RAS ONCOGENE MUTATION IN MULTIPLE MYELOMA

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Multiple myeloma (MM) is a malignant neoplasm of differentiated B cells of unknown etiopathogenesis (1). While alterations of proto-oncogene loci, including mutations, amplifications, chromosomal translocations, or deletions have been found associated with several types of human tumors (2), no such association has been firmly established for MM. In a few MM cases the presence of rearranged (3–5) or mutated (6)c-myc oncogenes or elevated levels of c-myc RNA (5) have been reported. In addition, the simultaneous presence of a c-myc and a N-ras oncogene has been recently reported in a single MM cell line (7). However, these observations remain as isolated findings that have not been confirmed in studies involving large panels of cases.

In particular, the frequency of oncogenic activation of ras genes has not been established in MM. Specific mutations of the H-, K- or N-ras genes are considered to play a relatively frequent pathogenetic role in tumorigenesis, having been found in 10–15% of human malignancies (8). Tumors derived from different tissues display variations in the frequency, type (H-, K-, or N-) of ras gene and type of mutations involved. In the specific context of lymphoid malignancies, we recently reported that significant differences exist among tumors derived from the same tissue since mutations involving the N-ras gene have been found in ~20% of acute lymphocytic leukemia (ALL), whereas no mutations were found in non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukemia (CLL) (9).

These considerations prompted us to comprehensively investigate the frequency of activating mutations of ras genes in a relatively large panel of primary MM cases. In addition, we wanted to conclusively reassess the frequency of c-myc oncogene activation in this malignancy by determining the frequency of structural alterations of

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1 Abbreviations used in this paper: ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; PCR, polymerase chain reaction.

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the c-myc locus, including rearrangements and mutations. We report here that, while alterations of the c-myc gene are not detectable at appreciable frequency, mutated K- or N-ras genes represent a relatively frequent lesion with possible pathogenetic and prognostic relevance in MM.

Materials and Methods

Pathologic Samples and DNA Preparation. Pathologic samples from 43 patients at diagnosis and 13 at clinical relapse were collected during standard diagnostic procedures. Diagnostics and clinical staging were made according to the criteria described by Durie and Salmon (10). For evaluation of response to therapy and relapse the criteria established by The South Western Oncology Group (SWOG) (11) were followed. Mononuclear cell suspensions of >95% viability were prepared from bone marrow aspirates by Ficoll-Hypaque gradient centrifugation. DNA was purified by digestion with proteinase K, extraction with phenol-chloroform and precipitation by ethanol (12).

Oligonucleotide Synthesis. All the oligonucleotides used in this study were synthesized by the solid-phase triester method (13).

Polymerase Chain Reaction (PCR). To amplify sequences spanning 112-115 bp across codons 12, 13, or 61 of N-ras and codons 12 or 61 of H- and K-ras, we used the panel of oligonucleotide primers previously described (9). For each PCR reaction, 1 μg of DNA and 20 pmol of each of the two primers were added to a 50 μl reaction mixture containing 1 U of Taq DNA polymerase as previously described (14). 25 cycles of denaturation, annealing and extension (at 94°, 55°, and 72°C, respectively) were performed on an automated heat-block (DNA thermal cycler; Perkin Elmer Cetus, Norwalk, CT) according to manufacturer's specifications. To determine the nucleotide sequence within the first exon of the c-myc gene, two oligonucleotide primers [5' primer (sense): GCACTGGAACTTACAACACC; 3' primer (antisense): GGTGCTTACCTGGTTTTCCA] were used to amplify a 135-bp fragment spanning the sequences 2738-2871 (15) of the c-myc gene using the same conditions as described above except for the annealing temperature, which was 67°C.

Oligonucleotide Hybridization. Aliquots (2.5 μl) of each PCR mixture were transferred to replicate nylon filters (Gelman Science,) with a slot blot manifold (Schleicher & Schuell, Keene, NH) and hybridized sequentially or separately to a panel of 20-mer synthetic oligonucleotide probes identical to those reported by Verlaan-de Vries et al. (16). These probes are representative of the normal codons 12 and 61 (H-, K-, and N-ras) and codon 13 (N-ras), as well as of all the known activating mutations affecting each of these codons. For hybridization, oligonucleotide probes were labeled with ϒ-[32P]ATP (specific activity 3,000 Ci/mmol) by means of T4-polynucleotide kinase (New England BioLabs, Boston, MA) and separated from unincorporated nucleotides through a Bio-Gel P4 fine column (Bio-Rad Laboratories, Richmond, CA). Prehybridization, hybridization, and washing of filters were performed in a 3 M tetramethyl ammonium chloride salt solution as described (17).

Sequencing of PCR Products. Direct sequencing of PCR-amplified DNA fragments was done by a modification of the protocol originally described by McMahon et al. (18) and later modified by Collins (19).

Southern Blot Hybridization Analysis. 5 μg of DNA from each sample was digested with the appropriate restriction endonuclease (Bethesda Research Laboratories, Gaithersburg, MD) according to manufacturer's specifications, electrophoresed in an 0.8% agarose gel, denatured, neutralized, transferred to nitrocellulose filters, and hybridized according to the method of Southern (20). DNA probes were 32P-labeled by the random priming method (21). After hybridization filters were washed in 0.2 x SSC/0.5% SDS for 2 h at 60°C and then autoradiographed using intensifying screens (Quanta III; DuPont Co., Wilmington, DE).

DNA Probe. The organization of the c-myc locus was analyzed by hybridization with a human c-myc probe, MC413RC, representative of the third exon of the c-myc gene (22).

Results

Analysis of ras Gene Mutations. 56 MM bone marrow aspirates were selected for this study; 43 samples were obtained at the time of diagnosis (including 1 plasma
cell leukemia), and 13 unrelated samples were taken after treatment. All DNAs extracted from these specimens were amplified by the PCR method using pairs of oligonucleotide primers flanking the genomic regions spanning codons 12-13 or 61 of the H-, K- or N-ras genes (9). Each PCR product was then hybridized to a panel of synthetic oligonucleotide probes representative of all H-, K- and N-ras mutations described for human tumors with the exception of mutations in codon 13 of the K-ras gene (23).

Mutations were detected in 18 of 56 (32%) samples of which 12/43 (27%) were at diagnosis and 6/13 (46%) were after treatment (see Table I and Fig. 1). Two ras genes, K- and N-ras, were found to be mutated, although mutation of the N-ras gene, particularly at codon 61, was the most frequent finding. As previously shown for ALL (9), multiple mutations, sometimes involving different ras genes, are found in some MM cases. Note that in two cases contiguous codons of the same gene are found to be mutated. Since under our experimental conditions an allele containing two mutations cannot form a stable hybrid with any of the wild-type or single base pair mutated probes, the two mutations detected in this case must be present in two distinct alleles.

The lack of detectable mutations in the negative cases was not due to insufficient representation of malignant cells in the samples since all the cases were shown by histologic examination and Ig gene rearrangement analysis to contain more than

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<th>Table I</th>
<th>Summary of ras Oncogene Mutations in MM</th>
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* PCL, plasma cell leukemia.
Representative data of slot-blot hybridization analysis for mutations of K-ras and N-ras codons 12-13 in MM. DNA from MM biopsies was amplified in the region across codons 12-13 of the N-ras (A) and K-ras gene (B), spotted onto a nylon filter, and hybridized with the wild-type and mutated probes as indicated. MM DNAs in A: 1a, case 982; 2a, 1006; 1b, 1011; 2b, 1122. (B) 1a, case 1122; 2a, 1011; 3a, 964; 4a, 963; 5a, 981; 1b, 980; 2b, 985; 3b, 997; 4b, 1102; 5b, 982. Note the presence of two distinct N-ras mutations in DNA sample 1011 (1b, A).

the 5-10% fraction of malignant cells (not shown) that represents the threshold of sensitivity of the method under our experimental conditions (9). It remains possible, however, that mutations present in a minor subpopulation (<5% of total cells in the specimen) of malignant cells or mutations different from those described so far in human tumors, are present in these samples.

To confirm these findings and to determine the exact nature of the mutations, the nucleotide sequence of the involved regions was determined by direct sequence analysis of the PCR products (see Table I and Fig. 2). The presence of the mutations in the specific codons indicated by the hybridization analysis was confirmed in all cases, whereas no mutations were detected in the remaining cases within genomic regions spanning ~200 bp across codons 12-13 and 61 of the three ras genes. The presence of two, or in one case (plasma cell leukemia) three, distinct mutations was also confirmed. An heterogeneous pattern of mutations was observed, including different types of purine-purine, pyrimidine-pyrimidine, and purine-pyrimidine transitions. These changes led to the replacement of glycine or glutamine residues with one of several different amino acids. No specific difference in the pattern of mutations (type of ras gene, codon, or base) is detectable between cases at diagnosis and after treatment.

Analysis of Clinico-Pathologic Features of MM Cases Displaying ras Mutations. The heterogeneity of MM cases with respect to the presence of ras oncogenes prompted an analysis of possible correlations with well-defined clinico-pathologic characteristics of MM (10-11). No correlation was observed between the presence or type of ras oncogenes at diagnosis and a number of parameters listed in Table II, with the exception of a correlation between the presence of ras oncogenes and a partial or complete lack of response to therapy. Despite the relatively small sample size this correlation approached statistical significance (p = 0.06).
Figure 2. Direct DNA sequencing of amplified fragments containing codons 12/13 of the K-ras (A) and N-ras (C) genes and 61 of the N-mu gene (B) in representative normal and MM DNAs. The 5' or 3' primers used for the in vitro amplification were also used as sequencing primers (9). The normal nucleotide sequences correspond to human placenta DNA. Arrows point to bands corresponding to mutated base pairs.
Analysis of Structural Alterations of the c-myc Locus. Structural alterations of the c-myc locus have been typically found in B cell malignancies carrying chromosomal translocations [(8, 14), (2, 8), and (8, 22)] involving the c-myc gene and Ig loci (24). In addition, alterations of the c-myc gene have been reported in a few MM cases studied that lacked these translocations (3–5). These alterations include: (a) truncations of the c-myc gene within the first intron, the first exon, or within 5' or 3' flanking sequences; (b) mutations of sequences within a specific restricted region of first exon. The truncations can be detected by Southern blot hybridization of genomic DNA using restriction enzymes that cut outside c-myc sequences (e.g., Eco RI and Hind III), while most (60%) of the mutations are detectable as polymorphisms of a Pvu II site located near the 3' side of exon I (25–26). Recently, by sequencing genomic DNA amplified by the PCR method, we have demonstrated the presence of c-myc...
FIGURE 3. Direct DNA sequencing of a 135-bp fragment spanning the sequences 2387-2871 (15) of the c-myc gene. Representative sequences are shown of DNAs from normal PBL, from a Burkitt lymphoma biopsy (IM-9) showing a base pair mutation (see arrow), and from two MM samples (986, 988). The PvuII restriction site located at positions 2839-2845 within the first exon of the c-myc gene (15) is shown as reference point.

mutations in B cell malignancies that carry (8, 14), (2, 8), and (8, 22) translocations even in those cases where the chromosomal breakpoint is distant (>100 kb) from the c-myc locus (Murphy, J. P., manuscript in preparation).

In this report, we have used restriction analysis and nucleotide sequence analysis of PCR-amplified genomic DNA in order to conclusively assess the frequency of rearrangements and mutations in MM. No structural alterations were detectable in a panel of 17 MM cases studied (see Fig. 3 for representative sequencing data). This indicates that c-myc activation by currently known mechanisms does not represent an appreciably frequent event in MM.

Discussion

This report provides the first evidence of an oncogene activation event present at significant frequency in MM. Previous information on the presence of activated ras oncogenes in this type of neoplasia had been limited to the report of an activated N-ras gene in a single MM cell line (7) and to the report of frequent overexpression of the H-ras protein without evidence of structural alterations (27). Several studies had, however, reported the presence of structural alterations of the c-myc gene in different MM cell lines (3-5). The results of the present study indicate that rearrangements of the c-myc locus are not detectable at appreciable frequency in fresh MM biopsies, suggesting that they may have occurred during in vitro establishment of MM cell lines or that they may have occurred in vivo in a very small number of MM cases and possibly contributed to their ability to grow in vitro and thus be established as cell lines. In addition, mutations of the first c-myc exon analogous to those found in Burkitt lymphoma were previously detected as restriction enzyme polymorphisms in 10 of 16 MM biopsies analyzed (6). These results cannot be
confirmed by our findings, which must be regarded, in this respect, as conclusive since direct nucleotide sequencing of the involved region of the c-myc gene has been performed. In the absence of any detectable structural alterations it remains possible that increased expression of the c-myc gene, perhaps secondary to other genetic alterations, may play a pathogenetic role in MM, as suggested by the increased levels of c-myc RNA in some MM cases (5). However, this possibility has not yet been clearly documented in any other type of tumor and, in the absence of clear parameters for normal levels of c-myc expression, remains speculative.

The mechanism of occurrence of ras oncogene mutations in MM remains unknown. The pattern of mutations observed in MM appears to confirm towards a preferential involvement of the N-ras gene in hematopoietic malignancies (28-29). However, the pattern of codon involvement as well as type of base and amino acid substitutions appears significantly more heterogeneous than in other hematopoietic neoplasms (29) and in particular in ALL (9). This suggests, among several possibilities, that the mutations may be generated through multiple and/or relatively nonspecific mechanisms. Alternatively, it is possible role that a larger variety of mutated ras products are biologically active in the cells that represent the target of transformation in MM than in ALL.

Regarding the possible role of ras oncogenes in the pathogenesis of MM it is intriguing to note again that ras oncogene mutations display a highly specific pattern of distribution in lymphoid malignancies, as they are detected at significant frequency in ALL and MM but are apparently completely absent in NHL and CLL (9). The difference in the stage of differentiation of these tumor types suggests that mutated ras genes may be biologically active only at early (ALL) or late (MM) stages of lymphoid development. Alternatively, it is possible that the general frequency of point mutations may vary during different stages of B cell development. In fact, the highly mutational environment associated with antigen receptor gene rearrangements and with the proliferative response following primary antigen stimulation during early stages of B cell development has been often suggested as a pathogenetic mechanism in ALL (30). Analogously, it is possible that the presence of mutations in MM may also reflect an analogous mutational tendency associated with the Ig gene rearrangements involved in isotype switching and with the proliferative response following secondary antigenic stimulation.

We have recently shown that the introduction of activated H- or N-ras genes causes both the tumorigenic conversion and the plasmacytoid differentiation of human lymphoblastoid cells immortalized by the EBV, leading to the establishment of cell lines having morphologic, immunophenotypic, and functional characteristics of MM cell lines (31). Although EBV-immortalized cells do not represent the natural target for ras activation during MM pathogenesis, this experimental model, together with the results presented here, suggests that ras oncogenes may contribute to MM pathogenesis by leading to both the malignant transformation and the plasmacytoid differentiation of the clonal pre-B-cell populations that are thought to represent the precursors of MM cells (32-34).

Regardless of the mechanisms involved the results presented here indicate that MM represents an heterogeneous neoplasm with respect to a putative pathogenetic marker such as the ras oncogene. This heterogeneity prompted us to search for possible clinico-prognostic correlates among a variety of biological and clinical markers
of MM. Two findings have emerged. First, we found an increased frequency of ras oncogenes in MM samples obtained after treatment (46% vs. 27% at diagnosis), suggesting, among several related possibilities, the involvement of ras oncogenes in later stages of tumor progression or the mutagenic effect of certain chemotherapeutic agents. Although of limited value as a single observation, we note also that three distinct ras mutations were detected in the single case corresponding to the more advanced and clinically aggressive form of MM, i.e., plasma cell leukemia. Second, the presence of ras oncogenes at diagnosis correlates with poor therapeutic response. Although of only indicative statistical significance in this preliminary study, these correlations have potentially important clinical implications and warrant further studies of larger panels of MM.

Summary
The frequency of ras (H-, K-, and N-ras) and c-myc oncogenes was investigated in multiple myeloma (MM). By means of the polymerase chain reaction (PCR)/oligonucleotide hybridization method, DNA from 56 tumor biopsies was analyzed for the presence of activating mutations involving codons 12 and 61 of the H-, K-, and N-ras genes and codon 13 of the N-ras gene. Mutations, involving the N- or K-ras genes, were detected in 18 of 56 (32%) cases of which 12/43 (27%) were at diagnosis and 6/13 (46%) were after treatment. In some cases, multiple mutations affecting different ras alleles were detected. Direct nucleotide sequence analysis of PCR products indicated that a more heterogeneous nature of the base pair changes than previously shown for other tumors along with a preferential involvement of N-ras codon 61. The heterogeneity of MM cases with respect to the presence of ras oncogenes prompted an analysis of possible correlations with different clinico-pathologic characteristics of MM from which a correlation between the presence of ras oncogenes and a partial or complete lack of response to therapy emerged. The frequency of activating rearrangements or mutations of the c-myc gene were studied by Southern blot analysis and PCR sequencing, respectively. However, contrary to previous reports involving mostly MM cell lines, no structural alterations of the c-myc gene were found. These results indicate that ras, but not c-myc, oncogenes are activated in vivo in MM cells, representing the first oncogene alteration that has been associated at appreciable frequency with this type of malignancy. While the mechanism of occurrence and biological role of ras activation in MM remains to be elucidated, the preliminary correlations observed in this study between the presence of ras oncogenes and poor therapeutic response suggest that further investigations of the possible prognostic significance of these alterations are necessary.

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