Transferrin-Oligomers as Potential Carriers in Anticancer Drug Delivery

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Received April 23, 2004; accepted July 6, 2004

Purpose. To investigate if the cross-linking of transferrin receptor (TfR) induced by Tf-oligomers alters the endocytosis of receptor-ligand complexes in cultured tumor cells and hence increases intracellular drug release.

Methods. An average of 3.5 Tf molecules per aggregate were cross-linked either by using homobifunctional linker (1, 11-bis-maleimidotetraethyleneglycol) [$Tf_{3.5}$-$_{BM(PEO)4}$] or heterobifunction linker [succinimidyl 4-(-*p*-maleimidophenyl)-butyrate] ($Tf_{3.5}$-$_{SMPB}$). Cell surface binding and competition experiments with ^{125}I-Tf for TfR binding were studied to demonstrate that Tf-oligomers maintain specificity of the TfR-binding. To determine the degradation of Tf-oligomers in TfR-mediated endocytosis, cultured tumor cells were pulsed for 15 min with ^{125}I-Tf-oligomers and chased for 2 h at 37°C in the presence of excess unlabeled Tf. The chase medium was subjected to TCA precipitation to separate the intact and degraded Tf. To investigate if the alteration of TfR-trafficking facilitates the intracellular release of the drug from the Tf-conjugated form, methotrexate (MTX) was conjugated to Tf-oligomer (Agg-Tf-MTX) and its antiproliferative activity was compared with monomeric-Tf-MTX (Mono-Tf-MTX) in human colon carcinoma (Caco-2) cells, human breast adenocarcinoma (MCF-7) cells, wild-type Chinese hamster ovary (CHO) cells, and MTX-resistant CHO (CHO-MTX-RII) cells.

Results. TfR-mediated degradation of Tf-oligomers was higher than that of monomeric Tf in both Caco-2 and MCF-7 cells. The IC_{50} of Agg-Tf-MTX was lower than that of Mono-Tf-MTX in both tumor cell lines. The IC_{50} of MTX and Mono-Tf-MTX in CHO-MTX-RII cells was higher than that in wild-type CHO cells, whereas the Agg-Tf-MTX was almost identical in both the resistant and wild-type cells.

Conclusions. Cross-linking of TfR induced by oligomeric Tf binding alters the intracellular trafficking of Tf-TfR complexes, redirects them out of the recycling pathway, and targets them to intracellular degradation in cultured tumor cells. The alteration of TfR-trafficking facilitates the intracellular release of the drug from the Tf-conjugated form. Consequently, Agg-Tf-MTX is more effective than Mono-Tf-MTX as a TfR-mediated antiproliferative agent in tumor cells, as well as in MTX-resistant transport deficient cells. Therefore, Tf-oligomers are potentially effective TfR-targeting carriers for intracellular delivery of anticancer drugs.

KEY WORDS: anticancer drug carriers; intracellular drug release; methotrexate; TfR-targeting; transferrin aggregates.

INTRODUCTION

The new approach for the treatment of cancer is to limit the action of the drug to the cancer tissue. This can be accomplished by using a targeted drug carrier, which could deliver the drug to the tumor cells. Free and targeted ligand-bound drugs enter cells by different mechanisms. Free drugs enter the cell through transmembrane diffusion or mediated by membrane transporters, whereas drugs linked to a selective transport carrier are mostly internalized by the target cells via receptor-mediated endocytosis (1). After internalization, receptor-ligand complexes appear to follow multiple pathways, depending on the type of receptor involved (2,3). Ideally, the conjugated drug should be delivered to and taken up by the target cell efficiently and subsequently delivered to compartments where free drug molecules will be released into cytoplasm and reach the site of action.

Transferrin receptor (TfR) has long been considered an interesting drug carrier for intracellular drug targeting (4–7) due to its high expression on the surface of most malignant cells (8) and high efficiency of internalization (9,10) and fast recycling once internalized (11,12). It has been shown in rats that the cellular uptake of transferrin by tumors is correlated with the proliferation activity of the tumor cells (i.e., the faster the tumor growth, the higher the uptake of transferrin). In addition, the uptake of transferrin by tumors results in a loss of transferrin from the blood circulation, which is one of the causes of the anemia observed in malignant diseases (13–16). Therefore, the active targeting of transferrin to the tumor and the hypotransferrinemia seen in cancer patients make transferrin a rational drug carrier *in vivo*. Besides, results from phase I and phase II clinical trials of transferrin-CRM107 (point-mutated diphtheria toxin) conjugate in the treatment of malignant gliomas further supports the feasibility of using transferrin as a carrier in anticancer drug delivery *in vivo* (17). However, for drugs that need to be released from the drug carrier in order to be active, such as methotrexate (MTX) (18–20), TfR-mediated endocytosis might not be an ideal pathway due to its short intracellular residence time and lack of intracellular degradation. It has been shown that anti-TfR antibody-directed MTX delivery to TfR-bearing cells was not cytotoxic over the course of several days; however, cytotoxicity was obtained in conjunction with the carboxylic ionophore monensin treatment (21). The ionophore causes the antibody-drug conjugate to bypass the normal TfR-recyclic pathway, allowing sufficient drug molecules to reach and inactivate the intracellular target, dihydrofolate reductase. Therefore, it is of interest to investigate if the cross-linking of TfR induced by Tf-oligomers can alter the intracellular processing of endocytosed Tf-TfR complexes in cultured tumor cells and hence increase intracellular drug release.

Receptor cross-linking induced by multivalent-ligand has been demonstrated to alter the intracellular trafficking of several receptor-ligand complexes. For example, the multivalent apo-E of β-very low density lipoprotein (β-VLDL) binds to its receptor and is retained in tubular, surface connected peripheral compartments before it is delivered to lysosomes (22,23). In contrast, monovalent apo-B of low density lipoprotein (LDL) binds to the receptor and is targeted to the lysosome rapidly in mouse peritoneal macrophage cells (23). Furthermore, Mellman *et al.* showed that monovalent Fc receptors recycle back to the cell surface, while polyvalent immune complexes directed against Fc receptors target the complexes to the lysosomal compartment (24). It has been suggested that multivalent-Tf of a size greater than decameric aggregates are redirected out of the receptor recycling pathway (25). In this report, we investigated the TfR-mediated

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endocytosis, degradation, and drug targeting of Tf-oligomers, and compared with that of the monomeric Tf.

MATERIALS AND METHODS

Materials

N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 4-(*p*-maleimido-phenyl)-butyrate (SMPB), and 1,11-bis-maleimidotetraethyleneglycol [$BM(PEO)_4$] were obtained from Pierce Chemical Company (Rockford, IL, USA). Cell culture medium and reagents were purchased from Gibco BRL (Rockville, MD, USA). Methotrexate (MTX), *N*-hydroxylsuccinimide (NHS), *N,N′*-dicyclohexylcarbodiimide (DCC), and all other chemicals that are not specified above were purchased from Sigma (St. Louis, MO, USA).

Preparation and Characterization of Tf-Oligomers

Sulfhydryl-containing Tf was prepared by a similar procedure as previously described (26). The ratio of 3-(2-pyridyldithio) propionate to Tf was calculated as 3:1 by measuring the absorbance at 343 nm. The heterobifunctional linker (SMPB), with a 1.93-nm spacer arm, or the homobifunctional linker [$BM(PEO)_4$], with a 3.36 nm spacer arm, was used to cross-link the Tf molecules into Tf-oligomers with either a short or a long spacer respectively (Fig. 1). Iron-saturated Tf was incubated with a 5-fold molar excess of SMPB for 1 h at 25°C. Subsequently, the SMPB-modified Tf was reacted with sulfhydryl-containing Tf for 3 h at 4°C to produce Tf-oligomers with a shorter spacer arm. The longer hydrophilic spacer arm was prepared by incubation of sulfhydryl-containing Tf with a 3-fold molar excess of $BM(PEO)_4$ overnight at 4°C. The cross-linking products were fractionated by passing through a 1.5 cm × 56 cm Sephacryl S-300HR column (fractionation range: 10–1500 kDa). Absorbance of the fractions eluting from the column was measured at 280 nm to determine the position of the peak for Tf-oligomers. The column was calibrated with bovine serum albumin (66 kDa), Tf-diferric (80 kDa), human immunoglobulin G (150 kDa), ferritin (474 kDa), and blue dextran (2000 kDa). The size of Tf-oligomer with SMPB or $BM(PEO)_4$ spacer was estimated to have an average molecular weight of 280 kDa, corresponding to 3.5 transferrin molecules per aggregate. Fractions containing the aggregated Tf were pooled and concentrated to 1 ml by using Centricon YM3 membrane concentrators (MWCO 3000). The size of Tf-oligomer was further characterized by using 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). The Tf-oligomer products and monomeric Tf were iodinated using the chloramine-T method (27). The specific activities of ^{125}I-Tf and ^{125}I-Tf-oligomers ranged from 400 cpm/ng to 900 cpm/ng.

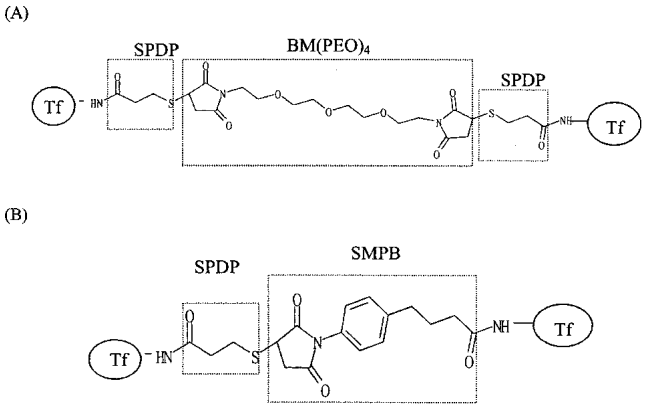

Fig. 1. The cross-linking of transferrin by using SPDP and (A) $BM(PEO)_4$ or (B) SMPB as cross-linker.

Preparation of Monomeric Tf-MTX (Mono-Tf-MTX) Conjugate and Aggregate-Tf-MTX Conjugate (Agg-Tf-MTX)

N-Hydroxylsuccinimide (NHS) ester of MTX was prepared as previously described (28). Briefly, NHS ester was added to MTX solution with stirring, followed by the addition of dicyclohexylcarbodiimide (DCC). The mixture was stirred for 1 h at 25°C and then 18 h at 4°C in the dark. For preparation of Tf-MTX conjugates, 4.5 mg NHS ester of MTX was added to 40 mg diferric Tf. This mixture was stirred at 4°C for 4 h in the dark and then centrifuged (9500 × *g*) for 20 min. The supernatant solution (Tf-MTX) was subsequently dialyzed against PBS at 4°C for 18 h in the dark. To cross-link Tf molecules, half of the Tf-MTX (20 mg in 2 ml PBS) was reacted with SPDP (1 mg in 50 μl DMF) to produce PDP-Tf-MTX, whereas the other half of Tf-MTX was reacted with SMPB (1.8 mg in 100 μl DMF) at 25°C for 1 h in the dark, followed by 18 h dialysis to produce MPB-Tf-MTX. PDP-Tf-MTX was then reduced by reacting with 25 mM dithiothreitol to generate HS-Tf-MTX. HS-Tf-MTX, after purification using a Sephadex G-50 column to remove excess DTT, was then reacted with MPB-Tf-MTX at 4°C for 4 h in the dark. The reaction was stopped by adding maleimide. This procedure was followed by 18 h dialysis in PBS. The product was purified using a Sephacryl S-300HR column. Absorbance of the fractions eluting from the column was measured at 280 nm to determine the position of the peak for both Mono-Tf-MTX and Agg-Tf-MTX. The column had been calibrated with various sizes of proteins, and the aggregated conjugate (Agg-Tf-MTX) and the monomeric conjugate (Mono-Tf-MTX) were separated. The number of molecules that were involved in cross-linking was determined to be 3.5 per aggregate and the

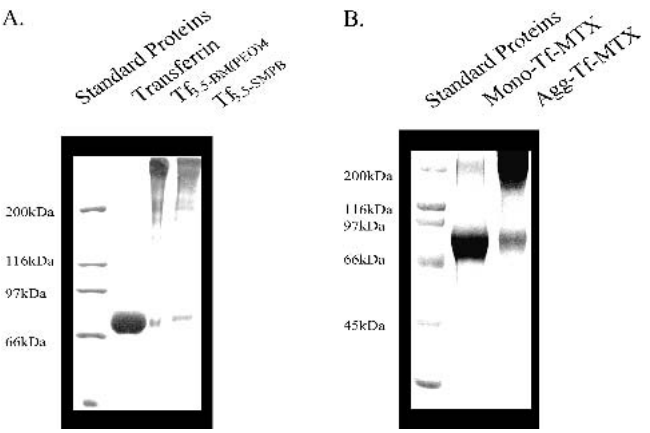

Fig. 2. SDS-PAGE analysis of (A) Tf-oligomers and (B) Tf-MTX conjugates. Samples were applied to 7.5% polyacrylamide gels with a 4% stacking gel. After electrophoresis, protein bands were detected by Coomassie blue stain and the molecular weight was estimated by comparison with standard proteins.

molar ratio of MTX to Tf was determined to be an average of 1 by measuring the absorbance at 370 nm for MTX. The size of Agg-Tf-MTX and Mono-Tf-MTX was further characterized by using 7.5% SDS-PAGE (Fig. 2).

Cell Culture

All experiments were performed using human colon carcinoma cells (Caco-2), human breast adenocarcinoma cells (MCF-7), Chinese hamster ovary (CHO) or MTX-resistant CHO (CHO-MTX-RII) cells (29). These cells were obtained from American Type Culture Collection (Rockville, MD, USA), except the CHO-MTX-RII cell line, which was obtained from Dr. W.F. Flintoff, University of Western Ontario (London, ON, Canada). The procedure used for culturing Caco-2 cells was adapted from the report by Pinto *et al.* (30). The cells were seeded at 50,000 cells/cm^2 in 6-well cluster plates or 12-well cluster plates (Costar, Corning, NY, USA). The seeded plates were incubated at 37°C in 5% CO_2-air and 90% humidity in an incubator. Confluent cell monolayers were obtained within a week after passage and ready for experiments. The procedure used for culturing MCF-7 cells was similar to Caco-2 cells. CHO and CHO-MTX-RII cells were grown in T-25 flasks (Corning) in α (–) minimum essential medium containing 10% fetal bovine serum.

Determination of Cell Surface Binding

Caco-2 cell monolayers grown on 12-well cluster plates were washed twice with serum free Dubecco's Modified Eagle's Medium (DMEM) at room temperature and then incubated with serum-free DMEM with 1 mg/ml bovine serum albumin (BSA) at 37°C for 1 h to deplete serum Tf. Subsequently, ^{125}I-Tf (3 μg/ml) or ^{125}I-Tf-oligomers (3 μg /ml) (either $Tf_{3.5\text{-}BM(PEO)4}$ or $Tf_{3.5\text{-}SMPB}$) was added to Caco-2 cells in each well at 4°C for 2 h in serum-free DMEM with 1 mg/ml BSA. Nonspecific binding was determined in parallel wells containing ^{125}I-ligand and a 100-fold excess of unlabeled Tf. Subsequently, cells were washed with cold PBS (pH 7.2) three times and solubilized by incubation with 1 N NaOH at 37°C for 10 min. After mixing the content of each well with a Pasteur pipette, the cell lysate was assayed for ^{125}I content by using a Packard gamma counter. TfR-mediated cell surface binding was calculated by subtracting nonspecific surface binding from the total surface binding.

Ability of Tf-Oligomers to Compete with ^{125}I-Tf for TfR Binding

^{125}I-Tf (80 ng/ml, i.e., 1 nM), with or without the addition of unlabeled Tf or unlabeled Tf-oligomers ($Tf_{3.5\text{-}BM(PEO)4}$, $Tf_{3.5\text{-}SMPB}$) (concentrations ranged from 0.1 to 500 nM), was added to Caco-2 cells at 4°C for 2 h in serum-free DMEM with 1 mg/ml BSA. Subsequently, cells were washed with cold PBS (pH 7.2) three times, solubilized in 1 N NaOH, and counted for ^{125}I-radioactivity by using a Packard gamma counter. The concentration of Tf, $Tf_{3.5\text{-}BM(PEO)4}$, or $Tf_{3.5\text{-}SMPB}$ needed to produce a 50% inhibition of ^{125}I-Tf (1 nM) binding was determined.

Determination of TfR-Mediated Degradation Induced by Tf-Oligomers

Caco-2 and MCF-7 cells were incubated with ^{125}I-Tf (3 μg/ml) or ^{125}I-Tf-oligomers (3 μg/ml) in serum-free DMEM (with 1 mg/ml BSA) at 37°C for 15 min. Nonspecific binding was determined in parallel wells containing ^{125}I-ligand and a 100-fold excess of unlabeled Tf. The unbound ^{125}I-ligand was then removed by three washes of serum-free medium. The cells were chased at 37°C for 2 h in the presence of excess unlabeled Tf to prevent reinternalization of ^{125}I-ligand. The chase medium from each sample was removed and assayed for radioactivity by gamma counter as total release from the cells. Trichloroacetic acid was used to precipitate proteins in the medium for determination of the intact protein released from the cells. Cell surface–associated ^{125}I-ligand was removed by a 5-min incubation in an acetic acid saline (0.2 M acetic acid, 0.5 M NaCl), pH 2.4 (31). Finally, cells were solubilized in 1 N NaOH and assayed for the radioactivity.

A similar procedure was used to investigate TfR-mediated degradation of ^{125}I-Tf and ^{125}I-Tf-oligomers in Caco-2 cells at 37°C for 24 h continuous exposure. The medium from the 24 h incubation was subjected to TCA precipitation to separate the intact and degraded Tf. The nonspecific degradation was determined in parallel wells containing ^{125}I-Tf and excess of unlabeled Tf. Results were presented as the percentage of degraded Tf in the incubated medium.

Effects of MTX, Mono-Tf-MTX, and Agg-Tf-MTX on the Growth of Cultured Cells

Caco-2 cells and MCF-7 cells were seeded at a density of 8000 cells/cm^2, and wild-type CHO and its MTX-resistant mutant, CHO-MTX-RII, cells were seeded at a density of 2500 cells/cm^2. All cells were allowed to grow for 24 h before the treatment. The cells were then incubated with various concentrations of either MTX or Tf-MTX conjugates in serum-free medium. After 24 h incubation, FBS was added to each sample to give a final concentration of 5%, and cells were exposed to the drugs for another 2 days. The cell proliferation was then determined by using the MTT assay. In some experiments, leupeptin or Tf was added together with MTX or Agg-Tf-MTX to test their protective effects.

RESULTS

Cell Surface Binding of Tf-Oligomers in Caco-2 Cells

The cell surface binding of Tf-oligomers was tested in Caco-2 cells. The results shown in Fig. 3 demonstrate that there was no significant difference between specific cell surface binding of $Tf_{3.5\text{-}SMPB}$ and monomeric Tf. On the other hand, cell surface binding of Tf with a longer spacer arm ($Tf_{3.5\text{-}BM(PEO)4}$) was 0.6-fold lower than cell surface binding of monomeric Tf. The nonspecific binding of both Tf-oligomers ($Tf_{3.5\text{-}BM(PEO)4}$, $Tf_{3.5\text{-}SMPB}$) were about 1-fold higher than that of the control.

Tf-Oligomers Maintain Ability to Compete with ^{125}I-Tf for TfR Binding

To determine the ability of Tf-oligomers to compete with monomeric Tf for TfR binding, Caco-2 cells were incubated with ^{125}I-Tf (80 ng/ml, 1 nM) in the presence of increasing doses of either unlabeled Tf or unlabeled Tf-oligomers ($Tf_{3.5\text{-}BM(PEO)4}$, $Tf_{3.5\text{-}SMPB}$) at 4°C for 2 h. Figure 4 shows that $Tf_{3.5\text{-}BM(PEO)4}$ had the lowest ability to compete with ^{125}I-Tf for TfR binding. The concentrations of $Tf_{3.5\text{-}BM(PEO)4}$ and

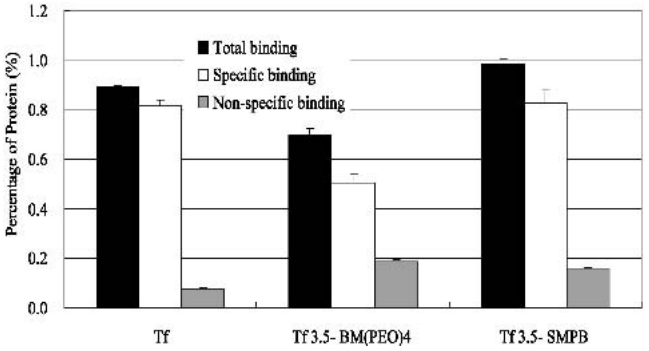

Fig. 3. Cell surface binding study of Tf-oligomers ($Tf_{3.5\text{-}BM(PEO)4}$ and $Tf_{3.5\text{-}SMPB}$). ^{125}I-Tf, ^{125}I- $Tf_{3.5\text{-}BM(PEO)4}$, and ^{125}I- $Tf_{3.5\text{-}SMPB}$ were added to Caco-2 cells in individual wells at 4°C for 2 h in serum-free DMEM with 1mg/ml BSA. Nonspecific binding (hatched column) was determined in parallel wells containing ^{125}I-ligand and excess unlabeled Tf. Specific binding (white column) was calculated by subtracting nonspecific binding from the total binding (black column). Data were interpreted as percentage of total treated ligand. Each column represents the mean of three measurements with error bars representing the standard deviation.

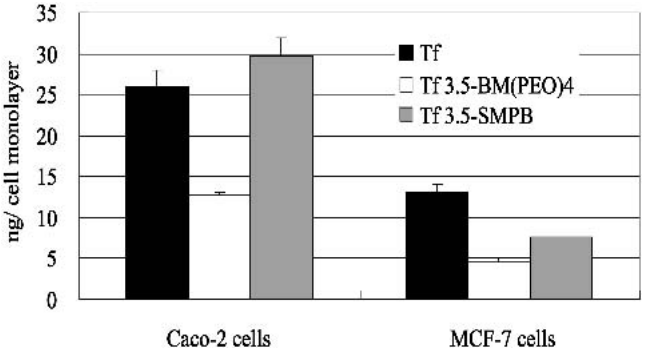

Fig. 5. TfR-mediated cellular uptake of $Tf_{3.5\text{-}BM(PEO)4}$ and $Tf_{3.5\text{-}SMPB}$ after a 15-min incubation. ^{125}I-Tf (black column), ^{125}I- $Tf_{3.5\text{-}BM(PEO)4}$ (white column), and ^{125}I- $Tf_{3.5\text{-}SMPB}$ (hatched column) were added to Caco-2 cells or MCF-7 cells in serum-free medium for a 15-min incubation at 37°C. Nonspecific uptake was determined in parallel wells containing ^{125}I-ligand and excess unlabeled Tf. The unbound ligand was removed by three washes of serum-free medium. Cells were then solubilized with 1 N NaOH and assayed for radioactivity. Each column represents the mean of three measurements with error bars representing the standard deviation.

$Tf_{3.5\text{-}SMPB}$ that could produce a 50% inhibition of ^{125}I-Tf (1 nM) binding was 6.6 nM and 3.5 nM, respectively, compared to that of monomeric Tf, which was 1.5 nM.

Increased Intracellular Retention and Degradation of Tf-Oligomers in TfR-Mediated Endocytosis

Pulse-chase studies were performed to investigate the influence of Tf-oligomers on the trafficking of TfR. After a 15-min pulse, the TfR-mediated uptake of $Tf_{3.5\text{-}BM(PEO)4}$ was 0.8-fold and 1.4-fold lower than that of monomeric Tf in Caco-2 cells and MCF-7 cells, respectively. The TfR-mediated cellular uptake of $Tf_{3.5\text{-}SMPB}$ after a 15-min pulse was not significantly different from that of monomeric Tf in Caco-2 cells, and it was 0.5-fold lower than that of monomeric Tf in MCF-7 cells (Fig. 5). These results are consistent with the results from surface binding study (Fig. 3). Table I shows the percentage of initially endocytosed ligands (after 15-min pulse). The total cell-associated $Tf_{3.5\text{-}BM(PEO)4}$ and $Tf_{3.5\text{-}SMPB}$ was 2-fold and 2.6-fold, respectively, of that of monomeric Tf in MCF-7 cells. The total cell-associated $Tf_{3.5\text{-}BM(PEO)4}$ and $Tf_{3.5\text{-}SMPB}$ was similar and was slightly higher than that of monomeric Tf in Caco-2 cells, respectively. There was no significant difference between the cell surface–associated Tf-oligomers ($Tf_{3.5\text{-}BM(PEO)4}$ and $Tf_{3.5\text{-}SMPB}$) and the cell surface–associated monomeric Tf. In the recycling medium, the percentage of degradation of $Tf_{3.5\text{-}BM(PEO)4}$ and $Tf_{3.5\text{-}SMPB}$ was 3.8-fold and 3.2-fold, respectively, of the control in Caco-2 cells. On the other hand, both the percentage of degradation of $Tf_{3.5\text{-}BM(PEO)4}$ and $Tf_{3.5\text{-}SMPB}$ in MCF-7 were about 2-fold of the control.

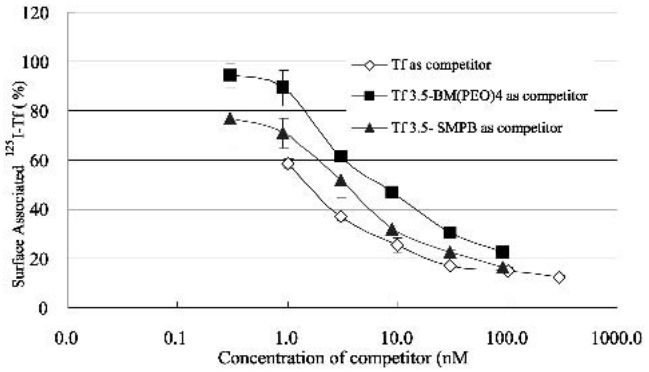

Fig. 4. Ability of Tf-oligomers ($Tf_{3.5\text{-}BM(PEO)4}$ and $Tf_{3.5\text{-}SMPB}$) to compete with the ^{125}I-Tf for TfR binding. Caco-2 cells were incubated with 80 ng/ml ^{125}I-Tf and graded doses of unlabeled Tf (open diamond), unlabeled $Tf_{3.5\text{-}BM(PEO)4}$ (closed square), or $Tf_{3.5\text{-}SMPB}$ (closed triangle) at 4°C for 2 h in serum-free DMEM with 1 mg/ml BSA. Each point represents the mean of three measurements with error bars representing the standard deviation.

Caco-2 cells were incubated with ^{125}I-Tf or ^{125}I-Tf-oligomers at 37°C. After 24 h, the incubation medium was subjected to TCA precipitation to measure the amount of TfR-mediated degradation of Tf. Again, this result (Fig. 6) was consistent with our pulse-chase studies that the specific degradation of Tf-oligomer was higher than that of monomeric Tf.

Agg-Tf-MTX Is More Potent Than Mono-Tf-MTX in Cultured Cells

The concentration that caused a 50% growth inhibition (i.e., IC_{50}) of Agg-Tf-MTX and Mono-Tf-MTX was 0.32 μM and 0.81 μM, respectively, compared to that of MTX, which was 0.07 μM in Caco-2 cells (Fig. 7a). The IC_{50} of Agg-Tf-MTX and Mono-Tf-MTX in MCF-7 cells was 0.37 μM and 0.57 μM, respectively, compared to that of MTX, which was 0.09 μM (Fig. 7b). The IC_{50} of MTX (1.54 μM) in MTX-resistant CHO cells was 25-fold of that in wild-type CHO cells (0.06 μM). The IC_{50} of Mono-Tf-MTX (1.66 μM) in MTX-resistant CHO cells was about 2-fold of that in wild-type CHO cells (0.73 μM), whereas the IC_{50} of Agg-Tf-MTX in MTX-resistant CHO cells (0.35 μM) was almost identical to that in wild-type cells (0.44 μM) (Fig. 8a).

Effects of Leupeptin and Transferrin on the Cytotoxicity of MTX or Agg-Tf-MTX Conjugate

Leupeptin (1 mM), a lysosomal thiol-protease inhibitor, partially protected the CHO-MTX-RII cells against the cyto-

Table I. The Effects of Tf-Oligomers ($Tf_{3.5\text{-}BM(POE)4}$ and $Tf_{3.5\text{-}SMPB}$) on TfR Trafficking[a]

Pulse chase studies (chase for 2 h)	Tf-oligomer	Total release[b]	Released intact protein	Released degraded protein	Total cell-associated[c]	Cellular uptake	Surface associated
Cell lines							
Caco-2	Control	71.9 ± 1.7	71.4 ± 1.8	0.5 ± 0.1 (0.7 ± 0.2)[d]	28.1 ± 1.7	25.8 ± 1.5	2.4 ± 0.2
	$BM(PEO)_{4(as\ spacer\ arm)}$	75.2 ± 1.7	73.2 ± 1.6	1.9 ± 0.2 (2.5 ± 0.3)[d]	24.9 ± 1.7	21.1 ± 1.8	3.8 ± 0.1
	$SMBP_{(as\ spacer\ arm)}$	65.1 ± 1.7	63.5 ± 1.7	1.6 ± 0.1 (2.4 ± 0.1)[d]	34.9 ± 1.7	31.2 ± 2	3.8 ± 0.3
MCF-7	Control	93.5 ± 0.2	91.9 ± 0.5	1.6 ± 0.3 (0.7 ± 0.3)[d]	6.5 ± 0.2	3.7 ± 0.5	2.8 ± 0.3
	$BM(PEO)_{4\ (as\ spacer\ arm)}$	86.3 ± 1.5	83 ± 1	3.3 ± 0.8 (3.8 ± 0.9)[d]	13.7 ± 1.5	9.8 ± 1.6	3.8 ± 0.2
	$SMPB_{(as\ spacer\ arm)}$	82.9 ± 1.5	80 ± 1.2	3 ± 0.3 (3.6 ± 0.4)[d]	17.1 ± 1.5	12.2 ± 1.1	4.9 ± 0.4

[a] Data were interpreted as percentage of initially endocytosed ligands (means ± SE, n = 3).
[b] Sum of released intact and released degraded protein.
[c] Sum of cellular uptake and surface-associated ligand.
[d] Values shown in the parentheses are interpreted as percentage of total release.

toxicity of Agg-Tf-MTX, while no protection against the cytotoxicity of MTX was observed (Fig. 8b). The presence of 50 μM Tf completely protected the CHO-MTX-RII cells against the cytotoxicity of Agg-Tf-MTX, while no protection against the cytotoxicity of MTX was observed.

DISCUSSION

Targeting of drugs or toxins to tumor cells by linking to Tf or anti-TfR antibodies has been a topic of investigation for many years (4–7,17). However, the short intracellular residence time for Tf-TfR due to the efficient recycling of the receptor to the cell surface and the lack of intracellular processing Tf-TfR in lysosomes are two major limitations for TfR-mediated drug delivery. Lysosomotropic agents have been used to enhance the delivery and potency of TfR-targeted conjugate by altering the trafficking of TfR (32,33). It was shown that monensin causes an accumulation of the receptor-bound Tf-toxin conjugate in a perinuclear region contiguous to the cisternae of the trans-Golgi network, in addition to inducing alkalization of normally acidic intracellular compartments (33). However, lysosomotropic agents such as monensin are toxic when administered *in vivo* (34,35), other strategies to alter trafficking of Tf-TfR are needed. One approach is to use Tf-oligomers for accomplishment of enhancing both the intracellular retention and degradation of the drug-Tf conjugates inside tumor cells. In this report, we investigate the effectiveness as drug-carriers of two different types of conjugates (i.e., monomeric Tf and $Tf_{3.5\text{-}SMPB}$).

Tf aggregates should have a higher affinity toward the cell surface due to multiple binding to the receptor. However,

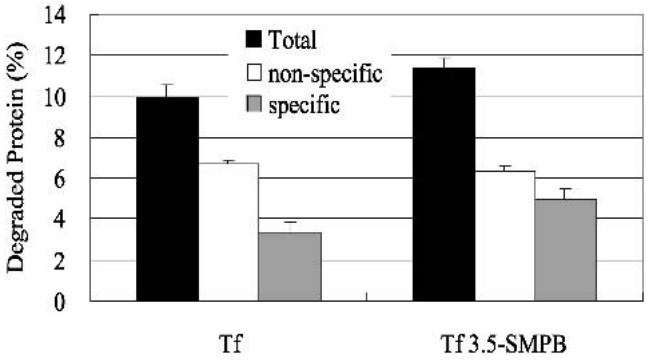

Fig. 6. TfR-mediated degradation. Caco-2 cells were incubated with ^{125}I-Tf and ^{125}I- $Tf_{3.5\text{-}SMPB}$ in individual wells in serum-free DMEM with 1 mg/ml BSA at 37°C for 24 h. Nonspecific degradation (white column) was determined in parallel wells containing ^{125}I-ligand and excess unlabeled Tf. The incubated medium was subjected to TCA precipitation to separate the intact and degraded Tf. TfR-mediated degradation (hatched column) was calculated by subtrating nonspecific degradation from the total degradation (black column). Each column represents the mean of three measurements with error bars representing the standard deviation.

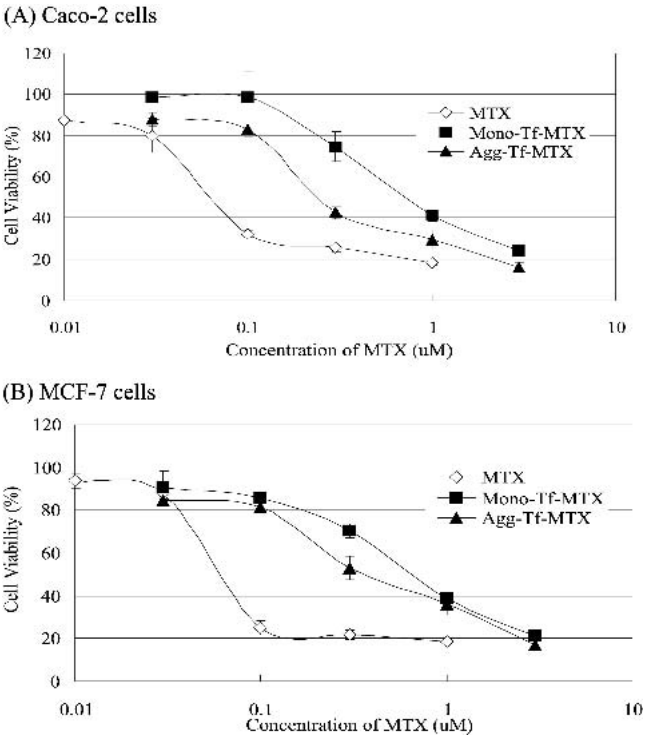

Fig. 7. Cytotoxicity of MTX, Mono-Tf-MTX, and Agg-Tf-MTX against the (A) Caco-2 cells and (B) MCF-7 cells. All cells were allowed to grow for 24 h before any treatment. The cells were then incubated with various concentrations of MTX (open diamond), Mono-Tf-MTX (closed square), or Agg-Tf-MTX (closed triangle) in serum-free medium. After 24 h incubation, FBS was added to each sample to give a final concentration of 5%, and cells were consecutively exposed to the drugs for another 2 days. The proliferation of cells after 4 days of growth was determined by MTT assay. Each point represents the mean of three measurements with error bars representing the standard deviation.

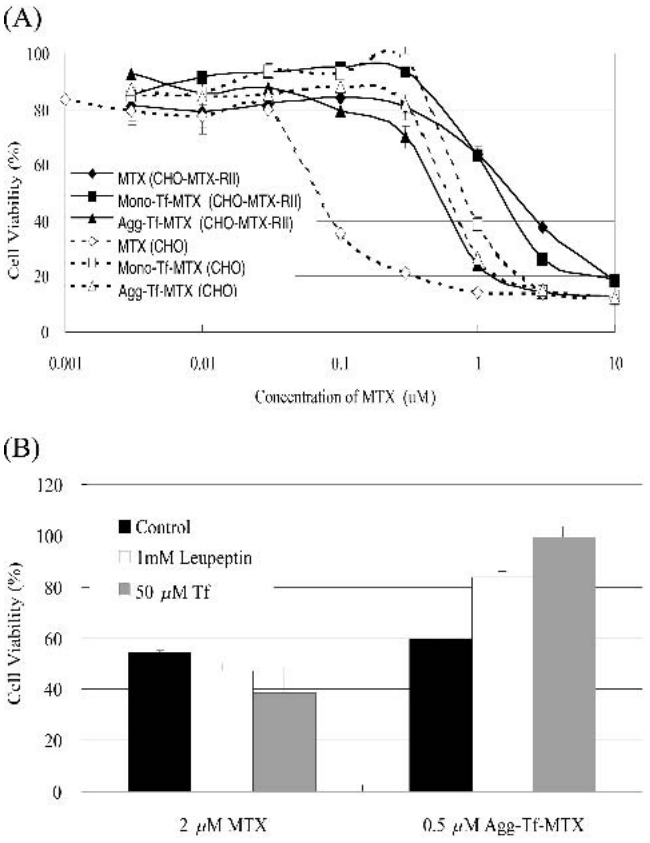

Fig. 8. (A) Cytotoxicity of MTX, Mono-Tf-MTX, and Agg-Tf-MTX against the CHO cells (dashed lines) and CHO-MTX-RII cells (solid lines). All cells were allowed to grow for 24 h before any treatment. The cells were then incubated with various concentrations of either MTX (diamonds), Mono-Tf-MTX (squares), or Agg-Tf-MTX (triangles) in serum-free medium. After 24 h incubation, FBS was added to each sample to give a final concentration of 5%, and cells were consecutively exposed to the drugs for another 2 days. The proliferation of cells after 4 days of growth was determined by MTT assay. (B) Effects of leupeptin and transferrin on the cytotoxicity of MTX and Agg-Tf-MTX in CHO-MTX-RII cells. The procedure is the same as described in (A) except leupeptin (white column) or transferrin (hatched column) was added together with MTX or Agg-Tf-MTX to test their protective effects. Each point or column represents the mean of three measurements with error bars representing the standard deviation.

our results showed that none of the Tf aggregates had a significant higher ability than monomeric Tf to compete with ^{125}I-Tf for TfR binding. One of the possible reasons could be the lack of cell surface fluidity when the experiment was performed at 4°C. Therefore, TfR was less accessible to the oligomers. Another possible reason is that the chemical modification of Tf by cross-linking reagents may cause the decrease of the intrinsic binding capacity of each Tf unit in the aggregate. Nevertheless, our results showed that both of $Tf_{3.5\text{-}BM(PEO)4}$ and $Tf_{3.5\text{-}SMPB}$ still maintained the specificity and capacity of TfR-binding (Figs. 3 and 4).

Marsh *et al.* (25) investigated the trafficking of multivalent-Tf, which was composed of approximately 10 Tf molecules (Tf_{10}), and demonstrated that Tf_{10} was retained in the pericentriolar recycling compartment (PCRC) in CHO cells four times longer than Tf. Our results showed that, after a 2-h chase, cell associated $Tf_{3.5\text{-}BM(PEO)4}$ and $Tf_{3.5\text{-}SMPB}$ was about 2- to 3-fold that of monomeric Tf in MCF-7 cells, whereas in Caco-2 cells, cell-associated $Tf_{3\text{-}BM(PEO)4}$ and $Tf_{3\text{-}SMPB}$ was similar and slightly higher than that of monomeric Tf, respectively. In addition, the amount of intracellular retention of both types of Tf-oligomers was 2- to 6-fold that of Tf in polarized Caco-2 cells (data not shown). It can be inferred that intact Tf-oligomers are retained in subapical compartment (SAC) in polarized cells, which is related to PCRC in nonpolarized cells (36–38).

On the other hand, Marsh *et al.* reported that multivalent-Tf aggregates, which are composed of greater than 10 Tf molecules per each particle, were redirected out of the recycling pathway and degraded. The results of our two independent experiments (Table I and Fig. 6) are consistent with their results. Interestingly, we showed that the degradation of Tf-oligomers was 2- to 4-fold higher than that of monomeric Tf in Caco-2 cells or MCF-7 cells, even though a relatively small aggregate (i.e., an average of 3.5 Tf molecules per aggregate) has been used. In addition, the TfR-mediated degradation occurred in Tf-oligomers with either a long [$BM(PEO)_4$] or short (SMPB) cross-linker. It has been shown that the down-regulation of Tf-TfR complexes, which is induced by dimeric Tf, correlated with the cross-linking of TfR (39), and it was consistent with a previous report that TfR was rapidly degraded after monoclonal antibody cross-linking (40).

It has been shown that MTX-carrier conjugates require the intracellular breakdown to liberate the pharmacologically active MTX (18). Therefore, the rapid recycling of TfR does not provide efficient release of the active drug inside the target cell. Our pulse chase studies showed that the cross-linking of TfR induced by Tf-oligomers altered the intracellular trafficking of receptor-ligand complexes. This alteration led the complexes to compartments with an increase of intracellular retention or degradation. Tf-oligomers appear to be able to alter the intracellular TfR trafficking without the use of lysosomotropic agents in TfR-mediated drug targeting. These findings indicate that Tf-oligomers are better drug carriers than the monomeric Tf. Results from the growth inhibition studies further support our hypothesis that Tf-oligomers are more effective in TfR-mediated drug targeting. As shown in Fig. 7, Agg-Tf-MTX was significantly more potent than Mono-Tf-MTX as an antiproliferative agent in both tumor cell lines (Caco-2 and MCF-7 cells). It is noteworthy that the efficacy of Agg-Tf-MTX in MCF-7 cells was similar to that in Caco-2 cells, even though the ratio of $Tf_{3.5\text{-}SMPB}$ vs. monomeric Tf retention in MCF-7 cells is higher than that in Caco-2 cells (Table I). In addition, the high intracellular retention of both monomeric Tf and $Tf_{3.5\text{-}SMPB}$ in Caco-2 cells (26–31% vs. 7–17% in MCF-7 cells) did not increase the antiproliferative efficacy of Tf-MTX-conjugates. This finding suggests that there exists a late endosomal compartment in the trafficking of endocytosed TfR in Caco-2 cells. This compartment, which lacks proteolytic activity and appears to be specific to Caco-2 cells, may play a role in either the storage or the transcytosis of the TfR in intestinal epithelial cells (26,41).

The importance of the intracellular degradation on the antiproliferative activity of Tf-MTX conjugates is further demonstrated in the MTX-resistant CHO cell line, CHO-MTX-RII, which is deficient in MTX transport. The IC_{50} of MTX and Mono-Tf-MTX in CHO-MTX-RII cells was 25-fold and 2-fold, respectively, of that in wild-type CHO cells,

whereas the IC_{50} of Agg-Tf-MTX was almost identical in both the resistant and wild-type cells (Fig. 8a). These results not only show that Agg-Tf-MTX is more potent than Mono-Tf-MTX in overcoming the drug resistance, but also suggest that the inhibition of the cell growth in cultured tumor cells by Agg-Tf-MTX conjugates is mostly due to the intracellular release of MTX. However, the extracellular release of MTX contributes partially to the cytotoxicity caused by Mono-Tf-MTX.

Leupeptin, an inhibitor of several lysosomal proteases, was used to examine the role of lysosomal degradation in the cytotoxic effect of Tf-MTX conjugate. Leupeptin at 1 mM did partially protect CHO-MTX-RII cells from the inhibitory effect of Agg-Tf-MTX conjugate, suggesting again the requirement of intracellular degradation of Tf-MTX conjugates for their pharmacological effect. The incomplete protection might be due to the fact that not all of the lysosomal enzymes involved in the degradation of Tf are leupeptin-sensitive thiol-proteases. Tf at 50 μM (100-fold excess of Tf) completely protected CHO-MTX-RII cells against the cytotoxicity of Agg-Tf-MTX, but not of free MTX (Fig. 8b). This result provides further evidence of the TfR-specificity of the cytotoxicity of Agg-Tf-MTX.

In summary, our results demonstrate that cross-linking of TfR induced by oligomeric Tf binding alters the intracellular trafficking of Tf-TfR complexes, redirects them out of the recycling pathway, and targets them to intracellular degradation in cultured tumor cells. The alteration of TfR-trafficking facilitates the intracellular release of the drug from the Tf-conjugated form. Consequently, Agg-Tf-MTX is more effective than Mono-Tf-MTX as a TfR-mediated antiproliferative agent in tumor cells, as well as in MTX-resistant transport deficient cells. In this report, an average of 3.5 Tf molecules per aggregate has been used in our studies. Therefore, it would be of interest to investigate if the drug efficacy is further increased by using larger size of Tf-aggregates or Tf-oligomers with star-shape structure (42).

ACKNOWLEDGMENTS

This work was supported by NIH grants GM63647 and HL 64365. We thank Dr. Wayne F. Flintoff of the University of Western Ontario for making available to us the methotrexate-resistant cell line.

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