Alteration and Abnormal Expression of the c-myc Oncogene in Human Multiple Myeloma

By Peter Selvanayagam, Mark Blick, Franco Narni, Peter van Tuinen, David H. Ledbetter, Raymond Alexanian, Grady F. Saunders, and Bart Barlogie

Structural alterations of the c-myc oncogene in human Burkitt’s lymphoma and mouse plasmacytoma suggest that this oncogene is involved in several B cell neoplasms. The possibility of c-myc alterations in human myeloma has not been explored, probably because the low proliferative activity characteristic of this tumor impairs the propagation of representative cell lines for the performance of adequate cytogenetic studies. This report describes alterations in the c-myc locus with concomitant elevated expression of mRNA in the tumor cells of two of 37 patients with multiple myeloma. In one case, somatic cell hybrid studies revealed that the cloned rearranged DNA was entirely derived from chromosome 8, thus indicating a novel mechanism of c-myc activation different from that in Burkitt’s lymphoma. Seven other patients exhibited five- to 12-fold overexpression of c-myc RNA when compared with normal marrow cells. Elevated mRNA expression in about one fourth of our patients suggests that the c-myc oncogene has a pathogenic role in the evolution of multiple myeloma.

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MATERIALS AND METHODS

Bone marrow aspirates were studied in 37 patients, in eight at the time of diagnosis and in 29 during later advanced stages of disease. Informed consent was obtained before the bone marrow aspiration procedures. The degree of marrow plasmacytosis was usually marked (median, 35%). Myeloma protein was phenotyped by immunolectrophoresis, and cytogenetic studies were conducted on several samples (Table I). Bone marrow cells and placental tissue from normal healthy individuals served as controls. HL-60 cells with abundant c-myc mRNA were used as a positive control for RNA expression studies. Informed consent was obtained before bone marrow aspiration procedures.

Nucleic acid analysis. High-molecular weight DNA and total RNA were coextracted from bone marrow cells by the method of Eisman et al. DNA was digested with restriction enzymes as recommended by the manufacturer. Size fractionation in agarose gel and transfer to nitrocellulose filter were carried out by the method of Southern. For Northern blot analysis, 20 μg of total RNA was denatured in 1 mol/L glyoxal, 50% dimethylsulfoxide, and 10 mmol/L phosphate buffer, pH 7.0, at 50°C for one hour; size-fractionated in 1.2% agarose gel; and transferred to nitrocellulose filter by the method of Thomas. Hybridization of the DNA and RNA filters with 32P-labeled gene probes and subsequent washing were carried out according to the method of Maniatis et al. Autoradiography on x-ray films was performed at ~70°C for various durations. Gene expression was quantitated by densitometric scanning of autoradiograms.

Isolation of the rearranged gene. Tumor DNA digested with Hind III enzyme was fractionated in a sucrose density gradient, and the fraction enriched for the rearranged myc fragment was cloned into λX2001 phage vector. Screening of the library and purification of the recombinant phage were performed by published protocols. Somatic cell hybrid analysis. Construction and analysis of Chinese hamster–human and mouse-human somatic cell hybrids have been reported previously. G-banding analysis was performed on at least 20 cells in each hybrid and was followed by sequential G-11 staining of selected G-banded cells. Chromosomes were scored as absent if they were found in less than 20% of the cells scored.

Gene probes. The c-myc probe used in our experiments is a 1.6 kilobase (kb) Cla I–Eco RI fragment (MC 41'3rC) consisting of the third exon and the 3' flanking sequences of the human c-myc gene. To detect structural abnormalities at the 5' portion of myc, we used a 0.8-kb Pvu II fragment consisting of the first exon and the 5' flank of the c-myc gene. Immunoglobulin gene rearrangements were examined with human JH, Sγ, Cγ, Cδ, Cε, and Cκ probes. For determining the proliferative activity, an S-phase–specific gene encoding histone H3 was used.
Normal Marrow

Myeloma 1

Fig 1. Detection of monoclonal plasma cells in the bone marrow of a patient with IgAX myeloma. Bgl II- and Bam HI-digested DNA from myeloma 1 was hybridized to ^P-labeled JH and C probes which detected 5.5-kb and 10.5-kb rearranged Ig heavy- and k light-chain gene bands, respectively (arrows). The probes also revealed 4.2-kb heavy-chain and 12-kb light-chain germline gene bands, probably from the unrearranged alleles of the 75% plasma cells and normal hemopoietic cells. Hybridization to the C probe revealed a germline configuration (not shown). DNA from bone marrow cells of a healthy donor was used as control.

Table 1. Clinical, Cytogenetic, and Molecular Features in 37 Patients With Multiple Myeloma

<table>
<thead>
<tr>
<th>No.</th>
<th>c-myc</th>
<th>Ig</th>
<th>Chromosome 8</th>
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<tbody>
<tr>
<td></td>
<td>RNA Expression (± 15%)</td>
<td>DNA Rearrangement</td>
<td>L Rearrangement</td>
</tr>
<tr>
<td>1</td>
<td>76</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>10</td>
<td>+</td>
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<tr>
<td>3</td>
<td>65</td>
<td>12</td>
<td>-</td>
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<tr>
<td>4</td>
<td>90</td>
<td>10</td>
<td>-</td>
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<tr>
<td>5</td>
<td>21</td>
<td>8</td>
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<tr>
<td>6</td>
<td>26</td>
<td>8</td>
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<tr>
<td>7</td>
<td>21</td>
<td>8</td>
<td>-</td>
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<tr>
<td>8</td>
<td>86</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>43</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>1</td>
<td>-</td>
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<tr>
<td>11</td>
<td>73</td>
<td>1</td>
<td>-</td>
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<tr>
<td>12</td>
<td>48</td>
<td>1</td>
<td>-</td>
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<tr>
<td>13</td>
<td>80</td>
<td>1</td>
<td>-</td>
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<tr>
<td>14</td>
<td>77</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>44</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>16-25</td>
<td>&gt;10</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>26-37</td>
<td>&gt;10</td>
<td>1</td>
<td>-</td>
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</tbody>
</table>

Abbreviations: X, fold increase over normal marrow; nd, not detected; ND, not done; BJP, Bence Jones protein; % PC, plasma cells.

RESULTS

Analysis of Ig (and more recently of T cell receptor) gene rearrangement has helped determine the clonality and differentiation stage in lymphoproliferative disorders.23'30 When applied to bone marrow samples from 37 myeloma patients, discrete rearrangements of Ig heavy- and light-chain genes were observed in most cases and corresponded to immunoelectrophoretic results (Table 1 and Fig 1). The autoradiographic signal intensity of rearranged Ig gene bands corresponded with the degree of bone marrow plasmacytosis.

For c-myc gene analysis, DNA was digested with Bam HI, Eco RI and Hind III restriction enzymes and hybridized to the probe MC 41'3rC. One of the 37 samples (myeloma 1), digested with Hind III, showed a rearranged 14-kb c-myc fragment as well as the germline 11.6-kb band. This finding was corroborated by additional analysis with Kpn I and Bgl II, which revealed rearranged myc fragments of 15 and 9 kb in size, respectively (Fig 2A). Further analysis of the gene locus by using additional restriction enzymes localized the aberration to a site between the Xba I and Bgl II at the 3' flanking region of the gene (Fig 2B). In comparison with their germline counterparts, the rearranged myc fragments always exhibited a reduced signal intensity (Fig 2A). The germline bands had the same size as those from normal human placental DNA analyzed in parallel (data not shown).

To examine whether the rearranged myc DNA in myeloma was associated with Ig genes frequently observed in other B cell malignancies,11 filters were rehybridized to several Ig heavy- and light-chain gene probes. As seen in Fig 3, the rearranged myc fragment did not react with C and JH as well as S, Ck, Ck, Ck, and Ck probes (data not shown), thereby implying a novel rearrangement of c-myc in myeloma 1. To confirm this, the novel DNA situated at the 3' flank of the altered myc was isolated by means of cloning into a phage vector and probed with a panel of hamster-human
When DNA was digested with Pvu II enzyme and hybridized to the first exon-specific \textit{myc} probe,\textsuperscript{22} all 37 samples exhibited a germline configuration indicating the absence of structural changes characteristic of endemic Burkitt's lymphoma. There was no gene amplification evident in any of these other samples investigated.

The two cases with \textit{myc} DNA rearrangement showed increased transcriptional activity on Northern analysis (Fig 6A). Seven other patients, or a total of 24\%, also exhibited higher \textit{myc} RNA expression in comparison with that of normal bone marrow (Fig 6B). RNA overexpression ranged from five- to 12-fold using slot blot analysis (Table 1). Bone marrow cells from a healthy individual were chosen as reference tissue in the absence of significant quantities of normal plasma cells. Elevated \textit{c-myc} expression in our myeloma samples was not proliferation related because the expression of the S-phase-specific histone H3 gene was consistently lower in myeloma samples compared with normal bone marrow (Fig 6C). Although (8;14) translocations have recently been observed in human myeloma,\textsuperscript{29} including our two cases (see Table 1), high \textit{myc} expression was also noted with other chromosome 8 abnormalities; conversely, only one of the two cases with t(8;14) translocations was associated with elevated \textit{myc} transcriptional activity. Although there was no apparent clinical association with increased \textit{c-myc} expression, the IgA myeloma protein type was more frequent among such patients in comparison with the remaining 28 individuals with low \textit{myc} gene activity (67\% v 39\%, \textit{P} = 0.29).

**DISCUSSION**

There is growing evidence that cellular oncogenes are altered or amplified in several human malignancies. The \textit{c-myc} oncogene, which is closely associated with Burkitt's lymphoma,\textsuperscript{2} is also altered in other human B cell tumors.\textsuperscript{31} In human plasma cell myeloma, however, gross alterations of the \textit{myc} gene were infrequent, with only two of 37 patients showing rearrangement within the 30-kb region analyzed. Sumegi et al\textsuperscript{19} found no alteration of the \textit{c-myc} locus among 21 patients with myeloma.\textsuperscript{32} Recently, Gazdar et al observed \textit{c-myc} gene rearrangement in a myeloma cell line that they considered responsible for maintaining continuous growth in culture.\textsuperscript{33} Interestingly, this cell line as well as six of our nine patients with elevated \textit{myc} expression secreted IgA myeloma protein (Table 1). Similarly, recent cytogenetic studies from this laboratory revealed that all of four t(8;14) anomalies were associated with an IgA phenotype.\textsuperscript{31}

The \textit{c-myc} gene alteration in myeloma 1 with 75\% marrow plasmacytosis appeared to be tumor specific because the hybridization signal intensities of the rearranged \textit{myc} fragments (and of the rearranged Ig gene bands) were consistently less than those of their germline counterparts (Figs 1 and 2). This observation ruled out the possibility of a constitutive \textit{c-myc} aberration and indicated involvement of only one allele. The S' portion of the \textit{myc} gene, which is frequently altered in endemic Burkitt's lymphoma, was intact in myeloma. In contrast to similar alterations in a few variant Burkitt's lymphomas\textsuperscript{34} and mouse plasmacytomas\textsuperscript{34} with chromosomal translocations involving Ig light-chain genes, alteration at the S' flank of the \textit{myc} gene from...
Fig 3. Cohybridization analysis demonstrating the lack of Ig gene elements within the rearranged c-myc fragment in myeloma 1. The filter containing Bgl II-digested DNA was hybridized to the c-myc probe MC41'C and revealed a 9-kb altered myc fragment (center). After the c-myc probe was stripped from the filter, the filter was rehybridized to Cμ (left) and JH (right) probes. Similar negative results were obtained after hybridization to the Sμ, Cκ, and Cλ subunits of the Ig heavy-chain gene as well as the Cκ and Cλ light-chain gene probes (not shown). The less intense germline Cμ signal is probably due to deletion of the Cμ region during B cell maturation of myeloma plasma cells. Equal amounts of placental DNA from a normal individual were used as control.

Table 2. Somatic Cell Hybrid Mapping of the humm 1 Probe

| Hybrid | Probe | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X | Y |
|--------|-------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| C1 1A  | -     | - | - | + | + | + | - | - | - | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 1.4    | -     | - | - | + | + | - | - | - | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 8.2    | +     | + | - | - | + | + | - | - | - | - | -  | +  | +  | +  | +  | +  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| 16.1   | -     | + | + | - | - | + | - | - | + | +  | +  | -  | -  | -  | -  | -  | +  | -  | -  | -  | -  | -  | -  | -  | -  |
| MR2.2  | -     | - | - | - | + | - | - | + | +  | +  | -  | -  | -  | -  | -  | -  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| MR7.11 | -     | - | - | - | - | - | - | + | -  | +  | -  | -  | +  | +  | +  | -  | +  | -  | +  | +  | +  | +  | +  | +  | +  |
| MR1.21 | -     | - | - | - | - | + | - | + | +  | -  | -  | +  | -  | +  | +  | -  | +  | -  | +  | +  | +  | +  | +  | +  | +  |
| SA-5   | -     | - | - | - | - | - | + | - | -  | +  | -  | +  | +  | +  | -  | +  | -  | +  | -  | +  | +  | +  | +  | +  | +  |
| MH-18  | +     | - | + | - | - | + | + | + | +  | +  | +  | -  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |

*This hybrid contains the translocation chromosome 15qter → 15q15:17p13 → 17qter.
†This hybrid contains the translocation chromosome 9qter → 9q12:17p11 → 17qter.

Fig 4. High-molecular weight DNA from hamster-human and mouse-human somatic cell hybrids was digested with Bam HI and transferred to nitrocellulose filter. Hybridization conditions with the humm 1 probe was as described in Materials and Methods. The size of the human and mouse fragments hybridizing to the probe was determined by comparison with standard size markers resolved in parallel. Lanes 1 to 9 contain DNA from somatic cell hybrids described in Table 2. Discrete bands reacting with the humm 1 probe can be detected in lanes 3 and 9.
myeloma 1 did not show cohybridization with either light- or heavy-chain gene probes. Instead, somatic cell hybridization experiments revealed that the cloned rearranged DNA originated entirely from chromosome 8, thus suggesting inversion or deletion as a possible novel mechanism of myc gene deregulation. Similar alterations at the 3' portion of the myc gene, associated with enhanced transcriptional activity, have been reported in T cell leukemia lines. Enhancer elements are capable of controlling gene transcription in an orientation-independent manner over long distances, and the possibility that the novel DNA isolated from the 3' part of the rearranged myc gene in myeloma 1 has enhancer properties is being investigated. A second case of myc gene alteration with associated RNA overexpression likewise revealed a lack of Ig gene involvement. The high myc RNA expression in the absence of DNA rearrangement could be due to alterations at large distances from the analyzed area. The occurrence of elevated c-myc expression in about one fourth of our patients with or without demonstrable gene alterations indicates a possible pathogenetic role of the c-myc oncogene in multiple myeloma.

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REFERENCES


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