Identification of a Second Transforming Gene, ras<sup>a</sup>, in a Human Multiple Myeloma Line With a Rearranged c-myc Allele

By Timothy J. Ernst, Adi Gazdar, Jerome Ritz, and Margaret A. Shipp

Multiple myeloma is a disease characterized by a long, slowly progressive phase and a final, more aggressive one. Little is known about the mechanism of transformation of myeloma cells, although the clinical characteristics of the disease suggest a multi-step process. Recently, a myeloma cell line, NCI-H929, was isolated from a patient with aggressive preterminal disease and found to have a rearranged myc allele. This myeloma cell line has been further characterized in a focus formation assay to determine whether its unusual growth characteristics were associated with a second activated transforming gene. We now report that the NCI-H929 myeloma cell line has an activated ras<sup>a</sup> allele in addition to a rearranged myc allele. This is the first identification of an activated transforming gene in a multiple myeloma cell line; furthermore, the characterization of two independently activated oncogenes in this B cell malignancy has implications for both the pathogenesis and evolution of the disease.

Oncogenesis is a multi-step process that has been associated in certain instances with the successive activation of two different oncogenes. The evidence that two independently activated oncogenes are associated with specific malignancies comes from several sources including in vitro cell culture systems, established tumor cell lines, primary tumors, and experimental animal models. For example, the oncogenes v-myc and c-ras<sup>a</sup> together, have been shown to transform primary cells under circumstances in which neither gene alone had this effect. In addition, certain established malignant hematopoietic cell lines and primary tumors have been shown to have both an activated c-myc allele, as detected by amplification or chromosomal translocation, and an activated ras<sup>a</sup> allele, as detected by focus formation in transfection assay. Examples include the HL-60 human promyelocytic cell line with an amplified c-myc and an activated ras<sup>a</sup> and the AW-Ramos human Burkitt's lymphoma cell line with a translocated c-myc and an activated ras<sup>a</sup>. Furthermore, certain experimental animal models developed to assess the effect of aberrant myc expression in vivo have shown that factors other than dysregulated myc expression may be required for the development of certain lymphoid malignancies. These studies also indicate that transgenic animals with dysregulated myc and ras expression develop lymphoid malignancies ten times as often as do transgenic animals expressing either oncogene alone.

For these reasons, we were particularly interested in the recent characterization of a human myeloma cell line, NCI-H929, that has a rearranged c-myc allele. This complex c-myc rearrangement in NCI-H929 involves the 3′ untranslated region and is associated with the stabilization of chimeric c-myc transcripts. As NCI-H929 is one of the few true mature plasmacytoid tumor lines that have been successfully established in vitro, we postulated that its unusual growth characteristics could be the result of a second activated transforming gene. In this report, NCI-H929 myeloma cell line is shown to have an additional transforming gene detectable in a standard focus forming assay. Analyses at DNA, RNA, and protein levels indicate that this NCI-H929 transforming gene is ras<sup>a</sup>.

**MATERIALS AND METHODS**

**Cells.** The human myeloma cell line NCI-H929 was originally isolated from a malignant pleural effusion of a patient with an IgA kappa multiple myeloma. Phenotypic characteristics of the IgA secreting cell line [PCA-1 (+), T10 (+)] were identical to those of the primary tumor. NCI-H929 cells were grown in defined ACL-3 media. Murine NIH-3T3 cells were maintained in Temin's modified Eagles media with 10% newborn calf serum. The human fibrosarcoma cell line, HT-1080, and the human promyelocytic cell line HL-60 were grown in Dulbecco's modified Eagles medium (DMEM) with 10% fetal calf serum (FCS). Peripheral blood lymphocytes were isolated over Ficoll gradients as previously described.

**Transfections.** High molecular weight DNA from the NCI-H929 cell line was prepared as previously described. NIH-3T3 cells were plated at a density of 5 x 10⁴ cells per 60-mm culture dish. After incubation at 37°C overnight, the NIH-3T3 cells were transfected with 20 μg of NCI-H929 DNA per culture dish using the calcium phosphate precipitation method. Media was changed at two- to three-day intervals and foci of transformed cells were counted 14 days after transfection. Individual primary foci were isolated with cloning cylinders, trypsinized, and grown in mass culture. High molecular weight DNA was then prepared from the expanded cultures of each primary transformant and used in secondary transfection assays and Southern blot analysis.

**Southern blot analyses.** Fifteen micrograms of high molecular weight DNA from the expanded cultures of each primary transformant and from NIH-3T3 and HT-1080 cells were digested with the appropriate restriction endonucleases and were used in Southern analysis. P-labeled probes including the 300 basepair (BP) from the Division of Tumor Immunology, Dana-Farber Cancer Institute, Department of Medicine, Harvard Medical School, Boston, and Human Tumor Cell Biology Section, National Cancer Institute, Bethesda, MD.

Dr Shipp's current address is Laboratory of Immunobiology, Dana-Farber Cancer Institute, Department of Medicine, Harvard Medical School, Boston.

Address reprint requests to Timothy J. Ernst, MD, Division of Tumor Immunology, Dana-Farber Cancer Institute, 44 Binney St, Boston, MA 02215.

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**RESULTS**

Transformation of NCI-H929 DNA. The NCI-H929 DNA was assayed for transforming activity in a standard NIH-3T3 cell transformation assay. Five foci were observed in the primary transfection assay giving an efficiency of 0.04 focus forming units per microgram of NCI-H929 DNA. This transformation efficiency is similar to that obtained when NIH-3T3 cells are transfected with DNA containing activated oncogenes such as ras\textsuperscript{kk,kk}. The immunoprecipitates were fractionated in a 7.5% polyacrylamide gel, using a 7.5% gradient gel, which was then fixed in 50% ethanol/7% acetic acid, immersed in Enhance (New England Nuclear, Boston), dried, and exposed at −70°C using Kodak XAR film.

Identification of the NCI-H929 transforming gene detected by transfection assay. Although the NCI-H929 cell line and the primary myeloma from which it was derived have a complex rearrangement of the c-myc locus, c-myc genes activated by rearrangement do not morphologically transform NIH-3T3 cells. For this reason the NCI-H929 cells were thought to contain an additional transforming gene detectable in the NIH-3T3 transfection assay. As the morphologic characteristics of the primary and secondary H929-3T3 foci resembled those of foci induced by ras, we analyzed H929-3T3 DNA for the presence of human ras sequences.

DNA from primary H929-3T3 foci and NIH-3T3 cells were digested with a panel of restriction endonucleases including BamHI, BglII, EcoRI, and SstI, Southern blotted, and probed with the 375 BP HindIII ras\textsuperscript{a} fragment. BamHI, BglII, EcoRI, and SstI human ras\textsuperscript{a} bands were detectable in DNA from primary H929-3T3 transformants, whereas ras\textsuperscript{a} bands were not found in DNA from control NIH-3T3 cells (Fig 1).

In order to determine whether the H929-3T3 transformants synthesized human ras\textsuperscript{a} protein, immunoprecipitations were performed using Y13-259 and YA6-172, two anti-ras monoclonal antibodies with different specificities. Y13-259 is a broadly reactive reagent that recognizes the human ras\textsuperscript{kk} proteins, whereas YA6-172 identifies only the human ras\textsuperscript{kk} proteins. The broadly reactive Y13-259 antibody immunoprecipitates a p21 protein from both primary H929-3T3 transformants (Fig 2B), but does not precipitate a similar protein from control NIH-3T3 cells (Fig 2B). However, the 21 kd protein is not immunoprecipitated from primary H929-3T3 transformants when the ras\textsuperscript{kk} specific monoclonal antibody YA6-172 is used (Fig 2A). These results indicate that the p21 synthesized by H929-3T3 transformants is ras\textsuperscript{a}.

The fact that DNAs from the two H929-3T3 foci have unique restriction patterns and that transforming efficiencies in the primary and secondary rounds of transfection are similar suggest that ras\textsuperscript{a} is activated in NCI-H929 cells rather than as a consequence of the transfection procedure. In order to confirm that ras\textsuperscript{a} is transcribed in NCI-H929 cells, Northern analysis of NCI-H929, HL-60, and peripheral blood lymphocyte RNAs was performed using the ras\textsuperscript{a} probe. Whereas peripheral blood lymphocytes lack detectable ras\textsuperscript{a} mRNA, NCI-H929 cells contain a 4.5 kb ras\textsuperscript{a} transcript similar to that seen in HL-60 cells (Fig 3).

The activated ras\textsuperscript{a} gene in NCI-H929 is not associated with the gross karyotypic abnormalities. The fact that NCI-H929 has an activated ras\textsuperscript{a} gene is of particular interest given the location of ras\textsuperscript{a} on chromosome 1 (1 cen − 1 p21) and the noted karyotypic abnormalities of chromosome 1 in NCI-H929 cells. NCI-H929 cells have six copies of chromosome 1: two copies have an aberrant duplication of the long arm, and four copies have deletions of varying length in the short arm possibly involving the ras\textsuperscript{a} alleles. In order to

![Fig 1. Detection of human ras\textsuperscript{a} sequence in the H929-3T3 transformants. DNA from control NIH-3T3 cells (A), and two primary H929-3T3 foci (B-E, and F-I, respectively) were digested with EcoRI (A, B, F), BamHI (C, G), BglII (D, H), or SstI (E and I), Southern blotted, and probed with a 32P-labeled 375 BP ras\textsuperscript{a} HindIII fragment.](image-url)
RAS<sup>+</sup> ACTIVATION IN A MULTIPLE MYELOMA CELL LINE

Fig 2. Immunoprecipitation of ras<sup>+</sup> p21 protein from H929-3T3 transformants. Cell lysates from NIH-3T3 cells (A, B) and from the two primary H929-3T3 transformants (A2.3, B2.3) were immunoprecipitated using the ras<sup>+</sup> specific monoclonal antibody YA6-172 (A) or the ras<sup>+</sup> reactive monoclonal antibody Y13-259 (B).

Fig 3. Identification of a ras<sup>+</sup> transcript in NCI-H929. Twenty micrograms total RNA from (A) peripheral blood lymphocytes, (B) HL60 cells, and (C) NCI-H929 cells was Northern blotted and probed with <sup>32</sup>P-labeled 375 BP ras<sup>+</sup> HindIII fragment.

determine whether karyotypic abnormalities involving chromosome 1 were involved in the activation of ras<sup>+</sup> in NCI-H929 cells, the sizes of NCI-H929 ras<sup>+</sup> restriction fragments were compared with those of HT-1080. In the HT-1080 cell line, ras<sup>+</sup> activation results from a point mutation rather than overexpression secondary to a rearrangement. The 800 BP PvuII probe from the first ras<sup>+</sup> exon identifies BamHI, BglII, EcoRI, and SstI fragments containing both the first exon and the 5' flanking region. The ras<sup>+</sup> BamHI, BglII, EcoRI, and SstI restriction fragments from NCI-H929 and HT-1080 are identical in size, suggesting that there are no gross karyotypic abnormalities involving ras<sup>+</sup> in NCI-H929 cells. However, comparison of the BamHI, BglII, EcoRI, and SstI ras<sup>+</sup> restriction fragments from the NCI-H929 cell line with those of the two H929-3T3 transformants indicates that the NCI-H929 ras<sup>+</sup> restriction fragments are identical to those from one of the primary H929-3T3 transformants but different from those of the other (Figs 2 and 4). Although the sizes of BamHI, BglII, EcoRI, and SstI ras<sup>+</sup> restriction fragments from the two H929-3T3 primary transformants differ, the sizes of their p21 ras<sup>+</sup> gene products are identical (Fig 2). These results suggest that the differences in the ras<sup>+</sup> restriction fragment sizes of the second H929-3T3 transformant do not affect ras<sup>+</sup> coding regions; rather, they appear to result from incorporation of a shorter fragment of human DNA 5' to the ras<sup>+</sup> gene. Taken together, these data suggest that ras<sup>+</sup> activation in the NCI-H929 cell line is not secondary to a rearrangement.

DISCUSSION

We have shown that the NCI-H929 myeloma cell line has a second transforming gene that is detectable in transfection assay and have identified this gene at DNA, RNA, and protein levels as ras<sup>+</sup>. Although NCI-H929 has karyotypic abnormalities involving the short arm of chromosome 1, these do not appear to be associated with ras<sup>+</sup> activation. To date, the only mutations of ras<sup>+</sup> that are detectable in transfection assay involve codons 12 and 61. Therefore, it is possible that a point mutation at one of these positions is associated with ras<sup>+</sup> activation in NCI-H929.

The fact that a human myeloma cell line with a karyotypic abnormality involving c-myc has an additional activated ras<sup>+</sup> gene is significant for several reasons. While providing further evidence for a multi-step theory of carcinogenesis, it also adds insight into the pathogenesis of multiple myeloma, as well as a possible mechanism by which similar cell lines can be established in vitro.

Land et al<sup>13</sup> first demonstrated that two or more oncoproteins could co-operate to transform primary cells to a malignant phenotype in studies in which primary rat embryo fibroblasts, which were not transformed by myc or ras alone, could be transformed and made highly tumorigenic following co-transfection with myc and ras. Similar studies using murine pre-B cell lines indicated that v-myc and v-ras were also synergistic in lymphoid cells.<sup>4</sup> Murine pre-B cell lines that expressed both v-myc and v-ras rapidly induced tumors in syngeneic animals; furthermore, they grew to higher densities and had lower growth factor requirements than did cell lines expressing v-ras alone.<sup>4</sup>
voted ras gene under the control of a mouse mammary tumor virus (MMTV) promoter developed B cell malignancies. However, they noted that cross-breeding the two transgenic strains gave rise to mice with both myc and ras transgenes and a tenfold increase in their incidence of B-cell lymphomas. Our demonstration of an activated transforming gene in a myeloma cell line with a c-myc rearrangement provides evidence of yet another B-cell malignancy in which myc and ras may have synergistic action.

It is possible that the successful establishment of the NCI-H929 myeloma cell line was related to the presence of both activated myc and ras alleles. In this regard, it is of note that the original NCI-H929 tumor sample was obtained from a myeloma patient in an aggressive preterminal phase of her disease, characterized by rapid proliferation, massive extramedullary disease, and cytologic atypia. Furthermore, in the rare instances in which other human myeloma cell lines have been established, the tumor specimens have been obtained from patients with similar refractory preterminal disease and all have been derived from either malignant effusions or circulating plasma cells. Given the fact that multiple myeloma is characterized by a long relatively indolent phase and a final, more aggressive one, it is possible that the disease course is associated with the sequential activation of transforming genes such as myc and ras.

In this regard, a recent report indicates that although no rearrangement or amplification of the c-myc gene was found in 21 cases of multiple myeloma, c-myc amplification was found in two of three cases of preterminal plasma cell leukemia. Furthermore, serial evaluation of myeloma tumor specimens from a patient with refractory disease provided evidence for in vivo clonal evolution. These results suggest that an understanding of the pathogenesis and evolution of multiple myeloma may require sequential assessment of tumor specimens. They also indicate that an evaluation of the role of specific transforming genes in this disease will require several methods including both karyotypic analysis and transfection studies.

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Identification of a second transforming gene, rasn, in a human multiple myeloma line with a rearranged c-myc allele

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