Intracellular Disposition and Cytotoxicity of Transferrin–Mitomycin C Conjugate in HL60 Cells as a Receptor-Mediated Drug Targeting System

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A macromolecular conjugate of mitomycin C (MMC) with transferrin (TF) which possessed binding ability for TF receptor was synthesized. The conjugate (TF–MMC) was internalized into the human leukemia cell line HL60 cells and distributed into intracellular fractions, then exocytosed into an incubation medium. Although these phenomena were similar to those of TF, part of the internalized TF–MMC was degraded to a trichloroacetic acid (TCA)-soluble fraction. Therefore, the intracellular disposition of the conjugate was analyzed kinetically. The mean time of internalization of TF–MMC (7.14 min) was longer than that of TF (5.46 min). The mean exocytosis time of TF–MMC (22.1 min) was also longer than that of TF (13.0 min). Although elongation of both the internalization and exocytosis steps was responsible for the increase in recycling time of the conjugate, the binding process to the TF receptor in the internalization stage was found to be markedly retarded. The recycling times of TF–MMC and TF were 29.2 and 18.5 min, respectively. The mean decomposition time of TF–MMC was 76.3 min. Proliferation of HL60 cells was inhibited by TF–MMC in vitro. These results indicate that the TF-MMC was internalized via a TF receptor and a part of the internalized TF–MMC was degraded, so the released MMC might represent antitumor activity. TF–MMC was demonstrated to be a useful hybrid as a receptor-mediated targeting system.

Key words mitomycin C; transferrin; conjugate; endocytosis; HL60 cell; antitumor effect

Targeting of drugs or toxins to tumor cells by specific carriers has long been a topic of interest. Macromolecules such as antibodies, glycoproteins, polysaccharides, and synthesized polymers have been candidates as carriers to tumor sites.1)

Transferrin (TF) is a glycoprotein which delivers iron ions to actively growing cells.2) The high efficacy of the TF endocytosis process has tempted many investigators to ask whether this pathway could be exploited for the targeted delivery of drugs or larger molecules into cells.3) TF receptor levels in proliferating malignant cells have often been found to be far higher than in the corresponding normal cells. For this reason the TF receptor has been proposed as a target for cancer chemotherapy.4)

Mitomycin C (MMC), which binds to DNA, is an antitumor antibiotic. Its use in chemotherapy is limited by severe side effects because it is distributed to normal cells as well as tumor cells. Previously, we synthesized a transferrin–mitomycin C conjugate (TF–MMC) and showed that the conjugate bound specifically to TF receptors on Sarcoma 180 cells.5) In this paper, we describe the kinetics of the intracellular disposition of TF–MMC in human leukemia cell line HL60 cells. Moreover, the efficiency of TF–MMC as a receptor-mediated targeting system is discussed and its antitumor effect is also examined.

MATERIALS AND METHODS

Reagents Human holo TF was obtained from Sigma Chemical Co. (St. Louis, USA). MMC and RPMI1640 were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from Life Technologies (Gaithersburg, USA) and inactivated at 56 °C for 30 min before use. TF–MMC was synthesized according to the previous report.6) TF–MMC or TF (100 μg) was labeled with 125I (1 mCi, Amersham Corp., Tokyo, Japan) by the chloramine T method, in which the reaction time was limited to 1 min to avoid denaturation.7) Unbound 125I was removed by chromatography on a column of PD-10 (Pharmacia, Uppsala, Sweden). All other chemicals and reagents were of the highest grades commercially available.

Tumor Cells HL60 cells were kindly supplied by Dr. C. Yamashita (Formulation Research Institute, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) and grown in RPMI1640 supplemented with 10% FBS. Before the experiment, the cells were washed twice with ice-cold RPMI1640 supplemented with 1% bovine serum albumin (BSA/RPMI). In order to wash out the endogenous TF and to saturate the non-specific protein binding sites on the cell surface, the HL60 cells were incubated in the medium at 37 °C for 30 min, then the cells were again washed twice with ice-cold BSA/RPMI.

Binding HL60 cells (1.0 × 10^6 cells) in 0.25 ml of BSA/RPMI were incubated with 125I-labeled ligands (0.59—75 nM) at 0 °C for 100 min. After incubation, the cell suspension (0.2 ml) was added to an oil mixture (olive oil: dibutyln phthalate=1:4, 0.15 ml) and centrifuged at 5000 rpm for 2 min. A portion of the cell pellet was cut and the radioactivity of 125I to the cells was determined with a gamma counter (Aloka301, Tokyo, Japan). To correct nonspecific binding, assays were performed in parallel in the presence of 60 μM unlabeled TF.

Internalization Internalization was started by the addition of 125I-labeled ligand to preincubated HL60 cells. The cell suspension (1 × 10^7 cells) in BSA/RPMI (2.5 ml) was incubated in the presence of 37.5 nM 125I-labeled ligand at 37 °C. The internalization was terminated by the addition of ice-cold BSA/RPMI (1.0 ml) to a portion of the incubation mixture (0.2 ml). The cell suspension was centrifuged, then the cell pellet was washed three times with ice-cold BSA/RPMI. The radioactivity of the cell pellet was emitted by both bound and internalized ligand. In order to remove ra.
dioactivity from the cell surface, a portion of the incubation mixture (0.2 ml) was added to ice-cold 0.25 M acetic acid containing 0.5 M NaCl (1.0 ml). The cell suspension was centrifuged, then the cell pellet was washed three times with an acidic solution. The radioactivity of the internalized ligand was determined in a gamma-counter. In order to correct for nonspecific internalization, control experiments containing 60 μM unlabeled TF were run in parallel.

**Recycling**

Recycling of TF–MMC was studied according to the literature as follows. Washed HL60 cells (1 × 10^7 cells) were incubated with ^125^I-labeled ligand (37.5 nm) at 0°C in BSA/RPMI (2.5 ml) for 100 min. Cells were rinsed with ice-cold BSA/RPMI to remove the unbound ligand. BSA/RPMI preheated at 37°C was added to the cell pellet and reincubated at 37°C for another 0, 2.5, 5, 7.5, 10, 15, 20, or 30 min. At the end of the incubation, cell mixtures (0.2 ml) were added to ice-cold BSA/RPMI (1.0 ml) and centrifuged at 4°C and 1000 rpm for 5 min. The radioactivities of the supernatant and the pellet were determined. Trichloroacetic acid (TCA) was added to the supernatant at a final concentration of 10% and the aliquot was centrifuged at 12000 rpm for 10 min. The radioactivities of 10% TCA-soluble and insoluble fractions were measured. To distinguish the cell surface-bound ligands from the internalized ligands, 0.25 M acetic acid/0.5 M NaCl (1 ml) was added to the cell pellet. The mixture was centrifuged at 4°C and 1000 rpm for 5 min, and the radioactivity of the released ligands in the supernatant and the cell-associated counts were determined. In order to correct for a nonspecific endocytotic process, control experiments containing 60 μM unlabeled TF were run in parallel.

In order to compare the intracellular disposition between ^125^I-TF–MMC and ^125^I-TF, we used a kinetic model which assumes that all of these processes follow first order kinetics (Chart 1). It is essentially similar to the model proposed by Ciechanover et al. The model consisted of the following steps: uptake of the ligand–TF–receptor complex into the cell (k_1); exocytosis of the ligand out of the cell (k_2); decomposition of the ligand (k_3); dissociation of the ligand from the ligand–TF–receptor complex on the cell surface (k_4).

**Cytotoxicity**

Alamar Blue (Kanto Reagents, Tokyo, Japan) was used to determine cell proliferation. Briefly, HL60 cells (1 × 10^5 cells/ml, 0.1 ml) were inoculated into 96-well microplates (Nunc, Roskilde, Denmark). The cells were incubated with the test compound at specified concentrations in RPMI1640 containing 1% FBS at 37°C for 3 d, followed by an additional 3 h incubation with Alamar Blue (0.01 ml). Celluar proliferation induces a chemical reduction of the Alamar Blue. The absorbance of reduced Alamar Blue was determined by a microplate reader (Immuno-mini, NJ-2300, Inter Med Japan, Tokyo, Japan). Growth inhibition (G.I.) was calculated using eq. 1.

$$\text{G.I.} = \left(1 - \frac{S - B}{C - B}\right) \times 100\%$$

where S, B, and C represent the absorbances of the sample, the blank, and the control, respectively.

**RESULTS**

**Receptor Binding**

As shown in Fig. 1, the binding capacity of ^125^I-TF–MMC was almost 50% of that of ^125^I-TF. The equilibrium binding of ^125^I-TF–MMC and ^125^I-TF resulted in a saturation isotherm, the Scatchard's plot of the data gave a linear regression line (Fig. 2). The binding parameters were calculated according to eq. 2.

$$B = \frac{-nK_L}{1 + K_L}$$

where B is the amount of bound ligand, n is the number of binding sites, K is the association constant and L is the free ligand concentration. A curve fitting was done using the nonlinear least-squares program, MULTI. Table 1 lists the binding parameter obtained by computer analysis.

**Internalization**

37.5 nm ^125^I-labeled ligands were incubated with...
Table 1. Kinetic Parameter of Binding of $[^{125}]$TF–MMC and $[^{125}]$TF to HL-60 Cells at 0°C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$[^{125}]$TF–MMC</th>
<th>$[^{125}]$TF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding sites/cell (molecules/cell)</td>
<td>$1.16 \times 10^3 \pm 0.15 \times 10^3$</td>
<td>$2.58 \times 10^3 \pm 0.32 \times 10^3$</td>
</tr>
<tr>
<td>Association constant ($t^{-1}$)</td>
<td>$1.45 \times 10^3 \pm 0.32 \times 10^3$</td>
<td>$1.16 \times 10^2 \pm 0.23 \times 10^3$</td>
</tr>
</tbody>
</table>

Each value is the mean±S.D. which is calculated using a nonlinear least squares algorithm, Damping Gauss-Newton, in MULTI by eq.2.

Table 2. Kinetic Parameters of Internalization of $[^{125}]$TF–MMC and $[^{125}]$TF to HL-60 Cells at 37°C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$[^{125}]$TF–MMC</th>
<th>$[^{125}]$TF</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{app, int}$ (min$^{-1}$)</td>
<td>$0.103 \pm 0.021$</td>
<td>$0.213 \pm 0.075$</td>
</tr>
<tr>
<td>$L_c$ (molecules/cell)</td>
<td>$1.16 \times 10^2 \pm 0.09 \times 10^3$</td>
<td>$9.44 \times 10^2 \pm 0.83 \times 10^3$</td>
</tr>
<tr>
<td>$k_{int}$ (min$^{-1}$)</td>
<td>$0.140 \pm 0.064$</td>
<td>$0.183 \pm 0.060$</td>
</tr>
<tr>
<td>$T_{int}$ (min)</td>
<td>7.14</td>
<td>5.46</td>
</tr>
</tbody>
</table>

a) $k_{app, int}$ is the apparent rate constant of internalization. b) $L_c$ is the amount of internalization at the steady-state. c) $k_{int}$ is the rate constant of internalization. d) $T_{int}$ is the mean internalization time. $T_{int}=1/k_{int}$. The value of $k_{app, int}$ and $L_c$ is the mean±S.D. The value was calculated using a nonlinear least squares algorithm, Damping Gauss-Newton, in MULTI by eq.3. The $k_{int}$ value was estimated from the initial slope of a plot of Fig. 4. The value is the mean±S.D. of three experiments.

Fig. 3. Time Profiles of Specific Binding (A) and Internalization (B) of $[^{125}]$TF–MMC (A) and $[^{125}]$TF (B) in HL-60 Cells at 37°C

Each point and vertical bar represents the mean±S.D. of three independent experiments. Specific binding and internalization were simultaneously measured at the ligand concentration of 37.5 nM. Solid lines represent the computer-fitted profiles.

Fig. 4. Internalization versus Integrated Specific Binding of $[^{125}]$TF–MMC (A) and $[^{125}]$TF (B) in HL-60 Cells

Each point and vertical bar represent the mean±S.D. of three independent experiments. Integrated specific binding was estimated from the data in Fig. 3 by the trapezoidal method.

$[^{125}]$TF–MMC was similar to that of $[^{125}]$TF, the rate constant of internalization of $[^{125}]$TF–MMC was smaller than that of $[^{125}]$TF.

The rate of internalization ($k_{int}$) was estimated by eq. 4 according to the method described by Kato et al. 11)

$$I_t = k_{int} \int [L_R] \, dt$$

(4)

where $I_t$ is the amount of internalization at time $t$ and $[L_R]$ is the amount of cell-surface bound. The values of $k_{int}$ of $[^{125}]$TF–MMC and $[^{125}]$TF, which were estimated from the initial slope of a plot of $I_t$ vs. $\int [L_R] \, dt$ (Fig. 4), were 0.140 and 0.183 min$^{-1}$, respectively.

Recycling In the recycling experiment of $[^{125}]$TF–
MMC, the ratio of remaining radioactivity on the cell surface to the initially bound radioactivity decreased in time (Fig. 5A). The internalized radioactivity was maximal at 7.5 min after incubation at 37°C. At least 50% of the initial bound radioactivity was internalized within 7.5 min and then exocytosed. At 30 min, approximately 72% of the initially bound radioactivity was released from the cells. At that time, 9% of the initial bound radioactivity, which was not precipitable with 10% TCA, was released from the cells.

Kinetic parameters of the intracellular disposition of [125I]TF–MMC were obtained by computer analysis according to eqs. 5–8.

\[ A = A_0 e^{-t_0} \]
\[ B = k_1 A_0 \frac{k_2 + k_3 - k_4}{k_1 + k_2 - k_3 - k_4} (e^{-k_3 t} - e^{-k_4 t}) \]
\[ C = A_0 \left( \frac{k_1 k_2}{(k_1 + k_2 - k_3)(k_1 + k_2 - k_4)} e^{-(k_1 + k_2)t} \right) \]
\[ D = k_1 k_2 A_0 \frac{1}{(k_1 + k_2)(k_1 + k_3 + k_4)} \frac{e^{-(k_1 + k_2)t}}{k_1 + k_2 - k_3 - k_4} \]

Table 3. Kinetic Parameters of Recycling of [125I]TF–MMC and [125I]TF in HL60 Cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>[125I]TF–MMC</th>
<th>[125I]TF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate constant(a^{(\text{min}^{-1})})</td>
<td>0.0915 ± 0.0090</td>
<td>0.0641 ± 0.0143</td>
</tr>
<tr>
<td>(k_1)</td>
<td>0.188 ± 0.015</td>
<td>0.231 ± 0.019</td>
</tr>
<tr>
<td>(k_2)</td>
<td>0.0453 ± 0.0055</td>
<td>0.0768 ± 0.0082</td>
</tr>
<tr>
<td>(k_3)</td>
<td>0.0131 ± 0.0029</td>
<td></td>
</tr>
<tr>
<td>Mean time (min)</td>
<td>(T_{1/2})</td>
<td>10.9</td>
</tr>
<tr>
<td>Mean dissociation time</td>
<td>(T_m)</td>
<td>5.32</td>
</tr>
<tr>
<td>Mean uptake time</td>
<td>(T_u)</td>
<td>22.1</td>
</tr>
<tr>
<td>Mean decomposition time</td>
<td>(T_d)</td>
<td>76.3</td>
</tr>
<tr>
<td>Mean binding time</td>
<td>(T_b = T_m - T_2)</td>
<td>1.82</td>
</tr>
<tr>
<td>Mean recycling time</td>
<td>(T_r = T_u + T_2)</td>
<td>29.2</td>
</tr>
</tbody>
</table>

\(a^{(\text{min}^{-1})}\) Each value is the mean ± S.D. calculated using a nonlinear least squares algorithm, Damping Gauss-Newton, in MULTII by eqs. 5–10.

where \(A_0\) means the initially bound radioactivity.

In the case of [125I]TF (Fig. 5B), the bound [125I]TF decreased in time. The ratio of internalization reached a maximum at 5 min and then decreased. The ratio of the released radioactivity was increased over time. At 30 min, 85% of the initial bound radioactivity, which was TCA-precipitable, was released from the cell. The TCA-soluble fraction was less than 1% of the initial bound radioactivity. Since TF was not decomposed to a TCA soluble compound, we considered a kinetic model which ignored the decomposition. The kinetic parameters of [125I]TF were obtained according to eqs. 5, 9, and 10.

\[ B = k_3 A_0 \left( e^{-k_3 t} - e^{-k_4 t} \right) \]  \(9\)

\[ C = A_0 \left( \frac{1}{k_3 + k_4} - \frac{k_4}{k_3 + k_4} e^{-k_3 t} \right) \]  \(10\)

All the parameters were calculated by the least-squares program, MULTII, and are listed in Table 3. As shown in Fig. 5, the simulated curves were in fair agreement with the experimental values.

The rate constant of the uptake \(k_1\) of [125I]TF–MMC and [125I]TF were 0.188 and 0.231 min\(^{-1}\), respectively. The mean uptake time \(T_u\) of [125I]TF–MMC (5.32 min) was longer than that of [125I]TF (4.33 min). This finding suggests that a chemical modification of TF with MMC reduces the rate of uptake of the ligand–TF–receptor complex; however, the effect was relatively small.

Mean binding time \(T_b\) was calculated by subtracting \(T_2\) from \(T_{int}\). The values of \(T_b\) of [125I]TF–MMC and [125I]TF were 1.82 and 1.13 min, respectively. The values of \(k_1\) of both [125I]TF–MMC and [125I]TF were 0.0453 and 0.0768 min\(^{-1}\), respectively. The mean exocytosis time \(T_{exo}\) of [125I]TF–MMC (22.1 min) was longer than that of [125I]TF (13.0 min). The time required for the TF receptor to traverse an endocytic cycle (recycling time, \(T_r\)) was obtained from the sum of the individual mean time \(T_r = T_u + T_2\). The values of \(T_r\) of [125I]TF–MMC and [125I]TF were 29.2 and 18.5 min.
Ciechanover et al. reported that the cycle of TF and its receptor in a cell takes place rather quickly, and the cycling time from the binding of holo-TF to the secretion of apo-TF takes about 16 min in HepG2 cells; on the average, about 4 min are required for the binding of holo-TF, 5 min for the internalization of a complex, 7 min for its return to the surface and 16 s for release of apo-TF into the medium.\textsuperscript{[3]} In this study, the mean internalization time of [\textsuperscript{125}I]TF was similar to that previously reported in HepG2 cells. However, the recycling time of [\textsuperscript{125}I]TF-MMC (29.2 min) is longer than that of [\textsuperscript{125}I]TF (18.5 min), indicating that the chemical modification of TF influences its intracellular disposition. It was expected that the longer TF-MMC resides in the cell, the more cytotoxic TF-MMC becomes.

The extent of availability of endocytosis was calculated using eq. 11.

\[
F = \left( \frac{\int b \, dt}{\int b \, dt}_{\text{TF-MMC}} \right) - \left( \frac{\int b \, dt}{\int b \, dt}_{\text{TF}} \right),
\]

where \( F \) is the extent of availability. In the recycling experiment, the value of \( F \) was 1.1, indicating that the extent of endocytosis of receptor-bound [\textsuperscript{125}I]TF-MMC was almost similar to that of [\textsuperscript{125}I]TF. Moreover, the \( I_{\infty} \) of [\textsuperscript{125}I]TF-MMC is similar to that of [\textsuperscript{125}I]TF. It is thought that the extent of availability of TF-MMC as a receptor-mediated targeting system is similar to that of TF.

The other factor involved in the cytotoxic effect of TF-MMC is a release of MMC from the conjugate. The recycling experiment showed that [\textsuperscript{125}I]TF-MMC was internalized into the HL60 cells via the TF receptor, and part of the internalized [\textsuperscript{125}I]TF-MMC was decomposed into TCA-soluble fractions. The rate constant of the decomposition (\( k_d \)) was 0.0131 min\textsuperscript{-1}. The mean decomposition time (\( T_d \) of [\textsuperscript{125}I]TF-MMC was 76.3 min in the HL60 cells. In our previous paper, we indicated that the release rate of MMC from MMC-albumin conjugate was accelerated by the decomposition of the carrier protein.\textsuperscript{[5]} The release rate of MMC from the TF-MMC might be accelerated after its internalization into HL60 cells.

The cytotoxicity of TF-MMC was examined in HL60 cells which were incubated with the conjugate at different concentrations. The values of the IC\textsubscript{50} of TF-G-MMC and MMC were 1.6 \( \mu \)g MMC/ml and 0.41 \( \mu \)g/ml, respectively. This suggests that the TF-MMC has an inhibitory effect on the growth of HL60 cells.

The intracellular fate of TF-MMC is almost identical with TF, except for the degradation of TF-MMC. The intracellular fate of TF is well-known. TF binds to its receptor on the cell surface, the TF-receptor complexes are internalized in coated vesicles. The coated vesicles fuse with endosomes, an organelle with an internal pH of about 5—5.5.\textsuperscript{[14]} TF-MMC might be susceptible to a weakly acidic condition. From the stability experiments, TF-MMC was released MMC at pH 5.0 (unpublished data). It is thought that the TF-MMC might act as a prodrug of MMC in HL60 cells. However, the release rate of MMC was too late to release MMC completely in the cell. If the release rate under the weakly acidic condi-
tion could increase, the conjugate might be particularly useful in combating the tumor cell. In an albumin conjugate of MMC, maleic acid and aconitic acid acted as a pH sensitive spacer arm which rapidly released MMC at pH 5.0. Furthermore, poly-[N-(2-hydroxyethyl)-L-glutamine] conjugates of MMC, which used peptides as a spacer arm, released MMC rapidly at pH 5.5 and in the presence of lysosomal enzymes. In this study, TF–MMC was degraded to a TCA soluble compound. This finding suggested that part of the internalized TF–MMC was delivered to lysosomes. The introduction of these pH sensitive spacer arms might make the TF conjugate of MMC a more effective antitumor prodrug.

In conclusion, TF–MMC was internalized via a TF receptor, and a part of the internalized TF–MMC was degraded, suggesting that the release of MMC might represent antitumor activity. TF–MMC was demonstrated to be a useful hybrid as a receptor-mediated targeting system.

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