Concurrent mutations in two different ras genes in acute myelocytic leukemias

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ABSTRACT

DNA transfection analyses (tumorigenicity assay) and hybridization to mutation specific oligonucleotide probes established point mutations in codon 61 of both, N-ras and Ki-ras genes in fresh leukemic cells of an AML patient. Concurrent activation of N-ras and Ki-ras sequences by point mutations in codons 12 were demonstrated for AML cell line Rc2a. Moreover, using a rapid and sensitive dot-blot screening procedure based on the combination of in vitro amplification of ras specific sequences and oligonucleotide hybridization we could show that ras gene activation was not present in primary leukemic cells of the patient this cell line had been derived from, but rather occurred during later passages of Rc2a.

INTRODUCTION

The activation of ras genes by point mutation is one of the best studied mechanisms that convert proto-oncogenes to transforming sequences (1). The human ras gene family comprises the active members, Ha-ras, Ki-ras, and N-ras. Each gene encodes a 21 KD protein closely related to the others (2). While the precise physiological function(s) of p21 ras remain(s) to be elucidated, some of their biochemical and biological characteristics suggest that these proteins, by analogy with G-like proteins, are involved in transduction of receptor-mediated external signals into cells. Thus p21 ras proteins bind guanine nucleotides, exhibit intrinsic GTPase activity and are localized at the inner surface of the plasma membrane (3-5).

Cloning and sequence analyses of oncogenic versions of ras genes have demonstrated point mutations in codons 12, 13 or 61 of Ha-, Ki-, or N-ras resulting in substitution of amino acids in respective p21 proteins (1, 6). These molecular alterations are associated with a decrease in GTPase activity (7, 8). Yet the biological meaning of the latter modification is not settled thus far (9, 10).

Data suggesting that a cooperation of different oncogenes is essential for the manifestation of a transformed cellular phenotype fit well in the gene-

rally accepted view of carcinogenesis as a multistep process (2). Indeed such a cooperation has been established e.g. for ras and myc sequences both in vitro and in vivo (2, 11-13). However, not only mutated versions of p21, but also overexpression of normal ras sequences, result in cellular transformation (14-16). A combination of both modes of activation, i.e. point mutation and overexpression via gene amplification, has recently been demonstrated for the two Ki-ras alleles in a human gastric carcinoma (17). Herein we report for the first time a concurrent activation of two different ras genes (Ki- and N-ras) by point mutations in an AML cell line (Rc2a) as well as in fresh leukemic cells of an additional AML patient.

MATERIALS AND METHODS

Cell Samples

Different passages of cell line Rc2a as well as primary leukemic cells of the AML patient this line has been derived from (18) were generously provided by Dr. T.R. Bradley. These samples comprised 1) peripheral blood cells from the first presentation of the patient R.C. (frozen October 76), 2) peripheral blood cells taken just prior to patient's death, grown for 4 weeks with colony stimulating factor (CSF) as described (18) and then frozen (April 78), 3) the first cells grown without CSF, i.e. first passages of cell line Rc2a (frozen June 79), 4) a later passage of Rc2a grown without CSF (frozen February 80), and 5) a late passage of cell line Rc2a, originally obtained from Dr. Bradley and used in our laboratory since 1983.

Patient M.P. was a 12 yrs old male AML patient. Bone marrow showed 92% blasts of M5 morphology according to the FAB classification. The patient died two days after initiating chemotherapy from intracranial bleeding.

Peripheral blood cells of patient R.C. and bone marrow cells of patient M.P. (prior to chemotherapy) were purified over Ficoll gradients and used for further analyses.

Transfection Assay. High-molecular weight DNA was prepared according to standard methods. DNA transfer used a modified calcium phosphate precipitation method with NIH 3T3 cells as recipients as described in detail elsewhere (19). The tumorigenicity assay is based on the cotransfection of 3T3 fibroblasts with tumor DNA and a dominant drug-resistant selectable marker, pRSVneo, followed by G418 (600 µg/ml, GIBCO Laboratories) selection and injection of resulting 3T3 colonies into nude mice. NIH 3T3 fibroblasts were originally obtained from M. Wigler and subcloned by us as described previously (19). Transfection of 20 µg leukemic DNA with 500 ng of pRSVneo (20) onto

TABLE 1 SYNTHETIC OLIGOMERS

OLIGOMER	SEQUENCES	STRAND	AMINO ACID
N-12wt	GGAGCAGGTGGTGTGGGAA	s *	gly
N-12/7	-----GTT-----	s	val
N-61wt	TACTCTTCTTGTCAGCTGT	a	gln
N-61/5	-----TCG-----	a	arg
K1-12wt	CCTACGCCACCACTCCAAC	a	gly
K1-12/6	-----ATC-----	a	asp
K1-61wt	TACTCTTCTTGACCTGCTGT	a	gln
K1-61/p3	-----NTG-----	a	his
	N=A,G		
Ha-12wt	GTGGGCGCCGGCGGTGTGGG	s	gly
Ha-61wt	TACTCCTCCTGGCCGGCGGT	a	gln

*Sequences complementary to the coding (s, sense) or non-coding (a, anti-sense) strand. wt (wild type) sequence according to (34-38); p (pooled) oligomers, in which nucleotide N in the codon is substituted by different nucleotides as indicated.

a 9 cm dish seeded 1 day before with 2.5×10^5 NIH 3T3 cells gave approximately 5×10^2 G418 resistant colonies. Splitting and selection of transfected cells were performed as described by Fasano et al. (21). For each experiment cells obtained from 4000 colonies (4×10^6 cells) were injected subcutaneously into both flanks (2000 colonies per flank) of athymic, 4- to 8-weeks-old nude mice (NMR-I-nu/nu).

Southern Blot Analyses. Cellular DNAs were digested with restriction endonucleases (Boehringer Mannheim), electrophoresed on a 0.7% agarose gel, blotted and hybridized as described (19). Moreover, 2 μ g/ml pRSVneo DNA was added as competitor DNA to avoid cross hybridization with cotransfected pRSVneo sequences.

Inserts of the following ras probes were used: pEJ (hu-Ha-ras; 22), p640 (hu-Ki-ras; 23) and 0.9 kb PvuII fragment B (hu-N-ras; 24).

Synthetic Oligonucleotide Probes. The oligonucleotides (20-mers) were prepared by one of us (J.L.) via an asynchronous simultaneous synthesis strategy (25) using the solid-phase phosphite triester method. For Table I we selected those 20-mers that proved to be informative for the present study. A complete list of oligomers used for our analyses of ras gene mutations is available on request. The oligomers were end-labelled using $-^{32}\text{P-ATP}$ (New

England Nuclear, Boston, MA) and T_4 -polynucleotide kinase (Pharmacia). The kinased probes were separated from the unincorporated ATP by chromatography on Sephadex G 50 in TEN (100 mM NaCl, 10mM Tris-HCl, pH 7.5, 1 mM EDTA). Specific activities were greater than 2×10^6 cpm/pmol.

Oligomer Hybridization. DNA was digested with Pst I or EcoRI (Boehringer Mannheim, FRG) to detect mutations in the N-ras and Ki-ras genes, respectively, and fractionated by electrophoresis in 0.6% agarose. The gel was treated as described (26) to denature the DNA in situ and to immobilize the DNA by drying the gel. The dried gels were incubated with the different oligomer probes (N 12, 13, and 61, Ki 12, 13, and 61) in 5 x SSPE (1 x SSPE = 10 mM sodium phosphate, pH 7.0/0.18 M NaCl/1mM EDTA), 7% SDS (sodiumdodecyl sulphate), 5 x Denhardt's solution and 100 μ g/ml denatured salmon sperm DNA for 16 h at 50°C. The gels were washed in 2 x SSPE, 0.1% SDS at room temperature, incubated in 5 x SSPE, 0.1% SDS at 50°C for 30 min and subsequently for 10 min at the following temperatures: gels hybridized with N 12 and 13 or Ki 12 and 13 probes at 64°C and with N 61 or Ki 61 probes at 59°C.

Polymerase Chain Reaction. DNA amplification in vitro was performed as described by Saiki et al. (27) and Mullis and Faloona (28). The reaction mixture contained 150 ng chromosomal DNA, 100 ng of each of the amplimers (oligomers used for the chain elongation, see Table II), 1 mM of each of the dNTPs, 10 mM Tris-HCl pH 7.5, 5 mM NaCl, 10 mM $MgCl_2$ in a total volume of 30 μ l. The mixture was incubated for 5 min at 95°C to denature the DNA and the amplimers were allowed to anneal to the DNA at 37°C for 90 sec. The polymerase chain elongation reaction was started by the addition of 0.5 μ l (1 unit) of cloned Klenow polymerase (Pharmacia) and the mixture was incubated at 37°C for 90 sec. A new cycle of amplification was started by denaturing the DNA for 90 sec at 95°C. Routinely we perform 15 rounds of amplification with an outer set of amplimers followed by 15 rounds of amplification using an inner set of amplimers (28).

Dot Blot Hybridization. 1 μ l of the DNA amplified in vitro was spotted onto Nylon filters (Gene Screen Plus, New England Nuclear, Boston, MA). The filters were subsequently illuminated with a 254-nm UV lamp (1.6 kJ/m^2) to bind the DNA to the filter. The filters were prehybridized over night at 50°C in 5 x SSPE, 7% SDS, 100 μ g/ml sonicated, denatured salmon sperm DNA, and 5 x Denhardt's solution and subsequently hybridized for 3 h at 50°C in the same mixture containing approximately 1 ng ^{32}P -labelled oligomer probe. The filters were washed twice in 2 x SSPE, 0.1% SDS for 5 min at room temperature, followed by a 30 min wash at 50°C in 5 x SSPE, 0.1% SDS. Subse-

TABLE 2 LIST OF AMPLIMERS

AMPLIMERS	SEQUENCES	STRAND	TARGET
N-12/13 o *	CTTGCTGGTGTGAAATGACT	s ⁺	N-ras 12/13
N-12/13 o	ACAAAGTGGTCTGGATTAG	a ⁺	N-ras 12/13
N-12/13 i *	GACTGAGTACAAACTGGTGG	s	N-ras 12/13
N-12/13 i	AGCTGGATTGTCAGTGCCT	a	N-ras 12/13
N-61 o	GTTATAGATGGTGAACCTG	s	N-ras 61
N-61 o	AAGCCTTCGCCTGTCCTCAT	a	N-ras 61
N-61 i	GGTGAACCTGTTTGTGGA	s	N-ras 61
N-61 i	AAGCCTTCGCCTGTCCTCAT	a	N-ras 61
Ha-12/13 o	GAGACCCTGTAGGAGGACCC	s	Ha-ras 12/13
Ha-12/13 o	CGTCCACAAAATGGTTCTGG	a	Ha-ras 12/13
Ha-12/13 i	TGGATGGTCAGCGCACTCTT	a	Ha-ras 12/13
Ha-12/13 i	GACGGAATATAAGCTGGTGG	s	Ha-ras 12/13
Ha-61 o	CCGGAAGCAGGTGGTCATTG	s	Ha-ras 61
Ha-61 o	ACACACACAGGAAGCCCTCC	a	Ha-ras 61
Ha-61 i	AGACGTGCCTGTTGGACATC	s	Ha-ras 61
Ha-61 i	CGCATGTACTGGTCCCGCAT	a	Ha-ras 61
K1-12/13 o	TTTTTATTATAAGGCCTGCT	s	K1-ras 12/13
K1-12/13 o	GTCCACAAAATGATTCTGAA	a	K1-ras 12/13
K1-12/13 i	TGTATCGTCAAGGCACTCTT	a	K1-ras 12/13
K1-12/13 i	GACTGAATATAAACTTGTGG	s	K1-ras 12/13
K1-61 o	ACCTGTCTCTTGGATATTCT	s	K1-ras 61
K1-61 o	TGATTTAGTATTATTTATGG	a	K1-ras 61
K1-61 i	TTCCTACAGGAAGCAAGTAG	s	K1-ras 61
K1-61 i	CACAAAGAAAGCCCTCCCA	a	K1-ras 61

* According to (28) a set of amplimers, in which one amplimer (i, inner) is nested within the other (o, outer). ⁺Sequence complementary to the coding (s, sense) or non-coding (a, anti-sense) strand.

quently, the filters were stringently washed in 5 x SSPE, 0.1% SDS for 10 min. Finally, the filters were exposed up to 12 hrs to Kodak XAR films at -70°C using intensifying screens.

RESULTS

DNA transfection analyses

In a series of transfection analyses using the tumorigenicity assay we observed in AML cell line Rc2a as well as in leukemic bone marrow cells of patient M.P. a tumor induction in nude mice. In each experiment transfected cells were injected into both flanks of a single mouse, thus up to two tumors could develop per analysis. Five independent experiments were performed

Fig. 1 Detection of human N-ras and Ki-ras sequences in primary tumors derived from AML M.P. EcoRI digests obtained from DNA of human placenta (a), NIH/3T3 cells (b), MP-T₁ (c) and MP-T₂ (d) were blotted and hybridized to N-ras (panel A) and Ki-ras sequences (panel B). The N-ras probe cross-hybridizes with an endogenous 5.9 kb mouse N-ras fragment.

with Rc2a DNA resulting in eight different tumors (Rc2a-T₁ to Rc2a-T₈). One experiment performed with DNA obtained from patient M.P. induced two tumors (MP-T₁ and MP-T₂). Primary transfectants were tested for the presence of human ras genes (Ha-, Ki-, and N-ras).

Activated ras genes in patient M.P.

Southern blot analysis demonstrated the presence of human N-ras sequences in both M.P. transfectants (Fig. 1A). However, rehybridization of respective filters to a human Ki-ras probe showed likewise a strong positive autoradiographic band for MP-T₂ DNA as well as a faint hybridization signal for MP-T₁ (Fig. 1B). This variable autoradiographic intensity in both transfectants could have different explanations. One possibility might be that during the transfection process Ki-ras sequences became amplified in MP-T₂ but not in MP-T₁. Alternatively one has to take into account that tumors induced in nude mice are not necessarily monoclonal and may be composed of cells that

Fig. 2 Hybridization of mutation-specific oligomers to in vitro amplified DNAs obtained from bone marrow (a) and peripheral blood (b) of patient M.P. as well as transfectants MP-T₁ (c) and MP-T₂ (d) or human placenta (e). 5 ng of DNAs were spotted to Gene Screen Plus and hybridized to oligomers representing wild type (wt) and mutation-specific codon 61 N-ras (panel A) or respective codon 61 Ki-ras sequences (panel B).

have taken up either activated N-ras or Ki-ras sequences or both. No hybridization to Ha-ras sequences was observed (not shown). These data suggested the presence of oncogenic versions of both ras genes in leukemic cells of patient M.P. In order to verify these results and, moreover, to identify possible point mutations in respective DNAs we took advantage of a recently developed dot-blot screening procedure for the detection of mutated ras genes (27-29). This technique is based on a combination of in vitro amplification of ras-specific sequences and hybridization to mutation-specific oligonucleotide probes. As shown in Fig. 2A both transfectants derived from leukemic cells of this patient showed no hybridization to oligomers representing normal N-ras sequences around codon 61. However, a 20-mer with a mutated version of codon 61 sequences gave a strong hybridization signal with both transfectants. These findings indicate a point mutation in N-ras 61 corresponding to a substitution of arginine for glutamic acid in the respective p21 ras. A similar result was obtained for Ki-ras sequences (Fig. 2B). Again both transfectants showed no hybridization to a Ki-ras codon 61 wild type oligomers, whereas a positive signal was observed with 20-mers altered at the third position of codon 61 indicating a change from glutamic acid to histidine in p21 ras. MP-T₁ and MP-T₂ DNAs hybridized to normal N- and Ki-ras sequences around codons 12 and 13 (not shown). Taken together these results demonstrate a concurrent activation of both genes, N-ras and Ki-ras in AML cells of patient M.P. due to point mutations in codon 61.

Fig. 3 Hybridization of N-ras and Ki-ras specific oligomers to genomic DNA of transfectants derived from AML cell line Rc2a. DNAs obtained from human placenta (a), NIH/3T3 (b) as well as Rc2a transfectants T₁-T₈ (c-j) were digested, electrophoresed and hybridized in situ to oligomer probes representing mutated codon 12 sequences of N-ras (panel A) or Ki-ras (panel B).

Mutations in N-ras and Ki-ras in Rc2a

Surprisingly, Southern blot analyses exhibited a heterogeneous hybridization pattern for human N-ras sequences in transfectants obtained from Rc2a. Only four out of eight transfectants gave a strong hybridization signal, while Rc2a-T₂ and T₆ showed faintly visible autoradiographic bands and Rc2a-T₃ as well as T₈ DNAs lacked human N-ras sequences. Rehybridization of respective filters to a human Ki-ras probe revealed positive signals for all Rc2a transfectants except Rc2a-T₇. No hybridization to Ha-ras sequences was observed (data not shown). Since these results suggested an activation of two different ras genes in cell line Rc2a as has been previously shown for AML patient M.P. we decided to hybridize dried gels in situ to oligomer probes representing wild type or mutated sequences of N-ras and Ki-ras codons 12, 13 or 61. Indeed these studies detected mutated versions of N-ras codon 12 (corresponding to a change from amino acid glycine to valine) in Rc2a transfectants T₁, T₄, T₅ and T₇ (strong autoradiographic signal), T₂ and T₆ (weak signal), but not in Rc2a-T₃ and T₈ DNAs (Fig. 3A). Moreover, rehybridization of the filters to 20-mers representing Ki-ras sequences also detected point mutations in codon 12 (associated with a change from glycine to aspartic acid) in all transfectants except Rc2a-T₇ (Fig. 3B). These data

Fig. 4 Hybridization of mutation-specific oligomers to in vitro amplified DNAs obtained from Rc2a cells used for transfection experiments (a), peripheral AML cells of patient R.C. at diagnosis (b) and prior to death (c), early (1979) Rc2a passage (d) as well as a later (1980) passage (e). 5 ng of DNAs were spotted and hybridized to oligomers representing wild type (wt) and mutation-specific codon 12 N-ras (panel A) or respective codon 12 Ki-ras sequences (panel B).

matched exactly with the Southern blot analyses. Thus an activation of human ras genes could be established for all transfectants obtained from cell line Rc2a. While most transfectants showed mutations in codon 12 of N-ras as well as Ki-ras, some others exhibited mutations solely in N-ras (Rc2a-T₇) or Ki-ras (Rc2a-T₃, Rc2a-T₈). These results suggested a) that both oncogenes were activated in cell line Rc2a and b) that not in every transfection experiment both activated genes were concurrently transferred.

Ras mutations occurred in late passages of Rc2a

We finally addressed the question, at which state during the establishment of cell line Rc2a ras mutations might have occurred. We therefore decided to investigate primary leukemic cells of the AML patient this line had been derived from as well as early and late passages of Rc2a with the above mentioned dot-blot technique. We compared 1) peripheral leukemic cells from the first presentation of the patient, 2) peripheral cells taken prior to the patient's death, 3) first passages (from 1979) of cell line Rc2a, 4) later passages (from 1980) as well as 5) Rc2a cells we used for our transfection experiments (all cell samples were generously provided by Dr. T.R. Bradley). As is evident from Fig. 4, primary leukemic cells of patient R.C. as well as the first passages of cell line Rc2a lack mutations in N-ras or Ki-ras. However, sometime during 1980, Rc2a cells acquired point mutations in codon 12 of both genes. We would like to emphasize that Rc2a cells had already become growth factor (CSF) independent one year earlier (18). Thus both

events appear not to be related to each other. Moreover, it was not possible to associate these molecular alterations with any specific change in culture conditions during that period.

DISCUSSION

During transfection analyses aimed at detecting transforming genes in human hematopoietic neoplasias we investigated a variety of different AML cell lines as well as fresh AML cells including Rc2a and material obtained from patient M.P. In agreement with previous reports we observed a high incidence (40-50%) of ras gene activation, namely in N-ras (6, 19, 30-32). The present report demonstrates for the first time that point mutations in two different members of the ras gene family may occur within leukemic cells of a single AML patient or AML cell line. However, from our studies we cannot decide whether the cell samples investigated consist of two different cell types, one with a N-ras and another with a Ki-ras mutation or both mutations affect the genome of a monoclonal cell population. Moreover, even if the latter possibility is correct it would remain unknown whether the mutations of two ras genes in patient M.P. sufficiently explain the development of AML. Since leukemogenesis is a multistep process it appears to be essential that one activated oncogene is complemented by at least one other altered member of this heterogeneous group of genes (2). The cooperation of ras and myc sequences may be a case in point (2, 11-13). On the other hand recent data obtained from in vitro analyses of mutated ras versions indicate that concurrent point mutations in codon 12 and 61 could significantly increase the transforming ability of respective proteins as compared to increased expression of ras proteins altered solely either in position 12 or 61 (33). Along this line the present report may indicate that also mutations in two different ras genes, both bearing comparable, but in all likelihood slightly different biological functions show potentiating transforming ability.

While in patient M.P. the ras gene activations may be related to leukemogenesis, this possibility is ruled out for patient R.C. from whom cell line Rc2a has been derived. In the latter case cell passages prior to and after acquisition of mutated ras versions did not differ with respect to a variety of morphological, phenotypic, biochemical or cytogenetic parameters tested (18). However, in view of the molecular changes reported here different passages of Rc2a may offer a unique opportunity to study the meaning of mutated ras genes for the human myeloid lineage in much more detail.

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