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Differential Alterations of Dihydrofolate Reductase Gene in Human Leukemia Cell Lines Made Resistant to Various Folate Analogues

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In order to clarify a molecular mechanism of folate resistance in leukemia cells, we studied alterations of the dihydrofolate reductase (DHFR) gene in a human leukemia cell line, MOLT-3, and its sublines made resistant to methotrexate (MTX), trimetrexate (TMQ) and N^{10}-propargyl-5,8-dideazafolic acid (CB3717), alone or in combination. Major alterations of the DHFR gene were examined by Southern analysis of high-molecular-weight DNA. The presence of a base change (T→C) at nucleotide position 91 of the DHFR gene, which is reported to be responsible for the reduced affinity of the enzyme for MTX in an MTX-resistant human colon carcinoma cell, was examined by allele-specific oligonucleotide hybridization. In a 10,000-fold MTX-resistant subline (MOLT-3/$MTX_{10,000}$), the normal allele of DHFR gene had been amplified. In contrast, a 200-fold TMQ-resistant subline (MOLT-3/TMQ_{200}) and a 30-fold CB3717-resistant subline selected from MOLT-3/TMQ_{200} (MOLT-3/TMQ_{200}-$CB3717_{30}$) were shown to have the mutant allele. Furthermore, the mutant allele had been amplified in a 500-fold MTX-resistant subline, which was established by the continuous exposure of the MOLT-3/TMQ_{200} cells to stepwise increases of drug concentration and designated as MOLT-3/TMQ_{200}-MTX_{500}. On the other hand, a 40-fold-resistant subline to CB3717 alone (MOLT-3/$CB3717_{40}$) showed the normal allele without amplification. These data suggest that complex alterations of the DHFR gene are involved in the molecular mechanisms of folate resistance that can be differentially introduced into leukemia cells by exposure to various folate analogues, alone or in combination.

Key words: Folate(s)-resistance — Dihydrofolate reductase gene — Point mutation — Leukemia cell

Methotrexate (MTX[6]) is an inhibitor of dihydrofolate reductase (DHFR), which has been widely used in the treatment of human malignant tumors. However, the development of resistance to the drug is a major clinical problem.[1)] Recently a number of folate analogues have been developed in an attempt to overcome it, and pre-clinical and clinical studies of these new drugs are in progress. Trimetrexate (TMQ) is a quinazoline analogue which exerts antitumor activity by inhibiting DHFR.[2)] Unlike MTX, this lipophilic antifolate enters the cells via a route distinct from the classical reduced folate transport system.[3, 4)] N^{10}-Propargyl-5,8-dideazafolic acid (CB3717), another new quinazoline analogue, is structurally characterized by the propargyl group at the N^{10} position of 2-amino-4-hydroxypteroylglutamate, which has been demonstrated to be an important site for thymidylate synthase (TS) inhibition.[5)] Although CB3717 has mild inhibitory action on DHFR, its major mechanism of antitumor activity is TS inhibition.[6)] Because these folate analogues differ in target enzymes or transport systems, a combined or sequential treatment regimen with these non-classical antifolates may have therapeutic potential against cells which have developed resistance to MTX. We have shown a complex resistance pattern in a leukemia subline which had sequentially developed CB3717 resistance in TMQ-resistant cells (MOLT-3/TMQ_{200}-$CB3717_{30}$), suggesting a combination of drug resistance mechanisms in a single cell line.[7)] This intricacy in folate resistances in the TMQ-CB3717 double-resistant cells appeared to result from alterations at the gene level.

Although amplification of the DHFR gene is a well-known mechanism of MTX resistance in tumor cells,[1)] alterations of the DHFR gene in leukemia cells that have developed resistance to these newly developed folate an-

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[6] The abbreviations used are: MTX, methotrexate; DHFR, dihydrofolate reductase; TMQ, trimetrexate; CB3717, N^{10}-propargyl-5,8-dideazafolic acid; TS, thymidylate synthase; IC_{50}, 50%-inhibitory concentration of a drug for cell growth; PCR, polymerase chain reaction.

alogues have not been clarified. In this study, we established another cell line that was made doubly resistant to TMQ and MTX, and we investigated alterations of the DHFR gene in the cells after they had developed resistance to these folate analogue(s), alone or in combination.

For the establishment of a TMQ-MTX double-resistant subline, the MOLT-3/TMQ_{200} cells[8] were continuously exposed to increasing concentrations of MTX up to 2.0 μM for 4 months, resulting in a subline. The IC_{50} values (50%-inhibitory concentration of a drug for cell growth) were determined by MTT assay as previously described.[9] As the subline showed approximately 500-fold MTX resistance, as compared with the parent cells, MOLT-3,[10] the new subline was designated as MOLT-3/TMQ_{200}-MTX_{500}. (The IC_{50} values of MTX for MOLT-3, MOLT-3/TMQ_{200} and MOLT-3/TMQ_{200}-MTX_{500} were 0.004 ± 0.001 μM, 0.06 ± 0.01 μM and 2.0 ± 0.2 μM, respectively.) DHFR gene alterations were compared in two other cell lines: MOLT-3/$MTX_{10,000}$, a 10,000-fold MTX-resistant subline[11] and MOLT-3/$CB3717_{40}$, a 40-fold CB3717-resistant subline.[12]

Alterations of DHFR gene were analyzed in these cells by Southern hybridization as previously described.[13] The cDNA for the human DHFR gene (0.7 kb) (*Pst*I digestion) was kindly provided by Dr. G. Attardi, California Institute of Technology.[14] For Southern hybridization, DNA was digested with restriction endonuclease *Eco*RI, *Hin*dIII or *Bam*HI. Figure 1 shows the Southern blot profile of *Eco*RI-digested DNAs from MOLT-3 sublines hybridized with ^{32}P-labeled human DHFR cDNA. In parental MOLT-3 cells, an *Eco*RI digest results in bands of 23, 14, 5.8, 4, 1.8, and 1.7 kbp in size (lanes 1 and 3). The DHFR gene in MOLT-3/$MTX_{10,000}$ cells exhibited amplification (lane 2). The restriction pattern of the DHFR gene in MOLT-3/$MTX_{10,000}$ (lane 2) and MOLT-3/$CB3717_{40}$ (lane 5) was the same as that in the parent MOLT-3 cells. On the other hand, the DHFR gene in MOLT-3/TMQ_{200} cells (lane 4) and the two MOLT-3 sublines derived from the TMQ-resistant cells (lanes 6 and 7) exhibited an *Eco*RI restriction fragment length polymorphism. An extra 7.6 kbp fragment was demonstrated in MOLT-3/TMQ_{200} (lane 4) and MOLT-3/TMQ_{200}-$CB3717_{30}$ cells (lane 6), indicating that these cells have an altered DHFR allele lacking an *Eco*RI site. Since the 5.8 and 1.8 kbp fragments are joined at an *Eco*RI site located in the first exon of the gene, it is suggested that the 7.6 kbp fragment in MOLT-3 sublines arose through the loss of this site.[15] Furthermore, sequential development of the MTX resistance in MOLT-3/TMQ_{200} cells resulted in an amplification of the extra 7.6 kbp band without concomitant amplification of the 5.8 and 1.8 kbp fragments. This observation strongly suggests that the mutant allele of the gene was amplified in MOLT-3/TMQ_{200}-MTX_{500} cells.

It is likely that this amplified, altered gene may give rise to a variant enzyme which interacts differently with MTX, providing a growth advantage to the cells at a high

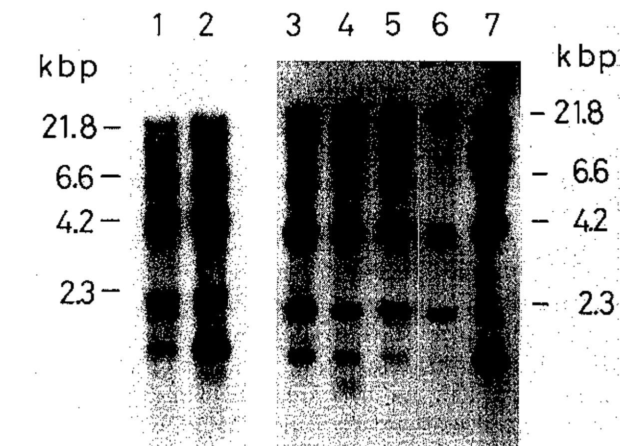

Fig. 1. Southern blot analysis of DHFR gene in MOLT-3 sublines. DNA (6 μg) from MOLT-3 sublines was digested with *Eco*RI and analyzed by Southern blotting for DHFR gene. Samples in lanes: 1, MOLT-3; 2, MOLT-3/$MTX_{10,000}$; 3, MOLT-3; 4, MOLT-3/TMQ_{200}; 5, MOLT-3/$CB3717_{40}$; 6, MOLT-3/TMQ_{200}-$CB3717_{30}$; 7, MOLT-3/TMQ_{200}-MTX_{500}.

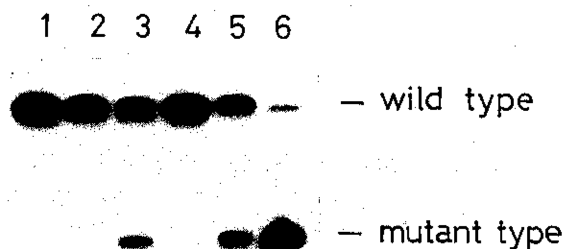

Fig. 2. Hybridization of *in vitro* amplified DNA from MOLT-3 sublines with allele-specific oligonucleotide probes for DHFR gene. Primers used to amplify DHFR gene were #1 5′-TTGT CAG GTG CCT TAG AGC TCG A (residues 777–799: sense strand) and #2 5′-CC ATT TAA CTA ACA TTT GCA CTG G (residues 936–959: antisense strand). Oligonucleotides used for detection of normal and mutant allele were #3 5′-TCT CTG GAA ATA TCT GAA TTC ATT C (residues 846–870) and #4 5′-TCT CTG GAA ATA TCT GGA TTC ATT C (residues 846–870), respectively. Each PCR sample (10 μl) was resolved on 1.8% agarose gel in tris-borate buffer and transferred onto Zeta-Probe Nylon membranes (Bio-Rad, Richmond, CA) overnight in 0.4 N NaOH. Identical filters were hybridized either with the wild-type- or the mutant-type-specific ^{32}P-labeled oligonucleotides probes. Samples in lanes: 1, MOLT-3; 2, MOLT-3/$MTX_{10,000}$; 3, MOLT-3/TMQ_{200}; 4, MOLT-3/$CB3717_{40}$; 5, MOLT-3/TMQ_{200}-$CB3717_{30}$; 6, MOLT-3/TMQ_{200}-MTX_{500}.

concentration of MTX. Recently, induction of a variant DHFR with decreased affinity for MTX was shown to occur in an MTX-resistant human colon carcinoma cell line.[15] A base change (T→C) at nucleotide position 91 of the DHFR gene produces a substitution of serine (TCC) for phenylalanine (TTC) at residue 31 of the enzyme, which results in a decrease in MTX affinity.[15,16] The base change produces an *Eco*RI restriction fragment length polymorphism of the DHFR gene, which is the same as that in the TMQ-resistant MOLT-3 sublines. Accordingly, we examined the presence of this point mutation of the DHFR gene by polymerase chain reaction and oligonucleotide hybridization techniques, as previously described with minor modifications.[17] Hybridizing the PCR product with an internal probe for the mutant allele revealed the presence of the point mutation in MOLT-3/TMQ_{200} cells (lane 3) and its two sublines (lanes 5 and 6) (Fig. 2). An increased signal for the mutant allele with a remarkable decrease for the normal one in MOLT-3/TMQ_{200}-MTX_{500} cells (lane 6) confirmed that the mutant allele had been amplified. Furthermore, the cells exhibited a proportional increase in DHFR transcripts (unpublished data). This observation indicates that the mutant DHFR gene was amplified once the TMQ-resistant cells gained an additional resistance to MTX.

The observation that the normal allele had been amplified in MOLT-3/$MTX_{10,000}$ cells is confirmatory of a previous finding of the homogenously staining region seen in the chromosomal analysis of this MTX-resistant MOLT-3 subline.[17] In MTX-resistant cells, the DHFR gene amplification, as well as decreased membrane transport for and reduced retention of the drugs in cells due to reduced polyglutamate formation, appears to provide a growth advantage to the cells at a high concentration of MTX.[1,11] In contrast, TMQ uses a transport route distinct from the classical reduced folate transport system and does not undergo polyglutamation.[2-4] This metabolic difference between the drugs could be relevant to the different alterations of DHFR gene seen in cells made resistant to MTX or TMQ.

In conclusion, the point mutation at nucleotide position 91 of DHFR gene, which has been reported in an MTX-resistant human colon carcinoma cell, was induced by exposure to TMQ, and this mutation was amplified by exposure to MTX in a human leukemia cell MOLT-3, while the MTX-resistant subline showed an amplification of the normal allele of the gene. The development of such complex DHFR gene alterations in cells made resistant to various folate analogues indicates the intricacy of the problem of drug resistance following multidrug chemotherapy. Since patients with malignancies have generally been treated with a series of chemotherapeutic agents, the molecular mechanisms of drug resistance seen in these patients as end results of such drug exposures could be extremely complex. Further studies will be required to clarify the nature of the altered gene product in the TMQ-resistant cells and the mechanisms that induce such alterations of the gene.

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