Communication

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Disulfide Spacer between Methotrexate and Poly(D-lysine)

A PROBE FOR EXPLORING THE REDUCTIVE PROCESS IN ENDOCYTOSIS*

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Poly(D-lysine) is taken up avidly by cultured cells through adsorptive endocytosis and can serve as a carrier to increase cellular uptake of other molecules. While direct conjugation of methotrexate to poly(D-lysine) yields a conjugate devoid of cytotoxic effects because poly(D-lysine) is not digested in lysosomes, the indirect conjugation using a triglycine spacer or a disulfide spacer strongly inhibits the growth of both the wild type and the methotrexate transport-defective Chinese hamster ovary cells. Cell treatment with 3 mM NH_4Cl or 50 μg/ml leupeptin prevents the effect of conjugate with the triglycine spacer, but not of conjugate with the disulfide spacer. On the other hand, preincubation with 2-mercaptoethanol abolishes the effect of the drug-disulfide conjugate in the methotrexate transport-defective mutant, but not the effect of the drug-triglycine conjugate. The disulfide conjugate shows an identical cytotoxic effect in α-minimal essential medium and RPMI 1640 media, even though cells grown in the latter have only half the glutathione content as cells grown in the former medium. We conclude that the reductive process through which methotrexate is released from the disulfide spacer (*a*) occurs inside cells and not at the cell surface, (*b*) requires neither acid pH nor lysosomal enzymes, and (*c*) is not mediated by a glutathione-disulfide exchange reaction requiring high glutathione concentrations. Although the cellular compartment in which this reductive process occurs is not yet identified, there are reasons to assume that it is prelysosomal.

The intracellular processing of endocytosed macromolecules involves several biochemical events in the course of a multistep transport sequence. For example, proteins can be partially degraded in prelysosomal vesicles (1), can undergo conformational changes (2) and dissociate from their receptors (3) in acidic endosomes (4), and can be totally hydrolyzed to amino acids in lysosomes (5). Another general reaction in the degradation of proteins is the reductive cleavage of the disulfide bonds. In cases such as insulin degradation (6), this reduction has been considered to be the first step in the sequential degradation of the proteins. Cells which are unable to reduce the disulfide bond will accumulate cystine inside lysosomes (7). This accumulation appears to be the major cause of the lysosomal storage disease known as cystinosis (8). In spite of its importance, the exact nature of this reducing reaction as well as its intracellular location are still largely unknown.

One of the difficulties in studying the cleavage of intramolecular disulfide bonds of endocytosed proteins is that it does not alter significantly the physicochemical properties of the macromolecules. Consequently, conventional methods that rely on the release of labeled amino acids, or on changes in either molecular weight or isoelectric point, are unable to detect a difference in the redox state of the cysteinyl residues. Furthermore, reduction or oxidation of isolated proteins can occur as an artifact during cell homogenization.

In this communication, we use a conjugate in which methotrexate (MTX[1]) is linked to poly(D-lysine) (poly(D-Lys)) through a disulfide spacer as a probe to study the reducing reaction in cultured CHO cells. Previously we have shown that direct linkage of MTX to poly(D-Lys) gives a conjugate that is totally devoid of cytotoxic effect, even though it is taken up equally well as poly(L-lysine) by cultured cells through endocytosis (9). MTX-poly(D-Lys) lacks cytotoxicity because, unlike poly(L-lysine), it can not be degraded intracellularly and thus does not generate small molecular active drugs (9). We have demonstrated that the introduction of appropriate spacers between MTX and poly(D-Lys) will render the conjugates cytotoxic. These spacers include a small peptide that can be digested by lysosomal proteases (10) and an acid-sensitive linkage that hydrolyzes spontaneously at the acid pH of endosomes-lysosomes (11). Poly(D-lysine) therefore offers a tool to study specific drug-carrier linkages that will be cleaved under different intracellular conditions. The drug-disulfide linkage described in this report offers a model for the study of the reducing process in endocytosis because the cleavage of the disulfide bond can be easily assessed by the growth inhibition due to the releasing of a cytotoxic drug in the intact cells.

MATERIALS AND METHODS

MTX was supplied by Lederle Laboratories. Poly(D-lysine) hydrobromide (M_r 60,000), triglycine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), leupeptin, and 3-(2-pyridyldithio)propionic acid *N*-hydroxylsuccinimide ester (SPDP) were obtained from Sigma. The tissue culture products were from GIBCO, Grand Island, NY. The CHO cell line CHOPro$^-$3 MtxRII 5-3, characterized as MTX-resistant due to transport defect (12), was given to us by Dr. W. F. Flintoff, University of Western Ontario, London, Ontario, Canada. Unless specified, these cells were grown in α-MEM medium with 10% fetal bovine serum. The preparation of MTX-poly(D-Lys) has been

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[1] The abbreviations used are: MTX, methotrexate; poly(D-Lys), poly(D-lysine); CHO, Chinese hamster ovary; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; SPDP, 3-(2-pyridyldithio)propionic acid *N*-hydroxylsuccinimide ester; α-MEM, α-minimal essential medium; PBS, phosphate-buffered saline, pH 7; MTX-GGG, MTX-triglycine conjugate; MTX-GGG-poly(D-Lys), MTX-poly(D-Lys) conjugate with a triglycine spacer; MTX-SS-poly(D-Lys), MTX-poly(D-Lys) conjugate with a 3-(aminoethyldithio)propionic acid spacer; MTX-poly(D-Lys), MTX-poly(D-lys) conjugate with a direct linkage.

described (9). The concentrations of poly(D-Lys) in various conjugates were determined by the trypan blue precipitation method (13).

Preparation of MTX-GGG-Poly(D-Lys)—To a solution containing 10 mg of MTX and 5 mg of triglycine in 1 ml of H_2O, pH 7, 10 mg of EDC was added. After 2 h, the reaction was diluted to 2 ml by H_2O, cooled in ice, and adjusted by 1 N HCl to pH 4.5. The precipitate was collected by centrifugation and redissolved in 2 ml of H_2O by slowly adding 1 N NaOH. The acid precipitation was repeated once, and the final product of MTX-GGG was dissolved in 1 ml of H_2O at pH 7. R_F values of thin-layer chromatography (methanol:acetone:acetic acid = 10:10:1) were 0.25 and 0.04 for MTX and MTX-GGG, respectively. The ratio of glutamic acid and glycine in the 6 N HCl hydrolysate was 1 to 3.3, indicating a 1:1.1 ratio of MTX to triglycine in the final product.

A 0.5-ml aliquot of MTX-GGG solution (10 mg/ml) was added to an equal volume of poly(D-Lys) solution (20 mg/ml). After mixed thoroughly, 10 mg of EDC was added. The reaction was kept at room temperature for 2 h with occasional mixing and was then separated by a Sephadex G-50 column equilibrated with PBS. The conjugate which emerged from the column at the void volume was collected and had an average of 5.5 mol of MTX/each mol of poly(D-Lys).

Preparation of MTX-SS-Poly(D-Lys)—To a solution of 45 mg of MTX and 30 mg of cystamine in 1.5 ml of H_2O at pH 7, 30 mg of EDC was added. Heavy precipitation formed during the mixing, and the reaction was allowed to proceed for 0.5 h on ice and then 0.5 h at room temperature. MTX-cystamine precipitate was isolated by centrifugation, washed twice with 2 ml of cold H_2O, and then converted to MTX-thioethylamide (MTX-cysteamine) by heating for 5 min with 2 ml of 10% 2-mercaptoethanol in a boiling water bath. During the reduction of the disulfide bond, the yellow solid of MTX-cystamine was slowly dissolved. The small amount of insoluble material in the final solution was removed by centrifugation, and the supernatant solution containing MTX-thioethylamide, 2-mercaptoethanol, and cysteamine was kept in a freezer. MTX-thioethylamide was purified by repeating precipitation in pH 4 for several times to remove the excess of 2-mercaptoethanol before coupling to SPDP-modified poly(D-Lys). R_F values from thin-layer chromatography (methanol:acetone:acetic acid = 10:10:1) were 0.25, 0.00, and 0.60 for MTX, MTX-cystamine, and MTX-thioethylamide, respectively.

SPDP-modified poly(D-Lys) was prepared by mixing 1.5 ml of poly(D-Lys) solution (20 mg/ml in phosphate buffer saline, pH 8) with 0.15 ml of SPDP (2 mg/ml in absolute alcohol). After 2 h at room temperature, the modified poly(D-Lys) was purified by extensive dialysis in PBS, pH 7, at 4 °C.

To prepare MTX-SS-poly(D-Lys), 5 mg of fresh purified MTX-thioethylamide was added to a solution containing 20 mg of SPDP-modified poly(D-Lys) in 1 ml of PBS. The reaction was allowed to stand overnight at 4 °C and was centrifuged to remove any precipitate. The conjugate was purified on a Sephadex G-50 column. The final product of MTX-SS-poly(D-Lys) had an average of 6.9 mol of MTX/each mol of poly(D-Lys).

Growth Inhibitory Effects of MTX-GGG-Poly(D-Lys) and MTX-SS-Poly(D-Lys)—Wild type of CHO cells or their MTX transport-defective mutants were seeded in 25-cm^2 culture flasks with 5 ml of growth medium at a density of 5×10^4 cells/flask. After 24 h of incubation, various amounts of MTX conjugates were added to each flask. Other agents, if required, were added at the same time. After a 3-day incubation, cells were fed with drug-free growth medium. One or 2 days after feeding, when cells in the control flasks had reached confluency, the cell number in each flask was counted with a Coulter counter. The numbers were compared with that of the untreated cells in control flasks.

In the pretreatment of 2-mercaptoethanol, a solution containing 0.1 mM MTX-SS-poly(D-Lys) and 3 mM 2-mercaptoethanol in PBS was incubated at 37 °C for 2 h. Five μl of this incubated solution was added to the culture flasks to give a final concentration of 0.1 μM MTX and 3 μM 2-mercaptoethanol in the 5 ml of medium. The cells in the flasks were processed as described above to determine growth inhibitory effects.

Intracellular Glutathione Determination—The MTX transport-defective mutant cells were grown in 25-cm^2 flasks and were fed 1 day before the glutathione measurement. Cells from four confluent monolayers were combined after the trypsinization and washed twice each with 5 ml of PBS. Cell pellets after centrifugation were lysed in 1 ml of 10 mM phosphate buffer, pH 7, with 5 mM EDTA, and the proteins in the cell lysates were precipitated with ice-cold 5% trichloroacetic acid solution. After centrifugation, the supernatant solutions were extracted five times each with 2 ml of ether. The final solutions after incubation at 37 °C for 10 min to remove the ether were used to measure glutathione concentrations by Ellman's reagent as described by Tietze (14).

RESULTS

The growth inhibition in both the wild type and the MTX transport-defective CHO cells by MTX and its poly(D-Lys) conjugates is shown in Fig. 1. In the wild type cells, the growth is inhibited by MTX and by the two spacer-linking conjugates, *i.e.* MTX-GGG-poly(D-Lys) and MTX-SS-poly(D-Lys), but is not influenced by MTX-poly(D-Lys) at concentrations up to 0.3 μM. In the MTX transport-defective cells, which are resistant to both free MTX and the poly(D-Lys) conjugate, the growth is also inhibited by the two conjugates with the triglycine and disulfide spacers. The ID_{50} values for MTX-GGG-poly(D-Lys) and MTX-SS-poly(D-Lys) are almost identical in the two cell lines, *i.e.* 43 and 42 nM in the wild type and 36 and 54 nM in the MTX transport-defective cells, respectively. The growth inhibitory effects of the two conjugates are due to the toxicity of MTX rather than that of the spacers or poly(D-Lys) moieties, because Leucovorin, a MTX antagonist, can completely protect the cells from the cytotoxicity of the conjugates (Table I).

When MTX-SS-poly(D-Lys) was preincubated with 2-mercaptoethanol before the addition to the cultures, the inhibitory effect of this conjugate in the transport-defective cells was abolished (Table I). This pretreatment did not change the effect of MTX-GGG-poly(D-Lys) (Table I). Treatments with either leupeptin (50 μg/ml) or NH_4Cl (3 mM) were effective to protect cells from the cytotoxicity of MTX-GGG-poly(D-Lys), but had no effect on the growth inhibition by MTX-SS-poly(D-Lys) (Table I).

The intracellular levels of glutathione in the MTX transport-defective cells grown in either α-MEM or RPMI 1640 medium were compared. When cells were cultured in α-MEM medium, the intracellular level of the reduced glutathione was 19.0 nmol/mg of cell protein. This quantity was significantly

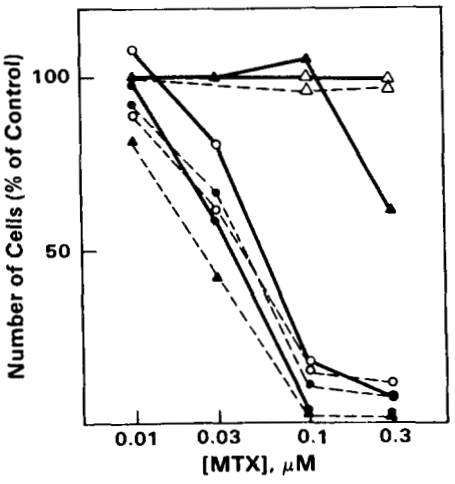

FIG. 1. **Growth inhibition of the wild type and the MTX transport-defective cells by increasing concentrations of MTX given as free drug or as poly(D-Lys) conjugates.** Both the wild type (– – –) and the MTX transport-defective (——) CHO cells were seeded at 5×10^4 cells/flask in α-MEM medium with 10% fetal bovine serum. After 24 h, the sparse monolayers were treated with MTX (▲), MTX-poly(D-Lys) (△), MTX-GGG-poly(D-Lys) (●), and MTX-SS-poly(D-Lys) (○). Cells were harvested and counted after 5 days as described under "Materials and Methods." The cell number in control flasks was 5.4×10^6 cells/flask for the wild type and 4.0×10^6 cells/flask for the MTX transport-defective mutants.

TABLE I

Effects of Leucovorin, 2-mercaptoethanol, leupeptin, and NH_4Cl on the cytotoxicity of MTX given either as MTX-GGG-poly(D-Lys) or as MTX-SS-poly(D-Lys) to transport-defective CHO cells

	Number of survival cells[a]			
	MTX-GGG-poly(D-Lys)		MTX-SS-poly(D-Lys)	
	0.1 μM	0.3 μM	0.1 μM	0.3 μM
	% of control			
No addition	14.0	3.9	16.9	3.2
Leucovorin (3 μM)		96.1		109.6
2-Mercaptoethanol[b]		2.9		103.2
Leupeptin (50 μg/ml)	84.3	46.4	11.2	3.4
NH_4Cl (3 mM)	102.6	34.0	17.6	4.0

[a] Number of cells in control flasks was 5×10^6 cells/flask (25 cm^2) set at 100%.

[b] Conjugates were pretreated with 2-mercaptoethanol as described under "Materials and Methods."

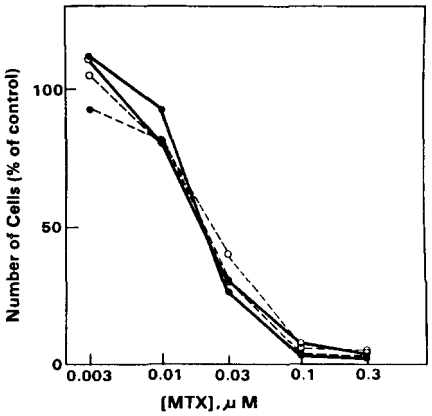

FIG. 2. **Growth inhibitory effects of MTX-GGG-poly(D-Lys) and MTX-SS-poly(D-Lys) on the MTX transport-defective CHO cells cultured in either α-MEM or RPMI 1640 medium.** Cells were treated with MTX-GGG-poly(D-Lys) (●) and MTX-SS-poly(D-Lys) (○) as described in the legend to Fig. 1, except that the experiments were also carried out in α-MEM (——) or RPMI 1640 medium (– – –). The cell number in control flasks was 5.6×10^6 cells/flask in α-MEM medium and 4.5×10^6 cells/flask in RPMI 1640 medium.

higher than that in the cells cultured in RPMI 1640 medium, *i.e.* 9.7 nmol/mg of cell protein. This difference in intracellular glutathione contents, however, was not reflected in the cytotoxicity of MTX-SS-poly(D-Lys), since both MTX-GGG-poly(D-Lys) and MTX-SS-poly(D-Lys) produced an identical growth inhibition when the MTX transport-defective cells were cultured in either medium (Fig. 2).

DISCUSSION

This paper demonstrates the effectiveness of two drug conjugates in which MTX is linked to poly(D-Lys) through two different types of spacer. The first conjugate, MTX-GGG-poly(D-Lys), as we reported previously, is effective in inhibiting the growth of MTX transport-defective CHO mutant cells (10). We confirm here that the triglycine spacer is indeed degraded by the lysosomal proteases. The second conjugate, MTX-SS-poly(D-Lys), is not susceptible to the lysosomal proteolysis and therefore must be cleaved by a reduction on the disulfide linkage.

The molecular mechanism and the intracellular location of this reductive reaction in the endocytotic pathway are largely undetermined. This reaction, however, is generally regarded as an important step in protein degradation. During the last decades, insulin degradation has been a subject of extensive study because of its potential involvement in the hormone function and regulation (6). Nevertheless, the intracellular location of the enzyme, glutathione-insulin transhydrogenase, which cleaves the disulfide linkage between the A and B chains of insulin, is still uncertain. Various localizations, including the plasma membranes (15), the endoplasmic reticulum (16), and the cytosol (17), have been suggested. Besides its role in the protein catabolism, the reductive cleavage of disulfide bonds is required for the intracellular activation of several functional proteins, including enzymes (18) and toxins (19). In protein toxins such as diphtheria toxin, the reduction of the disulfide linkage between the A and B fragments is considered essential for the A fragment to acquire an active conformation (19) and to escape from intracellular vesicles before it is degraded by lysosomal enzymes (20). This suggests that the disulfide bonds in proteins can be reduced at an early stage in the endocytosis process.

Because several oxidoreductases have been found to be associated with plasma membrane (15, 21) and because glutathione can be transported through plasma membranes to reach the outside of the cells (22), it is possible that some reduction of disulfide bonds may occur at the cell surfaces. That such a surface reaction is not a major factor in the effect of MTX-SS-poly(D-Lys) is indicated by the fact that the conjugate is as effective in wild type CHO cells as in their MTX transport-defective mutants. Since the mutant cells are not inhibited by either free MTX or MTX-thioethylamide, these two compounds would be equally ineffective if released by a reductive process at the cell surface. It could be argued that MTX-thioethylamide released at the cell surface could cross-link to sulfhydryl groups of membrane proteins and be carried with them across the cell membrane. We found, however, that pretreatment of MTX-SS-poly(D-Lys) by 2-mercaptoethanol abolishes its cytotoxicity. It does not appear likely that the same compound would be transported differently when released from MTX-SS-poly(D-Lys) at the cell surface or during 2-mercaptoethanol pretreatment. MTX-GGG-poly(D-Lys) also has comparable effects on the wild type and the mutant CHO cells, indicating that the two cell lines do not differ in the endocytosis of the poly(D-Lys) carrier. These considerations all suggest that the growth inhibitory effect of MTX-SS-poly(D-Lys) requires endocytosis of the conjugate and intracellular cleavage of its disulfide bond.

An intracellular cleavage of MTX-SS-poly(D-Lys) could be either enzymatic or nonenzymatic. In the latter instance, it would be expected that the cellular level of glutathione, the major known intracellular reducing agent in mammalian cells (22), would influence the efficiency of the disulfide cleavage. We have shown that a 2-fold difference in the level of intracellular glutathione can be achieved by growing cells in media that differ in their cysteine content. When grown in α-MEM medium, which contains 0.63 mM cysteine (23), the cellular level of glutathione was 19.0 nmol/mg of cell protein. When grown in RPMI 1640 medium, which contains only 0.24 mM cystine but no cysteine (24), the cellular level of glutathione was 9.7 nmol/mg of cell protein. Since the cells grown in these two media responded similarly to MTX-SS-poly(D-Lys), we conclude that intracellular cleavage of the conjugate does not require high levels of glutathione and it is not likely to be due to a nonenzymatic glutathione-disulfide exchange reaction.

Cysteamine has also been suggested as the physiological hydrogen donor for the nonenzymatic reduction of disulfide bonds inside the lysosomes (25). Intralysosomal levels of cysteamine can be changed by treating cells with lysosomo-

tropic amines like NH_4Cl. Such treatment is known to increase the lysosomal pH beyond the values required for optimal proteolysis and thus to inhibit lysosomal proteolysis (26), as evidenced in this paper by a decrease in the cleavage of MTX-GGG-poly(D-Lys). Increasing the lysosomal pH would have two effects relevant to the cleavage of disulfide bonds, namely to decrease lysosomal levels of cysteamine by decreasing its lysosomotropic capture (27) and to raise the lysosomal pH beyond the values required for an optimal activity of lysosomal thiol:protein disulfide oxidoreductase (28, 29). Since cell treatment with NH_4Cl did not influence the cleavage of MTX from its disulfide linkage, it can be inferred that neither lysosomal cysteamine nor lysosomal oxidoreductases play major roles in the cleavage of MTX-SS-poly(D-Lys). Although our results cannot rule out the possibility that the disulfide spacer in the conjugate is cleaved in lysosomes by a nonenzymatic and pH-independent reduction with a hydrogen donor other than glutathione and cysteamine, we think more likely that this reaction is enzymatic in nature and is taking place in some nonlysosomal compartments. Knowing that the interchain disulfide bonds in diphtheria toxin are reduced before it reaches lysosomes, we suggest, by analogy, that MTX-SS-poly(D-Lys) undergoes enzymatic reduction in a prelysosomal compartment.

We have shown that the uptake of polylysines in cultured CHO cells is unsaturable and is not receptor-mediated (30). Therefore, MTX-SS-poly(D-Lys) represents a probe for testing reductive reactions associated with nonspecific adsorptive endocytosis. However, the same drug linkage could be attached to ligands internalized by receptor-mediated endocytosis for a comparison of the reductive functions associated with other forms of protein transport.

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