth autonomy. When cloned in methylcellulose in the absence of IL-3, high, but not low, cell numbers seeded were able to produce colonies, a feature characteristic of autocrine tumors. Most importantly, antibody to IL-3 was able to block this colony formation in vitro. Lastly, when IL-3 cDNA was introduced into these cells by a retroviral vector (kindly provided by W. Ostertag), growth autonomy and tumorigenicity occurred together (unpublished data).

An important point to resolve was the temporal relationship between v-H-*ras* and IL-3 expression. Rigorous cloning experiments ruled out that the tumors arose from a preexistent, IL-3-producing cell, as tumors were derived from infected, transformation-competent clones, which were IL-3 dependent prior to infection. Figure 1 shows an analysis of IL-3 expression in transformation-competent cells and tumors derived thereof. IL-3 mRNA is measured by an RNA protection assay. The protected fragment

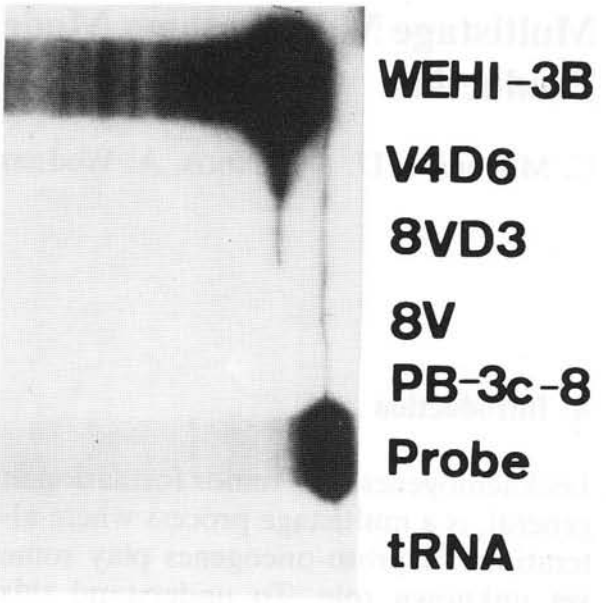

Fig. 1. Analysis of IL-3 mRNA by RNA protection assay. See text for explanation. The probe was a ^{32}P-labeled SP6 transcript of an IL-3 cDNA fragment. (Kindly provided by S. Gough)

(see WEHI-3B, an IL-3-producing line, used for positive control) was not detectable in the transformation-competent clone PB-3c-8, nor in its v-H-*ras*-expressing subclone (8V). IL-3 mRNA was detected in a tumor (8VD3) derived from 8V and in a second tumor analyzed (V4D6). These data show that the expression of IL-3 forms part of a progression step which has taken place in the animal.

Table 2 presents the temporal sequence of our present view by which normal bone marrow cells proceed to the mastocytoma stage. Essential steps are crisis (immortalization), acquisition of transformation competence, v-H-*ras* expression, and the last and important step establishing an autocrine loop involving IL-3. The molecular basis of immortalization, transformation competence and of inducing IL-3 expression are not known at this time.

As IL-3 gene expression becomes activated during mastocytoma formation, we wondered whether IL-3 in PB-3c cells is an inducible gene and tested various potential inducers. A Northern blot analysis (Fig. 2) shows that treatment of these cells with 5 μM calcium ionophore in-

Table 2. Stages of mastocytoma development

Step	Stage	Characteristics
	Bone marrow mast cells	Grow in vitro with IL-3
1. Crisis →	↓	
	Immortalized PB-3c line	IL-3 dependent, not transformable by v-H-*ras*
2. Acquisition of transformation competence →	↓	
	Immortalized transformation competent line	IL-3 dependent transformable by v-H-*ras*
3. v-H-*ras* →	↓	
	Premalignant line	Reduced IL-3 dependency, will progress in vivo
4. Unknown in vivo step →	↓	
	Mastocytoma	Growth autonomy in vitro, secretes IL-3 with autocrine stimulation

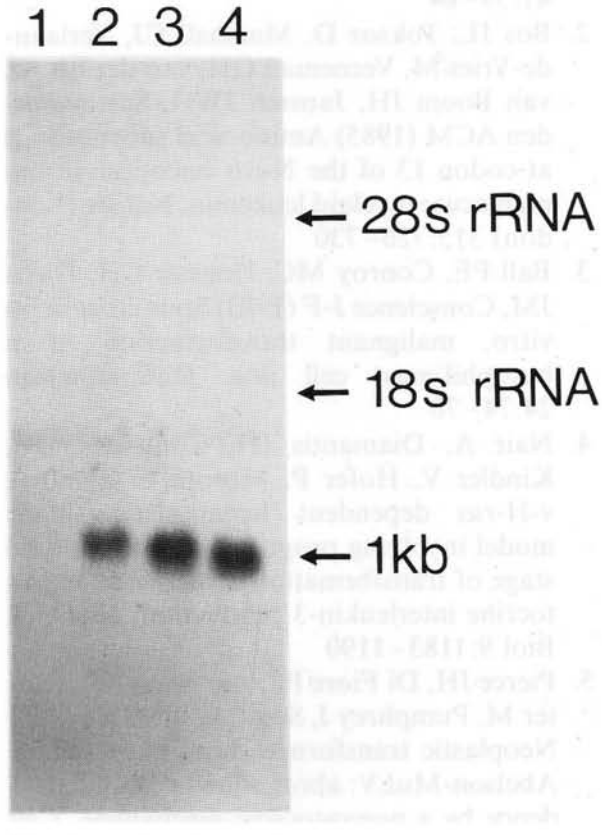

Fig. 2. Induction of IL-3 mRNA by Ca ionophore. *1* control, untreated PB3-c cells; *2* PB3 cells + Ca ionophore 5 μ*M*, 1 h; *3* PB3 cells + Ca ionophore 5 μ*M*, 2 h; *4* PB3 cells + Ca ionophore 5 μ*M*, 4 h. Each lane of the 1.1% agarose-formaldehyde gel contained 20 μg total RNA. The probe was as in Fig. 1

duces IL-3; peak values were seen after 2 h. These data show that the IL-3 locus in PB-3c is subject to regulation, and we are in a position now to analyze the regulating components. It will be interesting to see whether the same induction mechanisms are involved in Ca ionophore induction as in the tumor system.

We turned to cell fusion to study the mechanism by which IL-3 production had become activated in the mastocytomas. The rationale was that analysis of hybrids between IL-3-dependent PB-3c cells and autonomous mastocytomas should indicate whether activation involved a dominant or recessive mechanism. In the case of a dominant mechanism, hybrids should grow autonomously; in the case of a recessive mechanism, hybrids would require IL-3 for growth. A hypoxanthin, aminopterin, thymidin (HAT)-sensitive mastocytoma variant was selected with thioguanine and fused to PB-3c, which are sensitive to G418. (Tumors are resistant to this drug as they carry *neo* from the retroviral vector.) Hybrids were selected in the presence of IL-3 and then tested for growth in the absence of IL-3. Proliferation of sev-

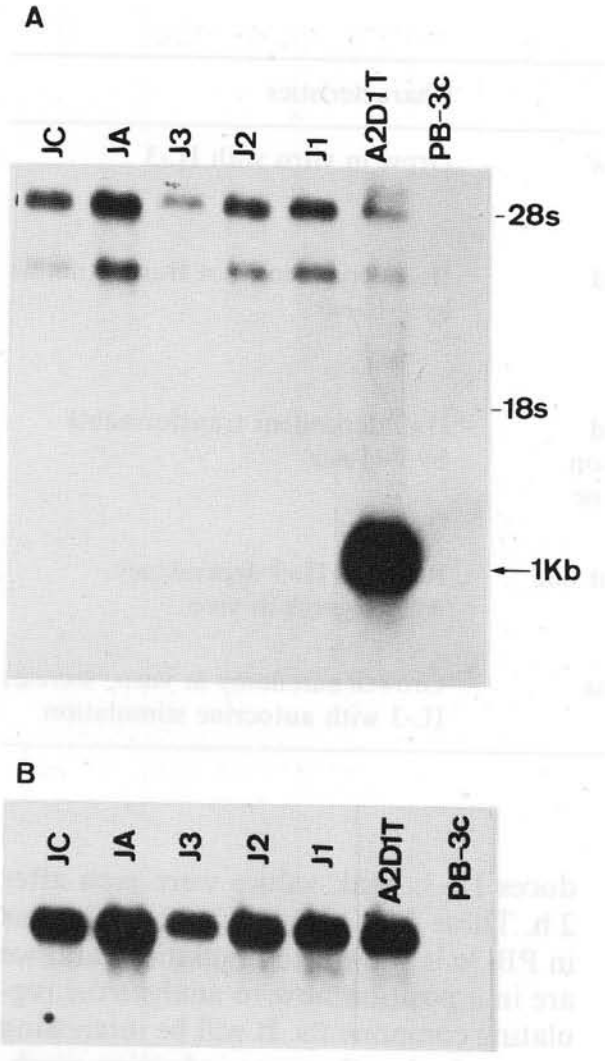

Fig. 3 A, B. Northern blot analysis of somatic cell hybrids. *Lanes* are explained in the text. The IL-3 probe **(A)** was the same as in Figs. 1 and 2. The v-H-*ras* probe **(B)** was a ^{32}P-labeled T7 transcript corresponding to 700 bp v-H-*ras* gene

eral independent hybrid cultures tested was clearly IL-3 dependent, indicating that growth autonomy in the tumors had occurred by a recessive mechanism. We next analyzed the levels of IL-3 mRNA in the hybrids by Northern analysis (Fig. 3 A). The parental tumor A2D1T revealed the expected band of about 1 kb, while no transcript was detectable in PB-3c cells. In five hybrid cultures (JA, JB, J1, J2, J3) IL-3 mRNA was undetectable (Fig. 3 A). When the same samples were analyzed using a v-H-*ras*-specific probe, tumor and hybrids expressed v-H-*ras* (Fig. 3 b). Taken together, these data indicate that cell fusion leads to downmodulation of IL-3 expression by a mechanism not involving downregulation of v-H-*ras* gene expression. This may reflect the activity of a suppressor gene in PB-3c cells, exerting a negative effect on IL-3 gene expression, and lack of this gene function in the tumor. Analysis of this system should not only provide insight into the mechanism by which IL-3 production becomes activated during tumor formation, but also into the mechanism of tumor suppression.

References

1. Senn H-P, Tran-Thang C, Wodnar-Filipowicz A, Jiricny J, Fopp M, Gratwhol A, Signer E, Weber W, Moroni C (1988) Mutation analysis of the N-*ras* proto-oncogene in active and remission phase of human acute leukemias. Int J Cancer 41:59–64
2. Bos JL, Toksoz D, Marshall CJ, Verlaan-de-Vries M, Veeneman GH, van der Eb AJ, van Boom JH, Janssen JWG, Steemvoorden ACM (1985) Amino-acid substitutions at codon 13 of the N-*ras* oncogene in human acute myeloid leukemia. Nature (London) 315:726–730
3. Ball PE, Conroy MC, Heusser CH, Davis JM, Conscience J-F (1983) Spontaneous, in vitro, malignant transformation of a basophil/mast cell line. Differentiation 24:74–78
4. Nair A, Diamantis ID, Conscience JF, Kindler V, Hofer P, Moroni C (1989) A v-H-*ras* dependent hemopoietic tumor model involving progression from a clonal stage of transformation competence to autocrine interleukin-3 production. Mol Cell Biol 9:1183–1190
5. Pierce JH, Di Fiore PP, Aaronson SA, Potter M, Pumphrey J, Scott A, Ihle JN (1985) Neoplastic transformation of mast cell by Abelson-MuLV: abrogation of IL-3 dependence by a nonautocrine mechanism. Cell 41:685–693
6. Rapp UR, Cleveland JL, Brightman K, Scott A, Ihle JN (1985) Abrogation of IL-3 and IL-2 dependence by recombinant murine retroviruses expressing v-*myc* oncogenes. Nature (London) 317:434–438