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Short communication

Hydrolysis of methotrexate-immunoglobulin conjugates by liver homogenates and characterization of catabolites*

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Summary. Methotrexate (MTX) linked to antitumor antibodies inhibits tumor growth better than free MTX, free antibody, or MTX linked to normal rabbit IgG (NRG), in spite of the less effective inhibition of the target enzyme dihydrofolate reductase (DHFR) by conjugated MTX. In addition to the demonstrated higher uptake of MTX linked to antitumor antibodies (compared with the uptake of free MTX or nonspecific IgG conjugates), a contributory factor to the superior tumor inhibitory action of MTX-IgG conjugates may be the prolonged release of active drug from the internalized conjugate. Therefore, we have investigated whether an MTX-IgG conjugate could be hydrolyzed to release free MTX or fully active MTX-containing fragments after incubation with liver homogenates and have characterized the catabolites according to the presence of free MTX and their capacity to inhibit DHFR. Catabolism was optimal at pH 4.6, activated by dithiothreitol, and inhibited by antipain and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, thus implicating lysosomal enzymes. Liver homogenates produced an MTX-containing, low-molecular-weight fraction that was isolated by gel filtration. Further purification of this fraction by DEAE-cellulose chromatography gave two MTX-containing peaks, neither of which migrated as free MTX on thin-layer chromatography or inhibited DHFR more effectively than the parent conjugate. However, the presence of amino acid residues in these catabolites could contribute to their observed prolonged intracellular retention and superior antitumor action.

Introduction

The folate antagonist methotrexate (MTX) is used extensively in the treatment of human cancers including leukemias and lymphomas [6]. However, this drug, like most cancer chemotherapeutic agents, is as toxic to rapidly proliferating normal cells (such as those in the bone marrow and intestinal epithelium) as it is to cancer cells [6]. Kulkarni et al. [7] have rendered MTX selectively toxic to tumor cells by its linkage to a rabbit IgG antibody against a tumor-associated antigen on the surface of mouse EL4 lymphoma cells. This conjugate was more effective in prolonging the survival of EL4-lymphoma-bearing mice than equivalent amounts of free MTX, free antibody, or MTX linked to normal rabbit IgG (NRG), even though free MTX was found to be more effective in inhibiting the target enzyme dihydrofolate reductase (DHFR) than MTX-IgG conjugates in vitro [7, 12]. In an attempt to delineate the mechanisms of action of conjugated MTX, it was found both in vitro and in vivo that tumor cells accumulated more MTX when it was conjugated to antitumor antibodies than when it was free or linked to a nonspecific IgG [10–12]. To further elucidate the mode of antitumor action, MTX-NRG was incubated with liver homogenates to determine whether the conjugate would be hydrolyzed to release free MTX or fully active MTX-containing fragments.

Material and methods

Preparation of MTX-NRG conjugate. MTX was conjugated to NRG by the active ester intermediate method described by Kulkarni et al. [7], using ^{3}H-MTX (specific activity 20 Ci/mmol, New England Nuclear, Boston, Mass, USA) to give a final specific activity of 5 mCi/mmol. Free MTX was first separated from the MTX-NRG conjugate by column chromatography with Bio-gel P-100 (Bio-Rad Laboratories, Richmond, Calif., USA) and then by dialysis overnight against 0.01 *M* sodium phosphate (pH 7.1) containing 0.45 *M* sodium chloride. The average incorporation of MTX was 5–6 mol MTX per mol NRG. Repeat chromatography of this conjugate after it had been stored for 1 week at 4° C and pH 7.1 gave a single peak of radioactivity that eluted at the position of the conjugate in the initial chromatography. This result shows that there was no dissociation of label from MTX or of the labeled MTX from the conjugate.

Preparation of liver homogenates. Freshly removed liver (2.2 g wet weight) from an adult female C57BL/6J mouse (i.e., the strain of origin of the EL4 lymphoma) was homogenized in 10 ml 0.1 *M* sodium acetate buffer (pH 4.6), using a Potter-Elvehjem homogenizer (10 passes at 2,000 rpm). The concentration of protein in the homogenate was 7–8 mg/ml.

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Assay of the hydrolytic activity of liver homogenates on the MTX-NRG conjugate. For determination of the hydrolytic activity of liver homogenates, a typical reaction mixture consisted of 0.5 ml MTX-NRG conjugate (20 μ*M* with respect to the bound drug), 0.5 ml liver homogenate, and 1.2 ml 0.1 *M* acetate buffer (pH 4.6), in a total volume of 2.2 ml.

To determine the effect of various agents on the hydrolytic activity, reaction mixtures contained one of the following: 92 μ*M* antipain, 860 μ*M* *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 92 μ*M* leupeptin, 92 μ*M* pepstatin, 92 μ*M* calcium chloride, 640 μ*M* iodoacetate. The reaction mixtures were incubated at 37° C for 28 h. At the end of the incubation, the mixtures were centrifuged at 10,000 *g* for 20 min and 1 ml supernatant was passed through a Bio-gel P-100 column (1 × 20 cm) and eluted with 0.1 *M* acetate buffer (pH 4.6). Fractions of 1 ml were collected and counted for radioactivity.

DEAE-cellulose chromatography. The low-molecular-weight radioactive fraction obtained from Bio-Gel P-100 chromatography was further purified by adsorption on a column (1.2 × 30 cm) of DEAE-cellulose equilibrated with 5 m*M* sodium phosphate buffer (pH 7.0) and elution with a linear gradient produced with 1 l 5 m*M* sodium phosphate buffer in the mixing chamber and 1 l 0.5 *M* sodium choride in the same buffer in the reservoir. Fractions of 19 ml were collected [1].

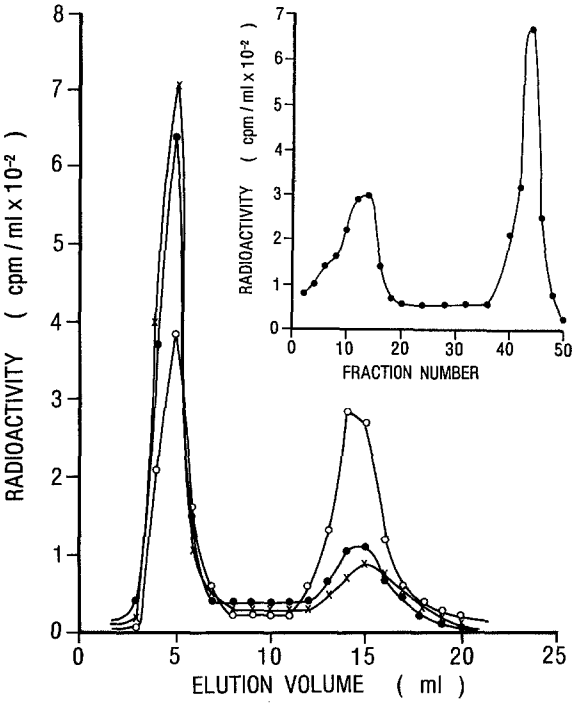

Fig. 1. Breakdown of MTX-NRG conjugate after incubation with liver homogenate alone (○) or in the presence of antipain (■) or *N*-α-*p*-tosyl-L-lysine (×). The reaction conditions and assay for the hydrolytic activity were as detailed in Materials and methods. *Inset.* Further chromatography of the low-molecular-weight fraction from MTX-NRG incubated with liver homogenate. The low-molecular-weight fraction was adsorbed on a column (1.2 × 3 cm) of DEAE-cellulose equilibrated with 5 m*M* sodium phosphate buffer, pH 7.0, and eluted with a linear gradient of 1 l 5 m*M* sodium phosphate buffer in the mixing chamber and 1 l 0.5 *M* NaCl in the same buffer in the reservoir. Fractions of 19 ml were collected. Free MTX elutes at approximately fraction 42

Thin-layer chromatography. Thin-layer chromatographic analysis was carried out on cellulose-coated glass plates with 0.1 *M* glycine containing 2% EDTA (pH 9.0) as the solvent system [9].

DHFR inhibition assay. Beef liver DHFR was obtained from the Sigma Chemical Co., St. Louis, Mo, USA, and diluted appropriately with distilled water just before use. Assay of DHFR activity was based on the direct spectrophotometric method of Peterson [8] and has been described in detail elsewhere [4].

Results

After incubation of the MTX-NRG conjugate with liver homogenates, a low-molecular-weight radioactive peak eluting slightly ahead of the position for free ^{3}H-MTX was observed when the incubation mixture was passed through Biogel P-100 (Fig. 1). The catabolic activity was influenced by the pH of the incubation medium. Using the conditions specified in Table 1, there was 51%, 74%, and 75% breakdown at pH 5.6, 4.6, and 4.0, respectively. Conversion to the low-molecular-weight fraction was also increased by dialyzing the homogenate overnight against buffer containing dithiothreitol (Table 1) but was inhibited by leupeptin, pepstatin, antipain, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, and iodoacetate (Table 1). Calcium chloride and EDTA had no effect on the activity.

DHFR inhibition by the low-molecular-weight fraction was on the same order as that of the original conjugate but half that observed with free MTX. Typical concentrations

Table 1. Effect of several inhibitors and an activator of lysosomal enzymes on the breakdown of MTX-NRG conjugate by liver homogenate

Inhibitors	Concentration (μ*M*)	% of original radioactivity in low mol.wt. peak	% inhibition
Nil (Control)	–	49	–
Leupeptin	92	23	53
Pepstatin	92	34	31
Antipain	92	23	53
N-α-*p*-Tosyl-L-lysine chloromethyl ketone	860	20	59
Calcium chloride	640	49	0
Iodoacetate	640	33	33
EDTA	640	49	0
EDTA + Dithiothreitol	640	62	27*
Dithiothreitol	640	59	20*

Samples of the MTX-IgG conjugate were incubated with mouse liver homogenate at pH 4.6 with the additions as indicated. After incubation, the reaction mixtures were subjected to gel filtration through a Bio-Gel P100 column and the recovery of radioactivity in the low-molecular-weight peak were measured. Details of the protocol are given in Materials and methods. In the case of activation by dithiothreitol, the homogenate was dialyzed against buffer containing the same dithiothreitol concentration overnight prior to the incubation

* The percentage of change represents activation rather than inhibition

giving half-maximal activity were 3 μM for free MTX and 6.3 μM for conjugated MTX. Further purification of this fraction by adsorbing it on a DEAE-cellulose column and eluting with a linear gradient of 0–0.5 *M* sodium chloride in phosphate buffer resulted in the emergence of two peaks, the second of which eluted at approximately the same ionic strength as free MTX and gave a blue quench spot, characteristic of MTX, under UV light. When subjected to thin-layer chromatography, using 0.1 *M* glycine containing 2% EDTA, this DEAE fraction migrated with an R_f of 0.4, whereas MTX itself had an R_f of 0.63 and the conjugate remained at the origin. DHFR inhibition by these peaks was also less than half that of free MTX.

Discussion

The cytotoxic action of MTX correlates with the excess of drug over the stoichiometric DHFR level and the duration of exposure [5]. Following the initial observation of Kulkarni et al. [7] and those of others using monoclonal antibodies [2, 3] that administration of MTX conjugated to antitumor immunoglobulins prolonged the survival of tumor-bearing mice more effectively than equivalent amounts of free MTX, free antibody or MTX linked to nonspecific immunoglobulins, it has been demonstrated that greater uptake by tumor cells of the drug conjugated to a specific antibody IgG (compared to free MTX or MTX conjugated to a nonspecific IgG) contributes to the greater antitumor effect of MTX-antibody IgG conjugates [10–12]. Another contributory factor could be the slow, sustained release from the conjugate of MTX or MTX-containing fragments, resulting in the prolonged maintenance of intracellular, active derivatives at a level higher than that of DHFR, i.e., the depot effect. As MTX-IgG conjugates are less effective in inhibiting DHFR than equimolar amounts of free MTX [7, 10–12], the depot effect could be further enhanced by the release from conjugates of free MTX or MTX-containing fragments that are more potent inhibitors of DHFR than the IgG conjugates of the drug. Using liver homogenates as a source of hydrolytic enzymes, we have now characterized the catabolites of a MTX-NRG conjugate. Catabolism was optimal at pH 4.6, activated by dithiothreitol, and inhibited by well-known lysosomal enzyme inhibitors. The involvement of lysosomal enzymes in the hydrolytic process confirms the results of a previous investigation demonstrating that the lysosomotropic agent chloroquine inhibited the intracellular catabolism of an MTX-anti-M21 antibody conjugate by melanoma M21 cells [12]. The separation of the low-molecular-weight gel filtration peak into two fractions eluting at different ionic strengths from DEAE-cellulose may reflect charge differences arising from differences in amino acids from the IgG that are still attached to MTX. This elution behavior and the R_f value of the second peak of TLC showed that neither ist free MTX. Also, DHFR inhibition was substantially less than that for free MTX.

This study shows that lysosomal enzymes are capable of hydrolyzing MTX-IgG conjugates releasing MTX-containing fragments that are as potent in inhibiting DHFR as the parent conjugate. Thus, in addition to the higher uptake of MTX in the form of an antibody IgG conjugate, the increase in cytotoxic potential of MTX-IgG conjugates in vivo would include slow, sustained catabolism, resulting in the prolonged maintenance of intracellular, active derivatives at a level higher than that of DHFR, as observed with human melanoma M21 cells preloaded with free MTX or MTX conjugated to antibody IgG [12]. The catabolic fractions contained neither free MTX nor a fragment that was a more potent inhibitor of DHFR than the parent conjugate, but the presence of amino acid substituents in these catabolites might delay their efflux and thus prolong intracellular retention.

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