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HIGHLY FREQUENT DETECTION OF TRANSFORMING GENES IN ACUTE LEUKEMIAS BY TRANSFECTION USING *IN VIVO* SELECTION ASSAYS

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Summary: DNAs from nine out of ten acute leukemia cases that were negative by in vitro focus forming assays exhibited transforming activity tested by in vivo selection assays in nude mice using transfected NIH3T3 cells. Of the nine cases, six cases contained activated N-ras genes, and one case exhibited activation of the c-K-ras gene. None of the ras gene family showed homology with the transforming genes derived from the other two cases. Our observations indicate that in vivo selection assays detect transforming genes including ras oncogenes at high frequency, and that activated N-ras genes are frequently detected in human acute leukemias.

One important approach to detect human transforming genes has involved DNA transfection. Human cellular transforming genes can be detected in tumors by their ability to induce the transformation of NIH3T3 cells (for review 1-3), a continuous cell line that is contact-inhibited and highly susceptible to DNA transfection (4). The use of in vitro focus forming assays to detect morphological transformation of NIH3T3 cells has shown that between 10 and 20% of human tumors contain activated form of either the c-H-ras-1 gene, the c-K-ras-2 gene or the N-ras gene (5-7). Activated N-ras genes have been detected in human leukemias with similar frequency (7-9). Attempt to study oncogenes in the residual tumors that have been negative by the transfection assay has great importance in the study of human carcinogenesis.

Activation of ras genes in human tumors so far analysed has been shown to result in a point mutation in either codon 12 or 61 of the gene (5-7). Recently, the use of a direct in vivo selection assay has detected a unique form of a mutation at codon 13 of the N-ras gene that has not

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been reported before (10,11). Using the in vivo selection assays, the present study was undertaken to detect transforming genes of a spectrum of human leukemias that have been negative by the in vitro focus forming assays (7).

MATERIALS AND METHODS

Sources of DNA: Fresh primary human leukemia cells were prepared from heparinized marrow from 10 acute leukemia patients: two with acute myelogenous leukemia (AML), three with acute monocytic leukemia (AMoL), one with acute myelomonocytic leukemia (AMMoL), and four with acute lymphocytic leukemia (ALL). High-molecular weight DNAs of marrow cells from these patients were shown to be negative by in vitro focus forming transfection assays as described previously (7). The normal marrow cells from eight healthy volunteers were obtained with informed consent and were examined as normal controls.

Transfection assays: High-molecular weight DNAs were extracted from buffy coat cells of heparinized marrow from the patients and the normal subjects. The transforming activity of the DNAs was assayed by transfection of NIH3T3 cells using an in vivo selection method as described (11). Briefly, 20 µg cellular DNA and 300 ng pSV2Neo were precipitated with calcium phosphate into each of five 60-mm plates seeded 1 day previously with 2×10^5 NIH3T3 cells. After 20-24 hr the co-precipitate was washed off and the cells were incubated in growth medium (Dulbecco's modified Eagle's medium supplemented with 10% calf serum) for 12-24 hr. Each dish was trypsinized and cells were divided into three dishes containing selective medium with 400 µg/ml G418. After 7-10 days of growth in selective medium, 1.5×10^6 cells were then injected into each of two inguinal subcutaneous sites of nude mice (BALB/c-nu/nu mice, 5 weeks old, female, background congenitally athymic). The DNAs from tumors arising in nude mice were used in a subsequent cycle of transfection assay (secondary transfection assay).

Analysis of DNAs from tumors in nude mice: DNAs isolated from tumors arising in nude mice were subjected to Southern blot analysis (12) for detection of human repetitive sequences and human proto-oncogene sequences. Ten micrograms of high-molecular weight DNAs were digested with restriction endonucleases, electrophoresed through 0.8% agarose gels, and blotted to nylon filters. The resulting blots were hybridized with a nick-translated ^{32}P-labeled DNA probe (7).

RESULTS

NIH3T3 cells transfected with each DNA sample were inoculated into four sites of two nude mice. The resulting tumors arose 30-60 days after inoculation of NIH3T3 cells transfected with nine out of ten preparations from leukemia patients. Of eight normal human DNAs tested in the same way, all failed to give any tumors within 80 days. The number of tumors and tumors containing Alu-sequences are shown in Table 1. All primary transformants but one derived from AML-2 DNA were shown to contain a large number of human repetitive sequences hybridized to the probe Blur-8 (Fig.

Table 1. Results of transfection assays

Cases	Primary transfection			Secondary transfection			Trans-forming gene
	No. of injected sites	No. of tumors	No. of tumors containing *Alu*-sequences	No. of injected sites	No. of tumors	No. of tumors containing *Alu*-sequences	
Myeloid leukemia							
AML-1	4	3	3	NT	-	-	N-*ras*
AML-2	4	2	1	2	2	2	N-*ras*
AMoL-1	4	4	4	NT	-	-	N-*ras*
AMoL-2	4	3	3	NT	-	-	N-*ras*
AMoL-3	4	1	1	4	2	2	non-*ras*
AMMoL-1	4	2	2	NT	-	-	K-*ras*
Lymphoid leukemia							
T-ALL-1	4	1	1	4	2	2	non-*ras*
T-ALL-2	4	1	1	2	2	2	N-*ras*
B-ALL-1	4	0	0	-	-	-	-
Null-ALL-1	4	3	3	NT	-	-	N-*ras*

NT, not tested.
non-*ras*, none of the *ras* gene family.

1A). Similar results were obtained when probed with total human genomic DNA (data not shown). In view of evidence relating transforming genes of a number of human tumors to ras genes, we analysed the transformant DNAs for these oncogene sequences. Human N-ras genes were detected in primary transformant DNAs derived from six leukemia cases (AML-1, AML-2, AMoL-1, AMoL-2, T-ALL-2 and Null-ALL-1 in Fig. 1B). In each of two cases (AML-2 and T-ALL-2) which gave only one primary transformant containing Alu-sequences, secondary transfection experiments confirmed the presence of a human activated N-ras gene (Table 1). The v-K-ras probe detected the presence of the human c-K-ras-2 gene in transformant DNAs of AMMoL-1 in which the 6.7 and 3.0 kb bands were detected (Fig. 1C). None of ras probes detected any fragments other than their endogenous mouse related fragments in transformant DNAs of AMoL-3 and T-ALL-1 (data not shown). In both cases, the results of secondary transfection assays are shown in Table 1. Secondary transformants that were independently obtained retained common EcoRI-digested fragments in each of AMoL-3 and T-ALL-1 cases (Fig. 2). Similarly, the retention of common fragments was observed when other restriction endonucleases were used for the analysis (data not shown). The present results indicate that DNAs of these two cases contained transmissible and dominant activated transforming genes distinct from ras genes.

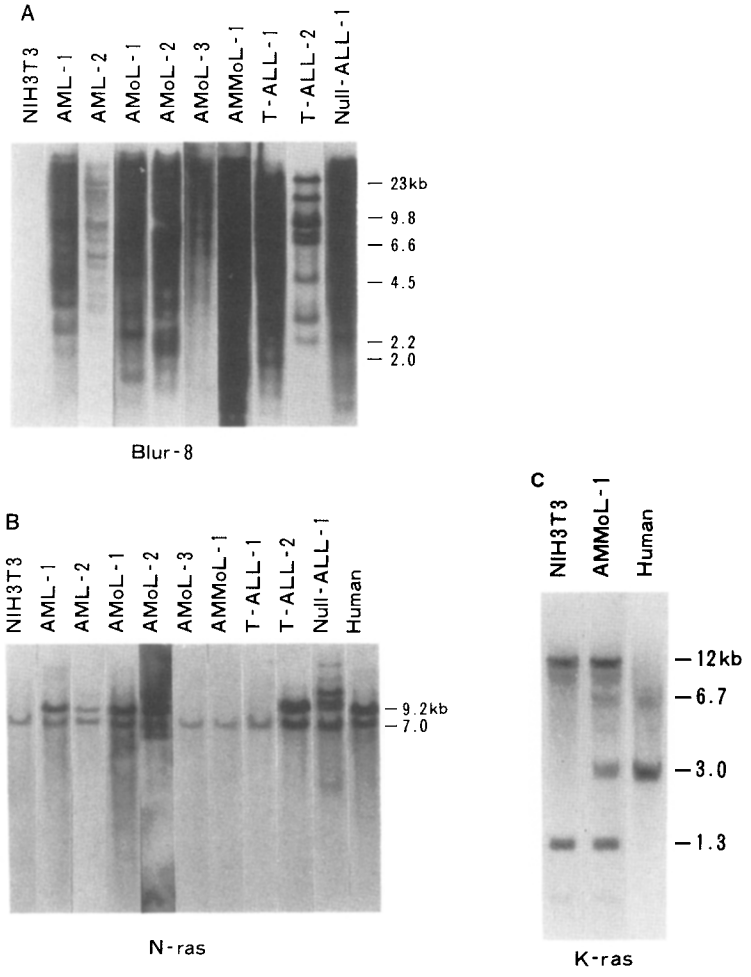

Fig. 1. Southern blot analysis of primary transformant DNAs. High-molecular weight DNA (10 μg) was digested with EcoRI, electrophoresed, blotted, and hybridized (A) to the Blur-8 probe, (B) to the N-ras probe, or (C) to the K-ras probe. DNAs from NIH3T3 cells and human placenta were used as controls.

DISCUSSION

A number of human tumors and tumor cell lines have been shown to contain oncogenes capable of transforming NIH3T3 cells (1-10). The combined results of several studies have indicated that human leukemias exhibit N-ras oncogenes detected by in vitro focus forming assays in around 10 to 30% of fresh leukemias or leukemia cell lines (7-9). In the present study using in vivo selection assays, 90% of fresh human acute leukemias (9 of 10 cases that were negative by in vitro focus forming assays) were found to exhibit transforming activity, and 60% of them (6 of the 10 cases) were shown to contain activated N-ras genes. Our observations

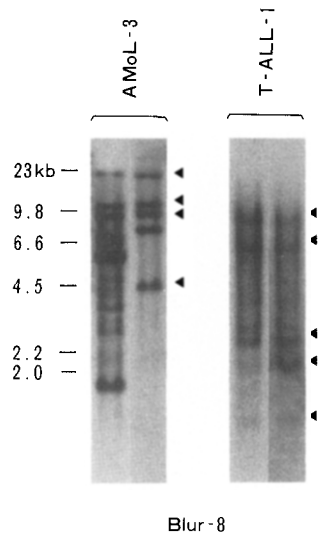

Fig. 2. Southern blot analysis of human repetitive sequences in secondary transformants derived from AMoL-3 and T-ALL-1. Two tumors independently obtained in secondary transfection assays were used for the analysis in each of the cases. High-molecular weight DNA (10 μg) was digested with EcoRI, electrophoresed, blotted, and hybridized to the Blur-8 probe. Migration of EcoRI-cleaved human DNA fragments shared by secondary transformants are indicated by arrows.

suggest that in vivo selection assays detect transforming genes including N-ras oncogenes at high frequency and that activated N-ras genes are frequently detected in human acute leukemias in either the myeloid or the lymphoid lineage.

There should be a bias towards activation of N-ras genes in human leukemias. The N-ras gene might be critically involved in the control of cell proliferation and differentiation in the hematopoietic system. Therefore, activation of the N-ras gene may lead to the neoplastic transformation of hematopoietic cells. The reproducible detection of specific transforming genes in experimental animal tumors strongly suggests that oncogene activation must be closely related to the carcinogenic agent responsible for the tumor (13-15). If these demonstrations apply to human leukemias, the N-ras gene may play a significant role in human leukemogenesis.

Recent reports using the in vivo selection assay presented evidence that a point mutation at codon 13, rather than at one of the common sites for activation at codon 12 or 61, is responsible for the acquisition of transforming properties by the N-ras genes in the AML cases (10) and in the patients with myelodysplastic syndrome (11). Another study demonstrated that the degree of transformation induced by each of human H-ras mutant at codon 12 was highly variable in morphology of transformed NIH3T3 cells (16). Furthermore, novel transforming genes, distinct from the ras genes reported, have been detected by analysing the transfected NIH3T3 cells that do not display a striking change in morphology (17), or by studying the tumors arisen in in vivo selection assays (18). These findings indicate that the in vivo selection assay can be developed to search for transforming genes from tumor cells that may not be readily detected by the standard focus forming assay. The secondary transformants of AMoL-3 and T-ALL-1 retain conserved Alu-related fragments. These observations suggest that transformation of NIH3T3 cells by AMoL-3 and T-ALL-1 DNAs is associated with transmissible and activated transforming sequences in the DNAs. However, whether these transforming genes are novel is a question that must await molecular cloning and sequence analysis.

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