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v-H-*ras* Gene Reduces IL-3 Requirement in PB-3c Mastocytes In Vitro Followed by Autokrine Tumor Formation In Vivo

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A. Introduction

The search for the physiological functions of proto-oncogenes has led to the notion that some code for products involved in control of mitosis as growth factor or growth factor receptor. This association of proto-oncogenes with mitotic control elements is consistent with the hypothesis that changes of various proto-oncogenes correlate with the multistep process of human carcinogesis, and there are numerous reports on specific proto-oncogene alterations in various human malignancies. What is not known, however, is the nature of the involvement of an altered proto-oncogene with the malignant process, i.e., the precise pathogenic role of the protein.

Of particular interest is the *ras* family of proto-oncogenes, H-, K-, and N-*ras*, which are activated by point mutations [1]. The ras-coded p21 proteins are located in the membrane, have a GTP-ase activity and show sequence homology to the G proteins, a family of proteins involved in the transmission of biological signals [2, 3]. It is thought that p21 proteins, by analogy with G proteins, mediate an external growth signal, by being associated with a specific growth receptor.

In acute myeloblastic leukemia (AML) cells one observes frequent activation of the N-*ras* gene [4–6]. As growth and differentiation of hematopoietic cells are controlled by a set of growth factors, it may be that in AML the activated N-*ras* gene exerts its putative pathogenic effect by being associated with the signal transmission of a growth factor necessary for myeloid cells.

To explore the role of *ras* genes on the growth regulation of hematopoietic cells, we have turned to a mouse model system. We report here that v-H-*ras* reduces the interleukin-III (IL-3) requirement of factor-dependent PB-3c mastocyte cells. Tumors derived from such cells grew in vitro without exogenous IL-3. In fact, we observed autokrine production of IL-3.

B. Material and Methods

I. Cells

PB-3c cells, a cloned line of normal, IL-3-dependent mouse mastocytes [7] were obtained from Dr. J.-F. Conscience. FDCP-1 cells [8], a myelomonocytic line of murine origin, requiring IL-3 or GM-CSF for growth were obtained through Dr. J.F. Delamarter. WEHI-3B cells, a myelomonocytic line producing IL-3 [9] were obtained from Dr. J.-F. Conscience. PB-3c and FDCP-1 cells were cultured in Iscove's modified Dulbecco medium (IMDM), supplemented with 50 μM β-mercapto-ethanol, 10% fetal calf serum, and IL-3 (see below).

II. Mitogenicity Assay

Cells were washed three times in IMDM lacking IL-3 and added to microtiter plates in 100-µl aliquots containing $2 \cdot 10^4$ cells. An IL-3 preparation (20 µl) was added to each

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well. Following 24 h incubation at 37 °C, 0.5 μCi of ^{3}H-thymidine (25 Ci/nmol, Amersham, TRK 300) was added to each well. After 6 h, incorporation was determined by a filtration procedure and scintilation counting. When factor production by tumor cells was studied, the more sensitive FDCP-1 cells were used as targets, incubation in the presence of 20% tumor cell culture supernatant was carried on for 40 h, after which labelling was done for 8 h.

III. Antibody Inhibition of IL-3

An antibody preparation from rabbit serum directed against mouse IL-3 was generously provided by Dr. J. Ihle. Concentration was 30 ng/ml. For control, a rabbit anti-rat Ig preparation was used; 70 μl aliquots containing 20 μl mitogenic culture supernatant (from an A3-derived cell), 10 μl antibody preparation, and 40 μl IMDM were incubated for 2 h in microtiter plates. Then, $2 \cdot 10^4$ PB-3c cells in 50 μl were added. After 24 h incubation, ^{3}H-thymidine incorporation was determined.

IV. Vector and Selection Procedure

Rash-1 cells were obtained from Dr. K. Marcu. They release a retroviral Zip vector [10] containing the v-H-*ras* gene inserted into the *Bam*H1 site and a gene for neomycin resistance. Supernatants from these cells transform NIH3T3 cells with a titer of $5 \cdot 10^3$ and transformed cells grow in the presence of G418 (data not shown).

To introduce the v-H-*ras* gene into PB-3c cells, $2 \cdot 10^6$ cells were incubated for 1 h in 2 ml rash-1 supernatant containing polybrene (16 μg/ml). Cells were spun and resuspended in growth medium. The next day, the selection for neomycin resistance was initiated by adding the drug G418. After about 3 weeks, infected PB-3c cells, but not control cells, contained viable cells growing in the presence of G418 at 1 mg/ml.

C. Results

In order to see whether introducing the v-H-*ras* gene into PB-3c cells would alter their

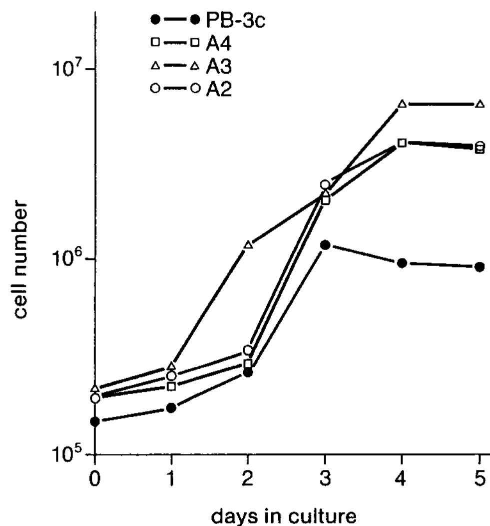

Fig. 1. Growth curve. Cells were cultured in the presence of IL-3 and numbers of cells were determined daily by Coulter counting

growth properties and/or IL-3 requirements, we infected these cells with a retroviral vector containing the v-H-*ras* gene and a gene for neomycin resistance as described in Methods. In this communication, we describe three infected cells, A2, A3, and A4. They express the *neo* gene, shown by growth in G418, and the v-H-*ras* gene, shown by elevated levels of p21 identified by immunoprecipitation with monoclonal antibody Y13-259 (data not shown).

Figure 1 shows the growth of A2, A3, and A4 compared with PB-3c. We observed an elevated saturation density of the infected cells, which showed a plateau at about $5 \cdot 10^6$ cells/ml, compared to PB-3c cells, which grew until 10^6/ml. This suggested that infected cells utilize IL-3 more efficiently and still grow when the factor becomes limiting. We therefore performed a titration of IL-3 on these cells. As can be seen in Fig. 2, the titration curve of infected cells showed a shift to the right, indicating a 20-fold increased sensitivity to IL-3. Furthermore, infected cells, but not PB-3c cells, were able to grow at $^1/_{20}$ of the saturating concentration of IL-3 (data not shown).

We next wished to determine whether these cells would give rise to tumor formation following inoculation into syngeneic mice. As expected, PB-3c cells were nontumorigenic. Infected cells, in contrast, produced slowly growing tumors at the site of inoculation (Table 1). Most active were A3

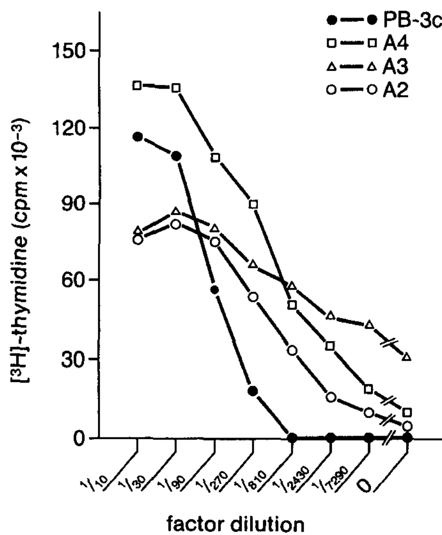

Fig. 2. Titration of IL-3. To 100-µl cultures containing $2 \cdot 10^4$ cells, 20 µl concentrated IL-3 preparation, diluted as indicated, was added. After 24 h, ^{3}H-thymidine incorporation during a 6-h pulse was determined. Values represent means of triplicate determinations

Table 1. Cumulative tumor incidence. Values show tumor incidence 5 months after subcutaneous inoculation into DBA/2 mice

Cell	No. of cells	Tumor/mice
PB-3c	10^6	0/5
	10^5	0/5
	10^4	0/5
A2	10^6	5/5
	10^5	4/5
	10^4	0/5
A3	10^6	5/5
	10^5	5/5
	10^4	5/5
A4	10^6	1/5
	10^5	0/5
	10^4	0/5

cells, where even 10^4 cells produced tumors in all mice inoculated.

To characterize these tumor cells further, we tried to establish them in culture. Generally, tumors, following surgical removal, yielded high numbers of cells which almost immediately took off in culture. To our surprise, their growth was now independent of IL-3 in all cases. Growth was observed also in the presence of G418, indicating that it was indeed the original cells which had become tumorigenic in vivo. We suspected that IL-3 independence might be the result of autoproduction of this factor by the tumor cells. We therefore tested supernatants from A2-, A3-, and A4-derived tumors for mitogenic activity. All cultures were positive (Table 2), however, to various degrees. The assay was performed on FDCP-1 cells, which are more sensitive to IL-3 than PB-3 cells, but the activity could also be demonstrated with PB-3 as target (data not shown). To see whether this activity was indeed IL-3, activity from a culture was preincubated with antibody to IL-3. Anti-IL-3, but not control antibody, was able to inhibit the mitogenic activity (Fig. 3).

Table 2. Mitogenic activity of culture supernatant; 20-µl culture supernatants were assayed on FDCP-1 cells cultured in microtiter plates as described in the legend to Fig. 2

Culture	c.p.m. $\times 10^3$
–	0.2
A2D1	3.4
A2D5	2.3
A2D6	6.4
A2D7	12.7
A3D6	38.1
A3D7	98.0
A3D8	8.2
A4D1	6.4

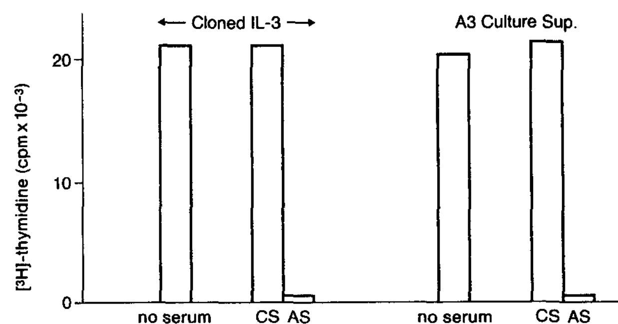

Fig. 3. Antibody inhibition. A mitogenic culture supernatant was incubated for 2 h with antibody and added to the cells. Cloned IL-3 (*left*) refers to recombinant *IL-3*, obtained from Dr. J. F. Delamarter, Biogen, and serves as positive control. A3 Culture Sup (*right*) denotes one of the A3-derived factor-independent lines. Mitogenic activity was determined as described in the legend to Fig. 1. *CS*, control antibody preparation; *AS*, anti-IL-3 preparation

D. Discussion

Introducing v-H-*ras* into IL-3 dependent PB-3c cells has allowed us to observe two phenomena. The first one is an altered growth behavior of v-H-*ras* containing cells, shown by a higher saturation density (Fig. 1). This is explainable by the increased response of these cells to limiting IL-3 concentration as shown in an IL-3 titration experiment (Fig. 2). As this effect on IL-3 utilization, observed immediately after selecting the cells with G418, correlates with increased p21 levels in these cells (data not shown) and is never found in cells selected with *neo* in the absence of v-H-*ras* (data not shown), we conclude that this effect is the result of the viral p21 protein. It may be that p21 acts as a G-like protein in association with a receptor for IL-3. But it may also be that v-H-p21 leads to an increase of number or affinity of the IL-3 receptor, or to the production of low levels of endogenous IL-3 in these cells which then could complement the exogenously added growth factor.

When infected cells were inoculated into syngeneic mice (Table 1), we observed tumor formation at the site of inoculation. It appears that the inoculated cells are not oncogenic per se but become so after going through at least one additional change. This conclusion is based on the observation that all tumors had become factor independent in vivo and remainded so following over 6 months of in vitro culturing. As we never observed tumor formation following inoculation of unselected PB-3c cells, we conclude that v-H-*ras* facilitates the transition from low IL-3 requirement to factor independence and tumorigenicity. The frequency of this transition is low, and differs amongst infected lines (compare tumor incidences in Table 1). Thus, v-H-*ras* plays a dual role. The early effect is direct, appears to operate in most if not all cells selected, affects IL-3 utilization, and is in its nature a premalignant change. Secondly, it conditions the cells, at some frequency, to proceed to a tumorigenic IL-3 independent phenotype.

The nature of IL-3 independence was found to be an autokrine mechanism involving a mitogenic factor (Table 2), antigenically related and possibly identical with IL-3 (Fig. 3). We wish to point out that this is the first experimental system where IL-3 autoproduction is observed. IL-3 production is a known property of T cells [11], and only non-T-cell line producing this factor is WEHI-3 where a retroviral LTR element was found to be integrated at the 5′-end of the *IL-3* gene [12].

The system described in this paper, consisting of the IL-3 dependent PB-3c mastocytes and their v-H-*ras*-induced progression from factor dependence, relaxed dependence to autokrine tumor growth represents a new and promising model to explore the multistep nature of carcinogenesis and the particular role of the *ras* oncogene.

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