Establishment and Molecular Characterization of a Novel Leukemic Cell Line with Philadelphia Chromosome Expressing p230 BCR/ABL Fusion Protein

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ABSTRACT

The cell line AR230 was established from the peripheral blood mononuclear cells of a patient with chronic myeloid leukemia and t(9;22) translocation bearing a variant type of BCR/ABL rearrangement. AR230 expresses a BCR/ABL fusion protein with a molecular mass of 230 kilodaltons (kDa) due to the insertion of 180 amino acids encoded by 3' exons of BCR (b4 to c3). An immune complex kinase assay showed that the 230-kDa BCR/ABL protein had autophosphorylation activity. Immunoprecipitation analysis revealed a stable complex of GRB2 and 230-kDa BCR/ABL proteins, indicating that the Ras activation pathway is involved in the process of transformation. AR230 expressed another short transcript consisting of a BCRc2/ABL junction, which is associated with a stop signal shortly after the junction. To our knowledge, this is the first cell line expressing a 230-kDa fusion product of BCR/ABL. AR230 will be useful for studying the biological function of divergent BCR/ABL proteins.

INTRODUCTION

Ninety-five % of the cases of CML[1] show the Ph chromosome. This cytogenetic hallmark is characterized by the translocation of the c-ABL proto-oncogene from chromosome 9 to the M-BCR of chromosome 22, and the subsequent production of an 8.5-kb chimeric BCR/ABL RNA species with an altered 210-kDa c-ABL protein (1). This altered protein is essential in the pathogenesis of CML and some acute leukemias.

In another well-known type of Ph chromosome, the translocation is associated with that of the c-ABL proto-oncogene to the minor breakpoint cluster region, which is outside of the 8.5-kb M-BCR. This type of translocation results in the production of an altered 190-kDa c-ABL protein (2, 3). Although the p190-type Ph translocation has been found primarily in ALLs, CML is also rarely associated with this type of translocation (4).

Recently, Saglio et al. (5) reported two CML cases with another type of Ph translocation. This variant type of Ph translocation has a breakpoint mapping outside of the 3' end of the 8.5-kb M-BCR gene and bears BCR/ABL transcripts with an additional 180 amino acids (BCR c3/ABL exon 2 junction). We report here a third case with such a variant type of BCR/ABL rearrangement and the establishment of a novel myeloid cell line (AR230) derived from this patient. The

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: CML, chronic myelogenous leukemia; M-BCR, major breakpoint cluster region; Ph, Philadelphia chromosome; CD, cluster of differentiation; ALL, acute lymphoblastic leukemia; PB, peripheral blood; MNC, mononuclear cells; BM, bone marrow; GTP, GTPase-activating protein; SH, src homology; kDa, kilodalton.

leukemic cells of this case and the cell line were associated with the expression of an additional truncated form of BCR/ABL with BCR c2 and the ABL exon 2 junction. The molecular features of the variant Ph translocation in the patient and the cell line are described and discussed.

MATERIALS AND METHODS

Case Report. A 50-year-old female presented with anemia (hemoglobin, 9.5 g/dl) and thrombocytosis (762 X 10^9 cells/liter) in April 1989. Her peripheral WBC was 8.8 X 10^9 cells/liter (1% monoblasts, 2% metamyelocytes, 4% stab neutrophil, 54% segments, 32% lymphocytes, 5% monocytes, 1% basophils, and 1% eosinophils). BM aspiration showed normal to slightly hypercellular BM containing 4.8% myeloblasts and increased megakaryocytes. Although she had no splenomegaly or prominent leukocytosis, a diagnosis of CML was made because cytogenetic analysis of BM MNCs revealed a single Ph chromosome with isochromosome 17q. Decreased neutrophil alkaline phosphatase activity (rate, 27%; score, 52) in the PB was also compatible with a diagnosis of CML. At the initial presentation, she was treated with one dose of 100 mg of Ranimustine, and the subsequent BM examination showed an appearance of normal female chromosomes with progressive hypoplasia without cytotoxic agents. There was no evidence of BM fibrosis throughout the clinical course. These clinical features were compatible with a diagnosis of atypical CML. In the terminal stage, she received small doses of cytosine arabinoside and IFN-alpha due to progressive hepatosplenomegaly and increased myeloblasts in PB. Nevertheless, the response was poor, and she died in September 1991.

Establishment of the Cell Line and Cloning of AR230. MNCs of PB at the terminal stage (September 1991. WBC, 18.5 X 10^9 cells/liter; myeloblast, 39%) were isolated and cultured in RPMI 1640 supplemented with 20% FCS in a 25-cm^2 flask at 37°C under 5% CO_2. After initiation of culture, continuous proliferation was observed, and cells were maintained in RPMI 1640 with 10% FCS. Four independent subclones were isolated by limiting dilution. All of the subclones were identical in terms of morphological, phenotypical, and molecular features. One of them was referred to as AR230 and subjected to additional study.

Cell Surface Marker Analysis. AR230 was immunologically characterized by indirect immunofluorescence and flow cytometry with the use of the anti-TdT mAb (Dakopatts, Copenhagen, Denmark) and the following mAbs in the T-cell, B-cell, and myeloid CD series: CD2 (OKT11), CD3 (Leu 4), CD4 (Leu 3), CD5 (Leu 1), CD7 (Leu 9), CD8 (Leu 2), CD19 (Leu 12), CD20 (B1), CD21 (OKB7), CD22 (B3), CD24 (OKB5), CD10 (J5), CD45 (LCA), CD11b (OKM1), CD13 (My 7), CD14 (My 4), CD33 (My 9), and CD34 (QBend 10).

Leukemic cells were also tested for HLA-DR (TC-8B1).

Cytogenetic Studies. MNCs of BM at the diagnosis and AR230 were cultured for 24 h without stimulation for a karyotype analysis. Chromosomes were analyzed with regular Giemsa stain and Q- or G-banding methods. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (6).

Southern Blot Analysis. Genomic DNA was extracted according to the standard method (4). Six µg of DNA were digested with three different restriction enzymes (BglII, HindIII, and EcoRI), electrophoresed on a 0.7% agarose gel, transferred to nylon filters, and hybridized to the α^-32P-labeled 5'-3' marker (7) and large bcr probes (provided by Dr. J. Groffen, Children's Hospital, Los Angeles, CA).
Reverse Transcription-PCR Assay. Total RNA was isolated by the acid guanidium-phenol-chloroform method with the use of BM MNCs at the diagnosis and cell line AR230 (8). The PCR assay was based on methods described previously (8). Briefly, 10 μg of total RNA were transcribed with reverse transcriptase. An aliquot of cDNA was amplified with the use of M-BCR exon 2 sense primer (5' - ATCCGCTGACCATCAATA-3') and ABL exon 3 antisense primer (5'-TCCCCATGTTGATTATAGCC-3'). The cDNA was also amplified with the use of M-BCR exon 6 sense primer (5' - ACCGCTATCGCTAATGTTG-3') and ABL exon 2 antisense primer (5'-GCTCAAAGTCAGATGCTACT-3'). PCR was performed for 30 cycles. The reaction mixture was electrophoresed, transferred to a nylon filter, and hybridized to the γ-32P-radiolabeled oligonucleotides corresponding to ABL exon 3 sense (5'-GTGAAAAAGCTCCTGACTTG-3') and ABL exon 2 junction (5' - GTGAAAGGCGCTTCTCTTATTGATG-3'). BCR exon 3/ABL exon 2 junction (5' - GTGAAAGGCGCTTCTCTTATTGATG-3'). BCR exon c2 sense (5' - TTCGGAGTCAAGATTGTGG-3'), or BCR exon c3 antisense (5' - ACAGTGAGGGGCACCTTG-3') sequences to identify amplified fragments.

Direct Nucleotide Sequence Determination. The PCR-amplified DNA fragments of M-BCR/ABL of AR230 were separated by agarose gel electrophoresis, cut out from the gel, and purified with GENECLEAN II (Funkasahi, Tokyo, Japan). Each of the purified fragments 1 and 2 (described below) was used as a template for direct sequencing to identify the BCR/ABL junction.

For sequence determination, Dynabeads M280-Streptavidin (Nihon Dynal, Tokyo, Japan) were used as described previously (9). PCR was performed with 5 pmol of M-BCR exon 6 sense primer and 5'-biotinylated ABL exon 2 antisense primer in a final volume of 50 μl. Forty μl of reaction mixture were added to 0.2 mg of Dynabeads M280-Streptavidin suspended in 10 mM Tris (pH 8.1) 1 mM EDTA and 1 mM NaCl, and the mixtures were incubated for 15 min at room temperature. The immobilized amplified fragments were subsequently incubated with 0.1 μM NaOH for 10 min, at room temperature. The immobilized single-stranded DNA fragments were subjected to a sequencing reaction by the dideoxy chain termination method with the use of Sequenase version 2.0 (U. S. Biochemical Corp., Cleveland, OH). The reaction mixtures were heated at 80°C for 2 min, and the supernatants were loaded on a 6% polyacrylamide sequencing gel.

Immune Complex Kinase Assay for BCR/ABL Protein. Immune complex kinase assay for BCR/ABL protein in AR230 was performed according to the method described previously (10). Briefly, anti-ABL antibodies were raised in New Zealand White rabbits by immunization with a synthetic peptide (Cys-Ser-Asp-Glu-Val-Glu-Lys-Glu-Leu-Gly-Lys). Cells were solubilized at 1 × 107 cells/ml in lysis buffer (0.15 M NaCl, 50 mM Tris-HCl (pH 7.4) 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA. 2 mM phenylmethylsulfonfluoride, 500 units/ml aprotinin, 0.1 mM ZnCl2, and 0.1 mM Na3VO4) at 4°C for 20 min. The lysates were clarified by centrifugation at 10,000 × g for 10 min, pretreated with preimmune rabbit sera for 15 min, and then with protein A-Sepharose (Pharmacia, Tokyo, Japan) bound to protein G-Sepharose beads (Pharmacia, Milwaukee, WI) directly or after 3-fold dilution with washing buffer (0.1 M Tris-HCl (pH 8.0), 0.5 M NaCl, 5 mM EDTA, 0.05% NaN3, and 0.1% NP40) at 4°C for 20 h. The beads were collected and washed extensively with washing buffer to remove unbound material. Bound proteins were analyzed by SDS-PAGE and detected by immunoblotting with anti-GRB-2 antibody, anti-phosphotyrosine antibody (PY20; Zymed, San Francisco, CA), or anti-ABL antibody with the use of an ECL Western blotting detection system (Amersham, Tokyo, Japan).

RESULTS

Cell Surface Market Analysis. Cell line AR230 was positive for CD13, CD33, and CD35 and weakly positive for CD11b but negative for CD34. Although the cell line was positive for CD4, other lymphoid-associated antigens, such as CD2, CD3, CD8, CD10, CD19, and CD20, were negative (Table 1). These findings indicate that AR230 has a myeloid phenotype.

Cytogenetic Analysis. Five metaphase chromosome of AR230 revealed 45, XX, inv(1)(p31-32p36) or add(1)(p31-32), –2, del(9)(q22) or der(9)(q;9)(q11-13;?), der(9)(q;9)(q11-13;p13-13;?), der(9)(q;9)(p13;9)(q22) or der(9)(q;9)(q11-13;?) or der(9)(q;9)(q11-13;?) or der(9)(q;9)(p13;9)(q22) or der(9)(q;9)(q11-13;?).

Molecular Analysis of the Ph Chromosome. Genomic Southern analysis of EcoRI-, BamHI-, BglII-, and HindIII-digested DNA blots with the use of 5'-bcr, 3'-bcr, and large bcr probes revealed no rearrangement of the M-BCR region in leukemic cells or in AR230 (Fig. 1). BCR exon 2 sense and ABL exon 3 antisense oligonucleotides were used as primers for the reverse transcription-PCR assay of BCR/ABL in snap-frozen leukemic cells at the time of diagnosis and AR230. Two fragments that were about 500 and 600 bases longer than p210-type BCR/ABL chimeric transcripts were detected in both of these samples. These fragments hybridized with the ABL exon 3 sense oligonucleotide probe, but not with the BCR exon 2/ABL exon 2 or the BCR exon 3/ABL exon 2 junction probe. To clarify the possibility that the two fragments included the 3'-BCR region, cDNA was amplified with BCR exon 6 sense and 5'-biotinylated ABL exon 2 antisense primers. This reaction gave rise to two amplified fragments, which corresponded to the detection with the use of BCR exon 2 and ABL exon 3 primers (Fig. 2). These two fragments were separated by agarose gel electrophoresis and purified for the analysis of nucleotide sequences. The longer fragment (fragment 1) was 540 bases longer than that of the BCR exon 3/ABL exon 2 junction and had an in-frame 3′-bcr, 3′-bcr, and large bcr probes revealed no rearrangement of the M-BCR region in leukemic cells or in AR230 (Fig. 1). BCR exon 2 sense and ABL exon 3 antisense oligonucleotides were used as primers for the reverse transcription-PCR assay of BCR/ABL in snap-frozen leukemic cells at the time of diagnosis and AR230. Two fragments that were about 500 and 600 bases longer than p210-type BCR/ABL chimeric transcripts were detected in both of these samples. These fragments hybridized with the ABL exon 3 sense oligonucleotide probe, but not with the BCR exon 2/ABL exon 2 or the BCR exon 3/ABL exon 2 junction probe. To clarify the possibility that the two fragments included the 3′-BCR region, cDNA was amplified with BCR exon 6 sense and 5′-biotinylated ABL exon 2 antisense primers. This reaction gave rise to two amplified fragments, which corresponded to the detection with the use of BCR exon 2 and ABL exon 3 primers (Fig. 2). These two fragments were separated by agarose gel electrophoresis and purified for the analysis of nucleotide sequences. The longer fragment (fragment 1) was 540 bases longer than that of the BCR exon 3/ABL exon 2 junction and had an in-frame junction between BCR exon c3 and ABL exon 2. Fragment 2 was 140 bases shorter than fragment 1 and had a junction between ABL exon 2 and BCR exon c2 (11). The nucleotide sequence of fragment 2, however, was associated with a frameshift at the junction and conse-

Table 1 Analysis of cell surface markers of AR230

<table>
<thead>
<tr>
<th>Antigen</th>
<th>% of positive cells</th>
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<td>CD2</td>
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<td>CD3</td>
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<td>CD6</td>
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* a*, <10% of cells were stained.

Table 1 Analysis of cell surface markers of AR230

- - , <10% of cells were stained.
proteins (Fig. 6). These results demonstrate that the GRB-2 protein forms a complex with p230 BCR/ABL tyrosine kinase in vivo as with p210 BCR/ABL and excludes the possibility that GRB-2 may be detectable due to association with normal c-ABL protein.

**DISCUSSION**

We encountered a CML patient with two variant types of BCR/ABL transcript. The novel myeloid cell line AR230 was established by repeated limiting dilution cloning of the patient’s MNCs. PCR analysis enabled identification of two fragments with higher molecular mass than was expected in the sample at the diagnosis and cell line AR230. One of the transcripts (fragment 1) was identified as the transcript of a BCR exon c3/ABL exon 2 junction. This fragment was 540 bases longer than that seen in the BCR exon 3/ABL exon 2 junction, which codes for a 210-kDa ABL protein. The 230-kDa hybrid BCR/ABL protein was shown to have kinase activity by an in vitro kinase assay (Fig. 4).

The other fragment (fragment 2) was shown to be the BCRc2/ABL chimeric transcript. This fragment was associated with a stop codon after a frameshift at the junction, which results in the loss of the COOH-terminus end of ABL protein, including SH3, SH2, and SH1. Accordingly, the BCRc2/ABL fragment showed no in vitro kinase activity.

**Fig. 1. Southern blot analysis of the BCR gene of AR230 and the clinical sample at diagnosis. Genomic DNA was digested with the indicated restriction enzymes and hybridized to the large ccr probe. Dashes, germline bands; P, patient sample; N, normal control.**

**Fig. 2. Agarose gel electrophoresis of the RT-PCR products of BCR/ABL chimeric mRNA. RT-PCR products were obtained with the use of BCR exon 6 sense primer and ABL exon 2 antisense primer and were hybridized with oligonucleotide probe corresponding to BCR exon c3 sequence (A) or BCR exon c2 sequence (B). Lanes 1 and 2, neoplastic cells at diagnosis; Lanes 3 and 4, AR230; Lane M, size marker (HindIII-digested PM2 DNA).**
The central portion of BCR appears to be similar to the dbl and with anti-ABL antibody due to the association of the stop signal at the head). Since the BCRc2/ABL fusion protein cannot be precipitated by the body and stained by anti-phosphotyrosine antibody (Fig. 5B arrowhead). Since the BCRc2/ABL fusion protein cannot be precipitated with anti-ABL antibody and stained by anti-phosphotyrosine antibody (Fig. 5B arrowhead). Since the BCRc2/ABL fusion protein cannot be precipitated with anti-ABL antibody due to the association of the stop signal at the eighth amino-acid residue from the junction, it is very unlikely that this smaller protein is the BCRc2/ABL protein. Rather, it is very likely that this band corresponds to the breakdown product of p230 BCR/ABL protein. This notion was supported by the result of the in vitro kinase assay, in which only p230 protein had kinase activity (Fig. 4).

It has been suggested that the BCR protein has three major functional domains based on its amino acid sequences. The NH₂-terminal portion reportedly includes serine/threonine kinase activity and interacts with the ABL protein mediated by SH2-binding domains (18). The central portion of BCR appears to be similar to the dbl and CDC24 genes and to act as a GDP/GTP exchanger (19). The COOH-terminal portion reportedly functions as a GAP for p21rac protein (20). Thus, the normal BCR protein has been demonstrated to have multiple functions in intracellular signal transduction pathways. In the commonly seen 210-kDa protein of BCR/ABL, the GAP domain of the BCR protein for p21rac is lacking (20). Although the 230-kDa BCR/ABL protein apparently includes a 3' region of BCR, it also has lost the GAP domain.

p210 and p190 BCR/ABL have been demonstrated to form stable complexes with GRB-2 in vivo (13, 14), indicating that the Ras signaling pathway plays an important role in the oncogenic activity of Ph translocation (15-17). Immunoprecipitation with anti-ABL or anti-GRB-2 antibody followed by Western blotting with anti-GRB-2 or anti-ABL antibody, respectively, showed in vivo stable complex formation between GRB-2 and p230BCR/ABL protein but not with normal c-ABL protein (Figs. 5A and 6). These results suggest that the Ras signaling pathway is involved in this type of variant Ph, as in typical Ph. Two fragments, p230 BCR/ABL protein and an unidentified smaller protein, were immunoprecipitated with anti-ABL antibody and stained by anti-phosphotyrosine antibody (Fig. 5B arrowhead). Since the molecular mass of the other hybrid protein has been shown to be 203 kDa, and it consists of BCR/ABL lacking ABL exon 2 (21), the last 17 amino acids of which are part of a stretch of 50 amino acids that form the SH3 region of the ABL protein. The SH3 domain is believed to have a negative regulatory effect on the kinase domain (SH1). The breakpoints of the ABL gene in this type of rearrangement are variable; some are located in the intron between exons 2 and 3 of the ABL gene (22, 23), and others are in the 3’ region of ABL exon 2, suggesting that ABL exon 2 has been skipped in the transcription of this BCR/ABL fusion gene (24). p203 BCR/ABL also forms a stable complex with GRB-2 in vivo. Thus, the Ph translocation has been revealed to give a 210-, 190-, 230- or 203-kDa BCR/ABL protein, depending on the chromosomal breakpoint and the type of gene expression. All of these fusion proteins bear a similar activity with regard to complex formation with the GRB-2 adapter protein, which participates in Ras activation. Additional study will be needed to reveal whether the other pathway Myc also operates in this variant BCR/ABL protein, which would support the notion that Ras and Myc operate via different pathways in BCR/ABL activation (25) overall.

To date, two types of variant BCR/ABL protein with autophosphorylation activity have been reported, including the p230 BCR/ABL described here. The molecular mass of the other hybrid protein has been shown to be 203 kDa, and it consists of BCR/ABL lacking ABL exon 2 (21), the last 17 amino acids of which are part of a stretch of 50 amino acids that form the SH3 region of the ABL protein. The SH3 region is believed to have a negative regulatory effect on the kinase domain (SH1). The breakpoints of the ABL gene in this type of rearrangement are variable; some are located in the intron between exons 2 and 3 of the ABL gene (22, 23), and others are in the 3’ region of ABL exon 2, suggesting that ABL exon 2 has been skipped in the transcription of this BCR/ABL fusion gene (24). p203 BCR/ABL also forms a stable complex with GRB-2 in vivo. Thus, the Ph translocation has been revealed to give a 210-, 190-, 230- or 203-kDa BCR/ABL protein, depending on the chromosomal breakpoint and the type of gene expression. All of these fusion proteins bear a similar activity with regard to complex formation with the GRB-2 adapter protein, which participates in Ras activation. Additional study will be needed to reveal whether the other pathway Myc also operates in this variant BCR/ABL protein, which would support the notion that Ras and Myc operate via different pathways in BCR/ABL activation (25) overall.

To our knowledge, only two cases with the BCRc3/ABL chimeric transcript have been reported. Although they were in the chronic phase of typical CML, no additional details were given (5). Our patient, however, showed atypical clinical features throughout the entire course. WBC at the initial presentation was not elevated, no BM fibrosis was seen, and one dose of 100 mg of Ranimustine given in the beginning of the disease induced subsequent hypoplasia of the BM, until the disease had further progressed 2 years later. Although the molecular mechanism that is responsible for the clinical features in this case is not clear, one of the characteristics of our patient was the presence of BCRc2/ABL protein with no tyrosine kinase activity. It would be interesting to learn whether a protein such as BCRc2/ABL with no tyrosine kinase activity might modulate the biological features of leukemic cells by functioning as a competitor of p230 BCRc3/ABL tyrosine kinase.

ACKNOWLEDGMENTS

We thank Dr. J. Groffen for the generous gift of the large bcr probe.


Fig. 5. A, Western blotting analysis for GRB-2 protein before (Lanes 4–6) or after (Lanes 1–3) immunoprecipitation with anti-ABL antibody. Cell lysates from U937 (Lane 1), K562 (Lane 2), and AR230 (Lane 3) were immunoprecipitated with anti-ABL antibody and Western blotted with anti-GRB-2 antibody according to the method described in the text. Cell lysates without immunoprecipitation are shown on Lane 4 (U937), Lane 5 (K562), and Lane 6 (AR230). B, phosphotyrosine staining of the immunoprecipitates with anti-ABL antibody. Immunoprecipitates of p140 normal c-ABL (Lane 1, U937), p210 BCR/ABL (Lane 2, K562), and p230 BCR/ABL (Lane 3, AR230) proteins were analyzed for phosphotyrosine using anti-phosphotyrosine antibody. Open arrowhead, breakdown product of p230 BCR/ABL protein.

Fig. 6. Western blotting for ABL protein before (A) or after (B) immunoprecipitation with anti-GRB-2 antibody. Immunoprecipitation was according to the method described in the text, using anti-GRB-2 antibody. The immunoprecipitates were blotted with anti-ABL antibody. p140-kDa normal c-ABL protein could be seen only after a longer exposure (open arrowhead). Lane 1, U937; Lane 2, K562; Lane 3, AR230.
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