Resistance Mechanisms to Methotrexate in Tumors

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Abstract

The mechanisms of intrinsic and acquired resistance to methotrexate (MTX) in human tumors are reviewed herein. In blasts from patients with acute lymphocytic leukemia, resistance mechanisms found are decreased uptake and increased dihydrofolate reductase (DHFR) activity. A major cause of intrinsic resistance to MTX in soft tissue sarcoma cells and in acute myelocytic leukemia appears to be a lack of drug retention, due mainly to low levels of polyglutamylation. A novel association between lack of the retinoblastoma protein and intrinsic MTX resistance has been found. This has been attributed to an increase in DHFR activity, due to an increased rate of transcription of this gene, stimulated by an increase in levels of free E2F, not sequestered by hypophosphorylated retinoblastoma protein.

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Introduction

In looking back on this field since Dr. Bruce Chabner was a postdoctoral fellow at Yale some 25 years ago, it’s been a fascinating series of developments, many of them not imagined or anticipated. Even in 1995, we are learning more about drug resistance, and particularly in tumors from patients, rather than from experimental tumors. Dr. Chabner and his colleagues have been major contributors to these advances, and in his new role as Clinical Director of the Cancer Center at the Massachusetts General Hospital, we look forward to even more innovative ideas for the treatment of patients with cancer.

In this short review, some of our current studies are described. Many talented investigators have contributed to these studies, including Dr. Bruce Chabner.

Mechanism of Action

Following the introduction of aminopterin for the treatment of childhood acute lymphocytic leukemia in 1948 [1], a less toxic analog, methotrexate (MTX) subsequently replaced aminopterin in the clinic in 1956 [2]. In the almost 50 years since the first patient was treated with aminopterin, the first antitumor agent found to be effective in treating leukemia, we have learned a great deal about the mechanism of action of this drug and mechanisms of resistance. This information has guided new drug development as well as resulted in more effective treatment schedules.

MTX is a potent inhibitor of dihydrofolate reductase (DHFR), a key enzyme for intracellular folate metabolism, and functions to regenerate tetrahydrofolate from dihydrofolate, a product of thymidylate synthase (Fig. 1). As a consequence of DHFR inhibition, intracellular levels of tetrahydrofolate coenzymes are decreased, resulting in inhibition of thymidylate and consequently DNA biosynthesis, as well as purine biosynthesis [3].

Drug resistance may occur rapidly in potentially curable tumors, and if cure is not achieved (e.g., 20%-30% of acute lymphoblastic leukemia [ALL] patients), leads to treatment failure. Resistance mechanisms to MTX have been extensively studied, mainly in experimental tumors propagated in vitro and in vivo. Five different resistance mechanisms have been described in cells that survive MTX treatment: 1) decreased accumulation due to impaired transport; 2) decreased retention as a consequence of lack of polyglutamate formation; 3) an increase in DHFR; 4) and altered (mutated) DHFR that binds MTX less avidly than the normal enzyme, and, most recently, 5) an increased level of a lysosomal enzyme, γ-glutamyl hydrolase, that hydrolyses MTX polyglutamates [4] (Fig. 1).

Decreased Transport

Transport resistance is a common mechanism of acquired resistance to MTX in experimental tumors both in vitro and in vivo [5-7]. Recently, the putative carrier for active MTX transport (the reduced folate transport carrier) has been isolated and the cDNA cloned [8, 9]. The study of MTX transport in tumor samples from patients has limitations, such as limited sampling, lack of internal controls and heterogeneity of tumors. An additional limiting factor to resistance assessment is the...
requirement of pre- and post-treatment samples for comparative analysis. A competitive displacement assay utilizing the fluorescent lysine analog of MTX (PT 430) was developed as a sensitive method of detection of transport resistance to MTX in cell lines as well as in blast cells from patients with leukemia [10]. After achievement of a steady-state level of intracellular PT 430 and subsequent incubation with the folate antagonists, MTX and trimetrexate (TMTX), which differ in the mode of carrier transport, produced characteristic displacement patterns of PT 430 [10]. We evaluated this assay for use in fresh blasts from patients with leukemia. Analysis of samples from 35 patients with untreated leukemia shows that 60% (60 ± 12) displacement of PT 430 occurs with MTX as well as with TMTX. When we analyzed blast samples from 32 relapsed patients who were considered to have acquired resistance to MTX, we found over half of the patient samples had less than 30% of displacement of PT 430 with MTX. Thus, decreased transport is a common resistance mechanism to MTX in relapsed ALL. This finding has important implications for clinical use of this drug and new drug development. The availability of cDNA clones for the reduced folate carrier should allow an elucidation of the molecular basis for transport defects. In acute non-lymphoblastic leukemia (ANLL), a disease considered to be naturally resistant to MTX with low clinical response. To explore the reasons for this natural resistance, we examined these tumors for mechanisms known to produce MTX resistance in vitro and compared these results to those obtained studying blasts from a sensitive neoplasm, childhood ALL. Patient tumor samples were evaluated for formation of MTX polyglutamates after a 24-h incubation with 10 µM [3H]-MTX and HPLC analysis. Although the total MTX (MTX plus MTX polyglutamate content) was almost the same in ANLL and ALL, we found lower levels of long chain polyglutamates in sarcoma cells and ANLL blast cells as compared to ALL blasts [11, 12]. The amounts of long chain polyglutamates formed by ANLL blasts were quite variable. Of interest, monoblastic ANLL (M5) cells formed as much MTX polyglutamates as the childhood ALL blasts [13]. We also evaluated adult ALL blasts for in vitro MTX polyglutamate accumulation. Adult pre-B ALL and T cell ALL blasts were found to accumulate fewer MTX polyglutamates as compared to childhood ALL blasts [14]. A lower content of MTX polyglutamates could be due to decreased synthesis by FPGS or an increased catabolism of MTX polyglutamates by γ-glutamyl hydrolase. We therefore are currently assessing activities of both of these enzymes in various leukemia blasts. A wide range of FPGS

**IMPAIRED POLYGLUTAMYLLATION**

Polyglutamylation of MTX is a metabolic process which has great pharmacologic importance. Long chain polyglutamates of MTX (n = 2 – 5) have an equal affinity for the target enzyme, DHFR, as does MTX itself and exit the cell much more slowly than MTX monoglutamates. The intracellular content of polyglutamate derivatives of MTX is controlled by a balance between folypolyglutamate synthetase (FPGS) and γ-glutamyl hydrolase activities [4].

Patients with ANLL and patients with sarcoma are considered to be naturally resistant to MTX with low clinical response. To explore the reasons for this natural resistance, we examined these tumors for mechanisms known to produce MTX resistance in vitro and compared these results to those obtained studying blasts from a sensitive neoplasm, childhood ALL. Patient tumor samples were evaluated for formation of MTX polyglutamates after a 24-h incubation with 10 µM [3H]-MTX and HPLC analysis. Although the total MTX (MTX plus MTX polyglutamate content) was almost the same in ANLL and ALL, we found lower levels of long chain polyglutamates in sarcoma cells and ANLL blast cells as compared to ALL blasts [11, 12]. The amounts of long chain polyglutamates formed by ANLL blasts were quite variable. Of interest, monoblastic ANLL (M5) cells formed as much MTX polyglutamates as the childhood ALL blasts [13]. We also evaluated adult ALL blasts for in vitro MTX polyglutamate accumulation. Adult pre-B ALL and T cell ALL blasts were found to accumulate fewer MTX polyglutamates as compared to childhood ALL blasts [14]. A lower content of MTX polyglutamates could be due to decreased synthesis by FPGS or an increased catabolism of MTX polyglutamates by γ-glutamyl hydrolase. We therefore are currently assessing activities of both of these enzymes in various leukemia blasts. A wide range of FPGS

**Figure 1. Mechanism of action and resistance mechanisms to MTX.** MTX enters cells by the reduced folate carrier (1), is polyglutamylated (2), and MTX or its polyglutamylated forms are potent inhibitors of dihydrofolate reductase (3). MTX polyglutamates are broken down to the monoglutamate form in lysosomes (not shown in the figure) by γ-glutamyl hydrolase and subsequently effluxed from the cell.
activity, proportional to FPGS mRNA levels, has been observed in leukemic blasts [15].

**Increased DHFR Enzyme Activity**

MTX is a tight-binding inhibitor of DHFR, and the concentration of MTX required to achieve inhibition of enzyme activity increases in direct proportion to the amount of the enzyme in the target cells. It is now well established that an important mechanism of resistance of cells to MTX is an increase in DHFR activity due to amplification of the DHFR gene [16]. Mouse, hamster and human MTX-resistant cell lines have been described with increased levels of DHFR activity due to gene amplification [16]. Following reports indicating that gene amplification is a common mechanism of resistance in cell lines exposed to gradually increased doses of MTX, four case reports in the literature appeared, one from our laboratory, indicating that low-level gene amplification occurs in tumor cells from patients treated with MTX, consistent with the expectation that a low level of amplification would be sufficient to cause clinical resistance to this drug [5]. In ALL, we assessed the frequency of DHFR gene amplification as an acquired resistance mechanism to MTX. DHFR gene amplification was determined with a DNA dot blot assay and confirmed by Southern and Northern analysis and DHFR enzyme activity. We found that low-level (two- to fourfold) DHFR gene amplification was detected frequently in relapsed ALL blasts (9/29 samples) [17].

**Mutant DHFR and Transcriptional or Translational Control**

Although mutations in the DHFR gene have been described in several MTX-resistant cell lines, in seven MTX-resistant blast samples from leukemia patients, we have not detected any mutant DHFR enzymes using DNA SSCP and sequencing DHFR cDNA (unpublished observations).

DHFR protein itself has a suppressive effect on translation of its own mRNA. When MTX binds to DHFR protein, this suppressive effect may be lost, allowing new enzyme synthesis [18, 19]. Thus far, limited data are available about the importance of these control mechanisms in clinical drug resistance.

**Tumor Suppressor Genes and Drug Resistance**

The effect of the retinoblastoma gene (Rb), a tumor suppressor gene, on DHFR expression is under active investigation in this laboratory. Lack of the retinoblastoma protein (pRb) may lead to MTX resistance as a consequence of an increase in DHFR mRNA expression and enzyme activity without gene amplification [20]. This increase has been linked to an increase in E2F, a transcription factor that is bound by hypophosphorylated retinoblastoma protein. In the absence of pRb, E2F levels increase in the cell, resulting in an increase in transcription of several genes involved in DNA replication, including DHFR [20].

Cell lines with mutated p53, a tumor suppressor gene, have the capacity to undergo gene amplification after antimetabolite exposure [21, 22]. We showed that low-level DHFR gene amplification in blasts from patients with ALL is also associated with p53 gene mutations [17]. Association of low-level DHFR gene amplification with p53 mutations in ALL blasts strengthens the concept that the loss of wild-type p53 function results in the loss of the checkpoint at the G1/S boundary and permits cells to enter S phase without repair of DNA damage caused by MTX.

**Strategies to Treat Tumor Cells Resistant to MTX**

Understanding mechanisms of resistance to MTX in the clinic may allow the development of new treatment modalities and new drugs. For example, the finding that defective transport of MTX is the most common resistance mechanism to MTX in relapsed ALL has led to interest in drugs like TMTX, which do not utilize the reduced folate transporter for uptake. TMTX has broad preclinical antitumor activity, and leukemia cells which are transport resistant to MTX are collateral sensitive to this drug [23]. However, clinical results with TMTX in relapsed ALL have been disappointing, as doses have been limited due to development of mucositis [24, 25].

The combination of TMTX and leucovorin (LV) is very active against pneumocystis carinii infections in AIDS patients without serious side effects [26]. While TMTX is transported by passive diffusion in this parasite, LV cannot rescue this organism because of the absence of the reduced folate carrier which is necessary for LV transport. With this combination, the side effects of TMTX on the host are eliminated, as normal host cells are protected by LV. In vitro cytotoxic studies with CCRF/CEM human lymphoblastic leukemia cells showed that cells resistant to MTX because of defective transport are not protected from the cytotoxic effects of TMTX by LV, but MTX-sensitive cells are protected from TMTX by LV. Recently, we have also shown that TMTX with LV “protection” is a nontoxic and effective treatment for MTX transport-resistant CEM cells propagated in severe combined immunodeficient mice [27]. In view of these in vivo data, we plan to test TMTX with LV in relapsed ALL patients who demonstrate resistance to MTX associated with impaired uptake of this drug.

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REFERENCES


