Jpn. J. Cancer Res. 81, 91–99, January 1990

Kinetics of Internalization and Cytotoxicity of Transferrin-Neocarzinostatin Conjugate in Human Leukemia Cell Line, K562

Yutaka Kohgo,[1] Hitoshi Kondo, Junji Kato, Katsunori Sasaki, Nobuyasu Tsushima, Takuji Nishisato, Michiaki Hirayama, Koshi Fujikawa, Naoaki Shintani, Yoshihiro Mogi and Yoshiro Niitsu

Department of Internal Medicine, Section 4, Sapporo Medical College, South-1, West-16, Chuo-ku, Sapporo 060

Human serum transferrin was conjugated with an anticancer-active polypeptide, neocarzinostatin, by using N-succinimidy1-3-(2-pyridyldithio)propionate. The conjugate consisted of 1.8 mol of neocarzinostatin per 1 mol of transferrin on average and retained cytotoxic activity against human tumor cells. This conjugate was capable of binding to the transferrin receptor of human myelogenous leukemia K562 cells and was internalized by endocytosis. The LD_{50} values of the conjugate and neocarzinostatin alone in the presence of excess native bovine transferrin were 0.20 μg/ml and 1.80 μg/ml, respectively, suggesting that the effect of the conjugate was greater than that of neocarzinostatin alone. A pulse-chase experiment using ^{125}I-labeled conjugate revealed that 25% of the internalized conjugate was degraded in lysosomes and the rest was recycled back to the cell surface without degradation. About 75% of this conjugate recycled back to the cell surface in 18.3 min (3.4 min for receptor binding and 14.9 min for recycling to the cell surface through the acidosomes), while the rest was delivered from the cell surface to the lysosome in 19.6 min. This phenomenon was confirmed by chasing the radioactivity in subcellular fractions separated by Percoll density gradient centrifugation. Therefore, it was concluded that this conjugate is internalized specifically by transferrin receptors and is at least partly transferred to and accumulated in lysosomal compartments, resulting in the inhibition of cellular DNA synthesis.

Key words: Transferrin — Transferrin receptor — Neocarzinostatin — Targetting — Conjugate

In an effort to obtain preferential uptake of antitumor agents into malignant tissue, we have explored the possibility of developing such agents by making use of transferrin receptor-mediated endocytosis. It is generally accepted that rapidly growing cells require more iron than resting cells for their growth and metabolism.[1] This is achieved through increases in the numbers of transferrin receptors on the cell membranes.[2] Iron-saturated transferrin binds the receptor and is internalized by endocytosis.[3] Then, the bound iron is released into the cytosol and apotransferrin recycles back to the surface with the receptor for reutilization.[4, 5]

The number of transferrin receptors increases several hundred-fold in various malignant cell lines and cancerous tissues.[6, 7] Thus, transferrin receptors have been considered as a possible recognition structure of tumor cells which could be used to deliver cytotoxic substances. Monoclonal antibodies against transferrin receptor bound covalently to toxins[8] and drugs,[9] and transferrin covalently-bound toxin[10] have recently been reported to show augmented cytotoxic effects against tumor cells.

We therefore prepared a conjugate of the commercially available anticancer polypeptide neocarzinostatin (NCS) with human transferrin in order to examine the feasibility of clinical application of this conjugate and to evaluate the intracellular mode of action.

MATERIALS AND METHODS

Cultured cell line K562, which had originated from a human erythroleukemia cell line, was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories Inc., North Ryde, Australia).

Preparation of transferrin-NCS conjugate Human transferrin was purified from pooled human plasma and saturated with iron as reported previously.[11] NCS was obtained from the Central Research Laboratory, Pola Chemical Industry, Yokohama. A fivefold molar excess of the cross-linking reagent, N-succinimidyl 3-(2-pyridyldithio)propionate (Pharmacia Fine Chemicals, Uppsala, Sweden) was added to both the transferrin and NCS in 0.1 *M* phosphate buffer, pH 7.5, and the reaction was allowed to proceed for 1 h at room temperature. The 3-(2-pyridyldithio)propionylated (PDP) NCS was reduced with 10 m*M* dithiothreitol (DTT) in a 0.1 *M* acetate buffer, pH 4.5, at 25°C for 30 min. The resulting thio group-bearing NCS, HS-NCS, was immediately passed through a Sephadex G-25 column. PDP-transferrin sim-

[1] To whom correspondence should be addressed.

ilarly prepared was also purified by gel filtration on a Sephadex G-25 column. Then, 0.1 mol of PDP-NCS was reacted with 0.01 mol of PDP-transferrin in the dark overnight at room temperature. The transferrin-NCS conjugate was separated from unbound NCS and unbound transferrin by gel filtration on a Sephadex G-100 column (Pharmacia, 1.7×100 cm) in phosphate-buffered saline (PBS), pH 6.0. The fractions showing the highest absorbance at 280 nm were pooled, concentrated, and used as transferrin-NCS. The biological activities of free NCS and transferrin-NCS conjugate were examined by measuring the growth inhibition of *Micrococcus luteus* on agar plates for standardization of NCS activity by the method of Fukuda.[12] Briefly, serial dilutions of transferrin-NCS and NCS standard solutions (30–50 U/ml) were prepared, and 0.25 ml of each dilution was dispensed onto a 24-well plate. The agar (1.8% Tryptosoy broth containing 1% Agarnoble) at about 50°C was added (0.75 ml per well) and quickly stirred. The mixtures were then allowed to stand until the agar solidified. One loopful of 10^7 bacteria/ml solution was placed in each well, and then the culture was incubated at 37°C for 12 h and examined for bacterial growth. The minimum concentration showing no bacterial growth was taken as the MIC (minimum inhibitory concentration), and the NCS activity of the complex was determined by comparing the MIC with that of the standard.

Radiolabeling of transferrin and transferrin-NCS Radioiodination of the proteins was carried out by the lactoperoxidase and glucose oxidase method using Enzymobeads (Bio-Rad, Richmond, VA). The iodinated transferrin and transferrin-NCS was separated from free iodine on a Sephadex G-25 column (1×10 cm) in PBS containing 1 mg/ml of bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO). The specific activity of transferrin and transferrin-NCS was usually about 1.5 μCi/μg protein.

Cytotoxicity assay K562 cells (200 μl; 5×10^6/ml) were incubated in RPMI 1640 medium supplemented with 1% FCS in a microtest plate (Nunc no. 167008) with NCS or transferrin-NCS at various concentrations in the range of 0 to 10.0 μg/ml. The concentration of transferrin-NCS conjugate was expressed as the amount of bound NCS. After 48 h of incubation, 1 μCi of ^{3}H-thymidine (specific activity, 22 Ci/mmol) was added to each well. After another 3 h, the cells were harvested onto glass filter membranes (Labo-Mesh filter LM101-10, Labo Science, Tokyo) by an auto cell harvester (Labo Science), and the radioactivity was counted in a Beckman scintillation counter. The cytotoxic activities of NCS and transferrin-NCS were compared by measuring cell viability by the trypan blue dye exclusion test after incubation for 48 h. All the assays were performed in triplicate and repeated twice.

Pulse-labeling study of K562 cells with ^{125}I-transferrin-NCS Washed K562 cells (1×10^6) were incubated with either ^{125}I-transferrin (20 n*M*) or ^{125}I-transferrin-NCS (20 n*M*) for 60 min at 4°C in RPMI 1640 medium with 0.1% BSA. Cells were then rinsed to remove radiolabeled ligands and reincubated at 37°C for another 0, 2.5, 5, 7.5, 10, 15, 20, or 30 min in RPMI 1640 medium with 0.1% BSA. At the end of the incubation, the cell mixtures were centrifuged at 300*g* for 5 min in an Eppendorf microtube and the radioactivities in the supernatant and the pellet were determined. Trichloroacetic acid (TCA) was added to the culture supernatant at the final concentration of 10% and the aliquot was centrifuged at 10,000*g* for 10 min. The radioactivities of the 10% TCA-soluble and insoluble fractions were measured. To distinguish cell surface-bound ligands from internalized ligands, 1 ml of 0.25 *M* acetic acid/0.5 *M* NaCl was added to the cell pellet. After 5 s, the mixture was centrifuged at 300*g* for 5 min and the radioactivity of the released ligands in the supernatant and the cell-associated counts were determined.[13]

Percoll density fractionation of internalized conjugate K562 cells were incubated at 4°C for 1 h with either 20 n*M* ^{125}I-transferrin or ^{125}I-transferrin-NCS, washed and warmed to 37°C for 5, 10, 15, or 20 min in serum-free RPMI 1640 medium under 5% CO_2-95% air. To end incubations, media were removed by centrifugation at 300*g* for 5 min and ice-cold PBS was rapidly added. Cells were homogenized by using a tight Dounce homogenizer (Thomas Instruments) in 1 ml of 0.25 *M* sucrose, 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0 (homogenizing buffer). After 20 strokes, the homogenate was centrifuged at 3,000*g* for 10 min to pellet nuclei and unbroken cells and 0.5 ml of the supernatant was layered over 9 ml of 20% isoosmotic Percoll (Pharmacia)-homogenizing buffer. After centrifugation (Hitachi, RPS40T rotor) at 40,000*g* for 1 h, the *in situ*-generated density gradient was collected from the bottom. Fractions were assayed for density by using density marker beads (Pharmacia) and for radioactivity by using a LKB autogamma-counter. β-Glucuronidase activity was determined for the localization of lysosomes.[14]

Kinetic model for recycling and degradation of transferrin-NCS The kinetic model in Fig. 1 depicts the essential parameters of the recycling of transferrin (Fig. 1-A) and transferrin-NCS (Fig. 1-B). It is essentially similar to the model proposed by Ciechanover *et al.*[15] Since transferrin and transferrin receptor can be simultaneously followed by the successive steps of the endocytotic cycle of the ligand, k_1 is the rate constant for binding of transferrin and transferrin-NCS to an unoccupied cell surface receptor $(R)_s$, k_2 is the rate constant for internalization of the receptor-ligand complex (R-Tf or R-Tf-NCS)$_s$, and k_3 is the rate constant for movement

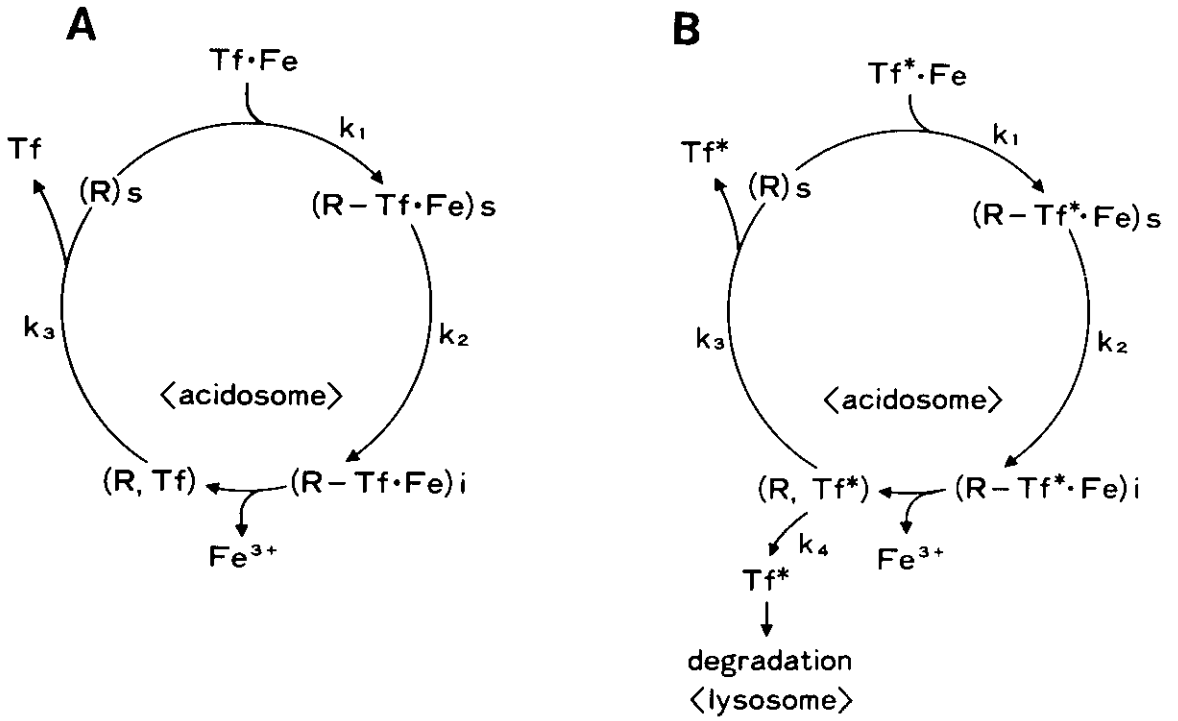


Fig. 1. Kinetic model for receptor-mediated endocytosis and recycling of transferrin (A) and transferrin-NCS conjugate (B). T, transferrin. T*, transferrin-NCS conjugate. $(R)_s$, unoccupied surface receptor. $(TR)_s$ or $(T^*R)_s$, surface transferrin-receptor or transferrin-NCS-receptor complex. $(TR)_i$ or $(T^*R)_i$, intracellular transferrin-receptor or transferrin-NCS-receptor complex. k_1, rate constant for binding of transferrin or transferrin-NCS to surface receptor. k_2, rate constant for internalization of surface transferrin-receptor or transferrin-NCS-receptor complex. k_3, rate constant for movement of the receptor-apotransferrin or receptor-apotransferrin-NCS to the surface. k_4, rate constant for movement of apotransferrin-NCS to lysosome. k_x, overall rate constant of transferrin or transferrin-NCS from receptor binding to recycling to cell surface. k_y, overall rate constant of transferrin-NCS from receptor binding to lysosome.

of the intracellular receptor-transferrin and receptor-transferrin-NCS complex from the acidosome (R-Tf or R-Tf-NCS)$_i$ to the surface. In practice, we were unable to separate k_3 from k_2 in these experiments, because both ligands and receptors share the same metabolic pathway through the acidosomes to the cell surface,[5] and thus we employ a single rate constant, $k_x=k_2+k_3$, to encompass both reactions.

On the other hand, Tf-NCS complex may undergo different processes. One pathway is the same as that of native transferrin ($k_x=k_2+k_3$). The other pathway is the process from acidosome to lysosome, of which the rate constant was designated as k_4. This factor is derived from the experiments using 10% TCA precipitation. Therefore, the single rate constant from surface-bound transferrin-NCS (R-Tf-NCS)$_s$ to lysosome $(Tf\text{-}NCS)_{deg}$ is designated as follows: $k_y=k_2+k_4$.

RESULTS

Characterization of transferrin-NCS conjugate During conjugation three molecules could be generated, transferrin-transferrin, NCS-NCS and transferrin-NCS. Formation of transferrin dimers was decreased by using a large molar excess of NCS. NCS dimers which might have been formed were separated from transferrin and transferrin-NCS conjugate by gel filtration. Since different levels of substitution in transferrin-NCS conjugate might be expected, we identified and quantitated these species by SDS/polyacrylamide gel electrophoresis. This analysis indicated that transferrin-NCS conjugate prepared by our method consisted of one to four moles of NCS per one mole of transferrin (Fig. 2). Since NCS has MW 12,000 and transferrin has MW 80,000 conjugates with 1, 2, 3, or 4 NCS mol/transferrin have molecular weights of 92,000, 104,000 116,000 and 128,000, respectively. Gel scanning analysis revealed that the major

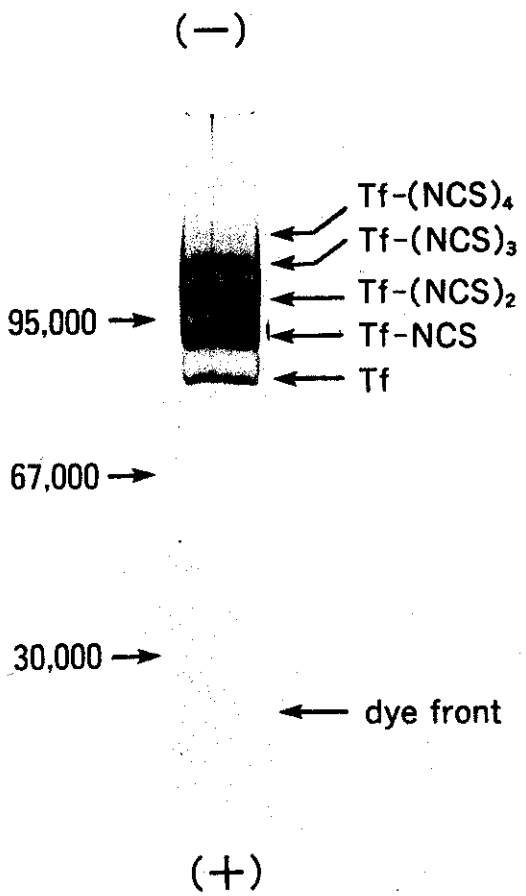


Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the purified transferrin-NCS conjugates. A sample of the conjugate (15 μg) was run on $NaDodSO_4$/5.6% polyacrylamide gel in the absence of 2-mercaptoethanol and the gel was stained for protein with Coomassie brilliant blue.

components of the conjugate are Tf-$(NCS)_1$ and Tf=$(NCS)_2$, while Tf-$(NCS)_4$ is very scarce. An average of 1.8 mol of NCS was bound to each transferrin.

Comparison of the effect of NCS and transferrin-NCS by cytotoxicity assay on K562 cells The effect of transferrin-NCS conjugates and NCS on cellular DNA synthesis of K562 cells was studied in terms of ^{3}H-thymidine incorporation (Fig. 3-A). The inhibitory effect of the conjugate compared with that of NCS was remarkable at a concentration of less than 1 μg/ml. Even at 0.2 μg/ml of NCS, when NCS itself had no inhibitory effect, the conjugates showed about 30% inhibition in the presence of 1% fetal calf serum, which contains an excess of bovine transferrin. The toxicity of the conjugates was also assessed by viable cell counts assay. It was evident that the conjugate is more toxic than NCS alone in the concentration range from 0.2 to 2 μg/ml (Fig. 3-B). The 50% lethal dose (LD_{50}) values of transferrin-NCS conjugate and NCS were 0.2 μg/ml and 1.8 μg/ml, respectively.

Internalization and degradation of transferrin-NCS To measure cellular uptake and degradation of transferrin-NCS conjugate, K562 cells were incubated with either ^{125}I-transferrin or ^{125}I-transferrin-NCS conjugate for 10 min at 37°C. The excess ligands were removed with ice-cold PBS, and cells were reincubated at 37°C for various time intervals. Based on the experiment shown in Fig. 6, the numbers of binding sites on the K562 cell surface for transferrin and transferrin-NCS are about 2.4×10^5 per cell and 1.9×10^5 per cell, respectively, with almost the same affinity (data not shown). Cells were removed by centrifugation, and the radioactivities in the supernatant and in the cells were measured, separately. The supernatant was treated with 10% TCA in order to elucidate whether lysosomal degradation had taken place. Normally, native transferrin recycles back extracellularly without degradation. Approximately 90% of the internalizad ^{125}I-transferrin (Fig. 4-A) and ^{125}I-transferrin-NCS (Fig. 4-B) was released into the medium in 30 min. More than 95% of the released peptide of ^{125}I-transferrin was TCA-precipitable, whereas 75% of the ^{125}I-transferrin-NCS was precipitable, suggesting that a considerable amount of transferrin-NCS was degraded in the lysosomal compartments.

Kinetics of transfer of transferrin-NCS between intracellular compartments Figure 5 demonstrates that transferrin and transferrin-NCS conjugate initially existed near the top of the Percoll density gradient before warming up to 37°C. When the cell-associated radioactivity after 5 min of incubation was analyzed on a colloidal silica gradient, transferrin and transferrin-NCS had nearly identical biphasic sedimentation profiles, with peaks at densities of 1.020 g/ml and 1.030 g/ml, respectively, corresponding to the localization of plasma membrane and prelysosomal acidosomes. After 10 to 20 min, the transferrin lost from these peaks was not present in other fractions, but began to appear in the culture medium. In contrast, the peak of transferrin-NCS broadened and a new peak cosedimenting with β-glucuronidase activity appeared at a density of 1.065 g/ml. These data indicate that more than 25% of the transferrin-NCS conjugate is eventually transferred to the lysosomes.

The rates of binding, recycling and degradation of transferrin-NCS The rate of binding of ^{125}I-transferrin and ^{125}I-transferrin-NCS to surface receptors at 37°C was measured in the presence of an inhibitor of ATP generation, sodium azide (10 m*M*). In the presence of the energy inhibitor at 37°C, the maximum amount of binding is similar to that obtained at 4°C, confirming that the

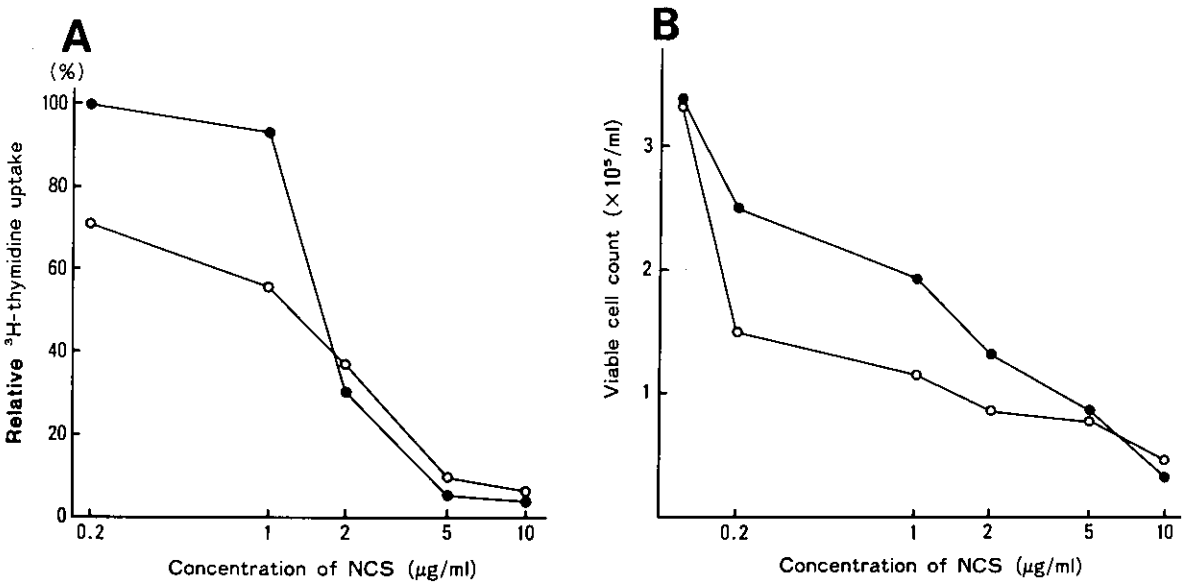


Fig. 3. A) Effect of transferrin-NCS on DNA synthesis in K562 cells. K562 cells were incubated for 48 h with various concentrations of NCS (●) or transferrin-NCS (○) in RPMI 1640 supplemented with 1% FCS at 37°C in humidified 5% CO_2-95% air. The concentration of transferrin-NCS was expressed as the amount of bound NCS. ^{3}H-Thymidine incorporation into cells was determined as described under "Materials and Methods." B) Cytotoxicity of transferrin-NCS conjugate. K562 cells were incubated as above and viable cells were counted at 48 h after the start of culture by the trypan blue dye exclusion test. The 50% lethal dose (LD_{50}) values of NCS and transferrin-NCS was 1.8 μg/ml and 0.2 μg/ml, respectively.

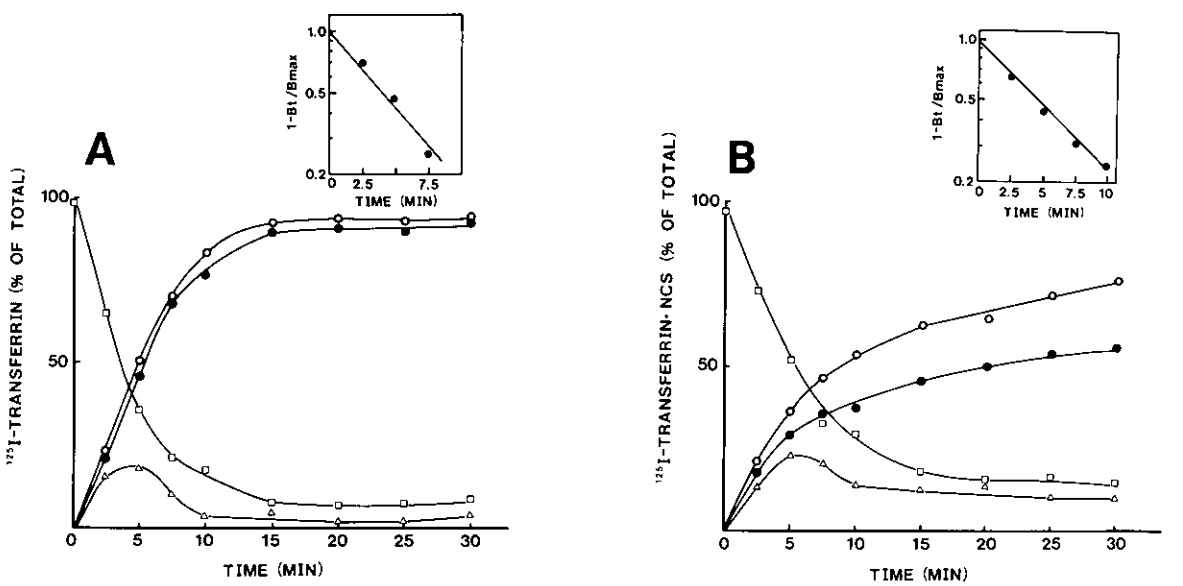


Fig. 4. Pulse-chase study with ^{125}I-transferrin (A) and ^{125}I-transferrin-NCS (B). Transferrin and transferrin-NCS were bound to K562 cells at 4°C. After washing to remove excess unbound ligand, cells were incubated at 37°C at the indicated times. At the end of incubation, the supernatant was quickly removed and cells were chilled on ice. The radioactivity of the supernatant (○) and that precipitated by adding 10% trichloroacetic acid (●) was determined. Cells were then treated with 0.25 *M* acetic acid/0.5 *M* NaCl and the radioactivity released from the cell surface (□) and that remaining in the cells (△) were also determined as described in "Materials and Methods." Inset, the accumulation of ^{125}I-radioactivity in the TCA-precipitable fraction of the medium (●) was plotted semilogarithmically as $1-(Bt/Bmax)$ versus time, where Bmax is the maximum amount of TCA-precipitable fraction, and Bt is the amount at time t.

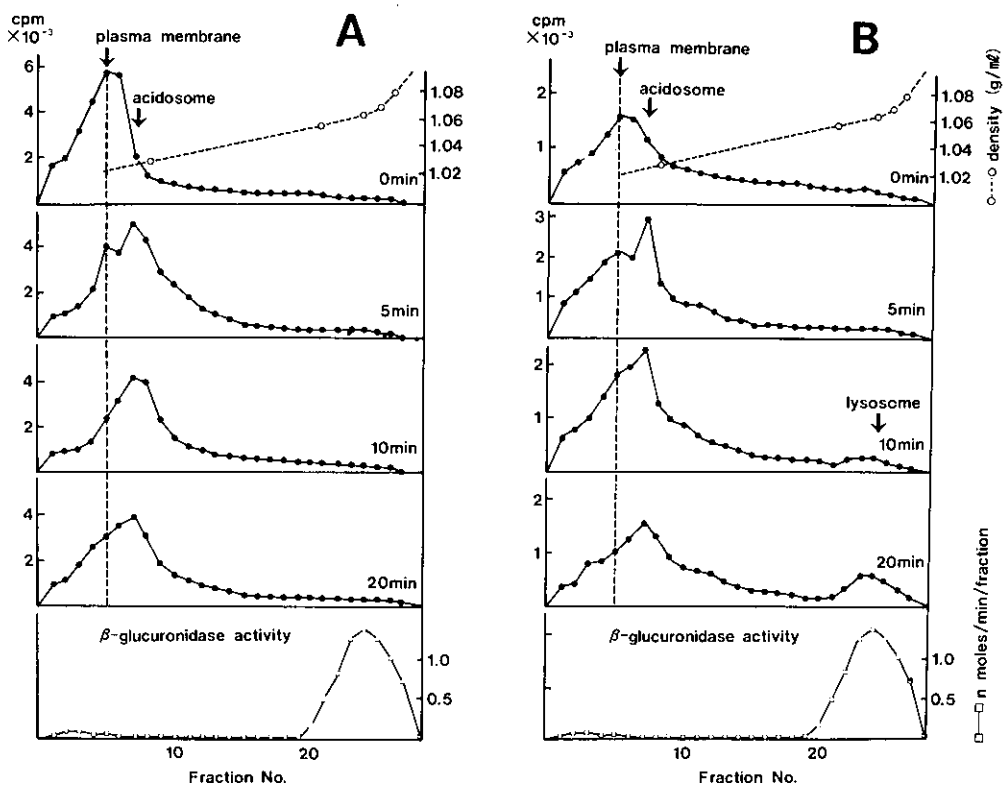
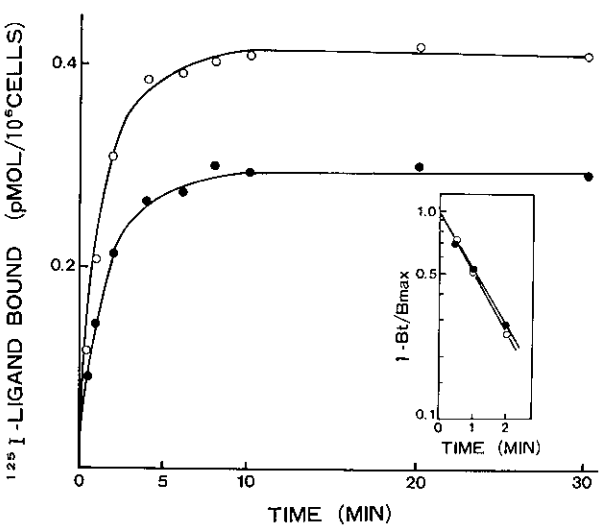


Fig. 5. Kinetics of transfer of ^{125}I-transferrin (A) and ^{125}I-transferrin-NCS (B) between intracellular compartments by Percoll density centrifugation. K562 cells were incubated with ^{125}I-transferrin or transferrin-NCS at 4°C, washed and warmed to 37°C for various times. After homogenization and fractionation on Percoll gradients, the levels of ^{125}I-radioactivity were determined and plotted. Density marker beads were used to determine the gradient density.



inhibitor does not affect the interaction between the ligand and the cell surface receptor (Fig. 6). Furthermore the cytotoxic effect of transferrin-NCS toward K562 cells was markedly suppressed at 37°C in the presence of sodium azide, suggesting that this conjugate is mainly taken up via receptor-mediated endocytosis (data not shown). Since $(k_1)^{-1}$ equals $t_{1/2}/\log 2$, the binding times of transferrin and transferrin-NCS were 3.3 min and 3.4 min, respectively (Table I). To measure $(k_x)^{-1}=(k_2+k_3)^{-1}$, the recycling time of transferrin and transferrin-NCS, a saturating amount of ^{125}I-transferrin or transferrin-NCS was bound at 4°C to the surface receptor followed by warming of the cells to 37°C. The

Fig. 6. Time course of binding of ^{125}I-transferrin (○) and ^{125}I-transferrin-NCS (●) to K562 cells in the presence of an energy inhibitor. Transferrin and transferrin-NCS were bound to K562 cells for the indicated times at 37°C in the presence of 10 m*M* sodium azide. Inset, semilogarithmic replot of the data as described in the legend to Fig. 4.

cells were incubated for various times and the culture supernatant radioactivity was counted. From a semilogarithmic plot of the data, we found the half-time ($t_{1/2}$) of transferrin recycling to be 4.00 min (Fig. 4-A, inset) and that of transferrin-NCS to be 4.05 min (Fig. 4-B, inset). As the rate constant of recycling ($k_x=k_2+k_3$) is $\log 2/t_{1/2}$, the k_x values of transferrin and transferrin-NCS are 0.075 and 0.067, respectively. Therefore the recycling time of transferrin is 13.3 min and that of transferrin-NCS is 14.9 min. The resulting supernatant was further treated with 10% TCA and the non-precipitable supernatant was counted as the lysosomal degraded fraction of transferrin-NCS (Fig. 7). Transferrin-NCS was degraded by 25% and the lysosomal degradation time of transferrin-NCS, $(k_y)^{-1}=(k_2+k_4)^{-1}$, was 19.6 min (Fig. 7, inset). However, there was no significant lysosomal degradation when incubating cells with transferrin, since more than 95% of the radioactivity in the culture supernatant was precipitable by 10% TCA.

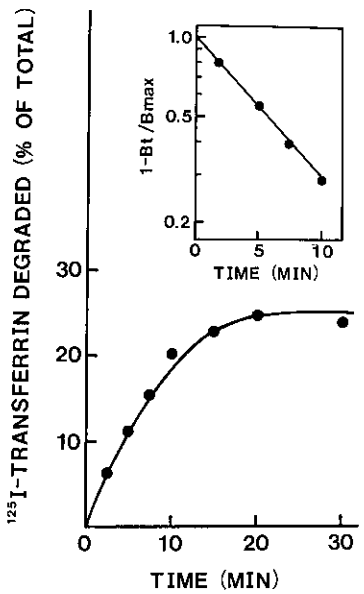


Fig. 7. Degradation of ^{125}I-transferrin-NCS. The accumulation of ^{125}I-radioactivity in the 10% TCA non-precipitable fraction (●) was determined by subtraction of the radioactivity of the TCA-precipitable fraction from that of the supernatant as described in the legend to Fig. 4-B. Inset, semilogarithmic replot of the data as described in the legend to Fig. 4.

DISCUSSION

Several attempts have been made to augment the chemotherapeutic effects of anticancer agents.[16-18] One approach is to use the antigenic specific site for targeting molecules of tumor cells by applying monoclonal antibodies.[19,20] However, this has several disadvantages such as neutralization of antibodies by circulating antigen, nonspecific binding to Fc receptor-positive cells, and an unclear mechanism of intracellular uptake of the bound antibodies. Another approach is the utilization of specific binding and internalization of ligand-drug conjugates. Models reported previously used ligands which corresponded to the receptors for epidermal growth factor,[21] insulin[22] and asialofetuin.[23] These ligand-receptor interactions are less specific in tumor cells and the application to cancer chemotherapy may be difficult.

On the other hand, transferrin receptors are abundant in cancerous tissues and reflect the potential of tumor growth.[6,7] It is therefore reasonable to assume that the

Table I. Parameters to Endocytosis of Transferrin and Transferrin-NCS

Parameter	Ligand	
	Transferrin	Transferrin-NCS
A. Binding of ligand to receptor		
k_1 (min^{-1})	0.30	0.29
Mean time (min)	3.3	3.4
B. Internalization and transfer of ligand through acidosome to cell surface		
$k_x=k_2+k_3$ (min^{-1})	0.075	0.067
Mean time (min)	13.3	14.9
C. Internalization and transfer of ligand through acidosome to lysosome		
$k_y=k_2+k_4$ (min^{-1})	—	0.051
Mean time (min)	—	19.6
Sum of $(k_1)^{-1}+(k_2+k_3)^{-1}$	16.6	18.3
Sum of $(k_1)^{-1}+(k_2+k_4)^{-1}$	—	23.0

transferrin receptor might be available as a target molecule for therapy.

Neocarzinostatin (NCS) is a single-chain polypeptide drug with a molecular weight of approximately 12,000 and exists as an apoprotein and an associated nonprotein chromophore.[24] Our choice of NCS was based on the following considerations. (1) Chemical alterations of apoprotein do not influence the active principle (chromophore). (2) No opening of covalent bonds between carrier and drug is required to release cytotoxic activity at the target. (3) It is easily crosslinked with another protein because of its solubility in water. (4) As NCS is widely used in the clinical field, its action is well-understood at the molecular level.

In the present experiments, we have prepared the conjugate of transferrin and NCS, an anticancer polypeptide. This conjugate bound to the transferrin receptor with almost the same affinity as native transferrin and ratained the cytotoxic activity. Since the cytotoxicity of the conjugate was greater than that of NCS alone against K562 cells (Fig. 3), we examined the intracellular metabolic pathway of the conjugate.

In K562 cells, uptake of transferrin-NCS is mediated through the receptor for transferrin, as shown in Fig. 4. The rates of binding and recycling of transferrin-NCS were not significantly different from those of native transferrin, suggesting that the initial metabolic processes such as recognition by the receptor and sorting to acidosomes were essentially similar to those of native transferrin (Figs. 4, 5). It was, however, shown by silica gradient centrifugation of the internalized ligand (Fig. 5) and by TCA precipitation of exocytosed radioactivity (Fig. 4) that 25% of the conjugate is transferred to the lysosomal compartment and degraded. The degradation of transferrin-NCS took about 20 min after binding to cell surface receptors. This delay is probably due to the delivery of the ligand to the lysosomes, as is the case with other ligands such as asialoglycoprotein[25] and epidermal growth factor.[26] In these experiments, we should take into account that the radioactivity count observed for transferrin-NCS would mainly reflect the transferrin portion, since the numbers of tyrosine residues, which are iodinated by the lactoperoxidase method, in transferrin and NCS are twenty-two and one, respectively. We think that this does not present a problem, because transferrin and NCS are covalently linked by SPDP as a conjugate and they should remain intact after the recycling process until degradation in the lysosome. It is of interest that the conjugates enter the lysosomal compartment through acidosomes after specific binding to transferrin receptors. Unlike other ligands, transferrin retained binding activity to its receptor even after entering the acidosomes, in which transferrin releases ferric iron and becomes an apotransferrin. Therefore, the sorting of the apotransferrin-NCS complex from acidosome to lysosome might be an important clue to explain the differential sorting of ligands from transferrin. Furthermore, this difference of intracellular metabolism between native transferrin and the conjugate may be an important factor to explain the increased cytotoxicity of the conjugate. The conjugate may be degraded in the lysosomal compartment, causing the release of active NCS.

In conclusion, we showed that the transferrin-NCS conjugate may be a useful tool for cancer chemotherapy. A preliminary *in vivo* study is under way to assess the effectiveness of the conjugate for the treatment of transplanted human colon cancer cells in nude mice.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Y. Koide, Pola Chemical Industry, Yokohama, Japan, for providing NCS and for examining the antibacterial activity of the conjugate by *M. luteus* inhibition assay. This work was supported in part by the Foundation for Promotion of Cancer Research, Japan.

(Received August 14, 1989/Accepted November 11, 1989)

REFERENCES

1) Aisen, P. and Listowsky, I. Iron transport and storage proteins. *Ann. Rev. Biochem.*, **49**, 357–383 (1980).
2) Trowbridge, I. S. and Omary, M. B. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc. Natl. Acad. Sci. USA*, **78**, 3039–3043 (1981).
3) Kohgo, Y., Niitsu, Y., Nishisato, T., Kato, J., Sasaki, K., Tsushima, N., Hirayama, M., Kondo, H. and Urushizaki, I. Externalization of transferrin receptor in established human cell lines. *Cell Biol. Int. Rep.*, **11**, 871–879 (1987).
4) Kohgo, Y., Niitsu, Y. Nishisato, T., Urushizaki, Y., Kondo, H., Fukushima, M., Tsushima, N. and Urushizaki, I. Transferrin receptors of tumor cells — potential tools for diagnosis and treatment of malignancies. *In* "Proteins of Iron Storage and Transport," ed. G. Spic, J. Momtruil, R. R. Chrichton and J. Mazurier, pp. 155–169 (1985). Elsevier/North-Holland Biomedical Press, Amsterdam.
5) Hopkins, C. R. and Trowbridge, I. S. Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. *J. Cell Biol.*, **97**, 508–521 (1983).
6) Hamilton, T. A., Wada, H. G. and Sussman, H. H. Identification of transferrin receptor on the surface of human cultured cells. *Proc. Natl. Acad. Sci. USA*, **76**, 6406–6410 (1979).
7) Niitsu, Y., Kohgo, Y., Nishisato, T., Kondo, H., Kato, J.,

Urushizaki, Y. and Urushizaki, I. Transferrin receptor in human cancerous tissues. *Tohoku J. Exp. Med.*, **153**, 239–243 (1987).

8) Trowbridge, I. S. and Domings, D. L. Anti-transferrin receptor monoclonal antibody and toxin-antibody conjugates after growth of human tumor cells. *Nature*, **294**, 171–173 (1981).
9) Yeh, C. G. and Faulk, W. P. Killing of human tumor cells in culture with adriamycin conjugates of human transferrin. *Clin. Immunol. Immunopathol.*, **32**, 1–11 (1984).
10) Raso, V. and Basala, M. A highly cytotoxic human transferrin-ricin A chain conjugate used to select receptor modified cells. *J. Biol. Chem.*, **259**, 1143–1149 (1984).
11) Aisen, P., Liebman, A. and Reich, H. A. Studies on the binding of iron to transferrin and conalbumin. *J. Biol. Chem.*, **241**, 1666–1671 (1966).
12) Fukuda, K. Study of targeting chemotherapy against gastrointestinal cancer: preparation of anticancer drug-monoclonal conjugate and investigation about its biological activities. *Akita J. Med.*, **12**, 451–468 (1985) (in Japanese).
13) Klausner, R. D., van Rensworde, J., Ashwell, G., Kemph, C., Schechter, A. N., Dean, A. and Bridges, K. R. Receptor-mediated endocytosis of transferrin in K562 cells. *J. Biol. Chem.*, **258**, 4715–4724 (1983).
14) Niemann, R. and Buddecke, E. Acceptor-specific glucuronyl transfer catalyzed by β-glucuronidase. *Biochim. Biophys. Acta*, **567**, 196–206 (1979).
15) Ciechanover, A., Schwartz, A. L., Dautry-Varsat, A. and Lodish, H. F. Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. *J. Biol. Chem.*, **258**, 9681–9689 (1983).
16) Torpe, P. E. and Ross, W. C. J. The preparation and cytotoxic properties of antibody-toxin conjugates. *Immunol. Rev.*, **62**, 121–158 (1982).
17) Jansen, F. K., Blythman, H. E., Carriere, D., Casellas, P., Gros, P., Laurent, J. L., Paolucci, F., Pau, B., Poncelet, P., Recher, H., Vedal, H. and Voisin, G. A. Immunotoxins; hybrid molecules combining high specificity and potent toxicity. *Immunol. Rev.*, **62**, 185–216 (1982).
18) Cumber, A. J., Forrster, J. A., Foxwell, B. M. J., Ross, W. C., Thorpe, P. E., Faulstich, H., Fiume, L., Domingo, D. L., Trowbridge, I. S. and Shier, W. T. Drug conjugates. *Methods Enzymol.*, **112**, 207–247 (1985).
19) Vitetta, E. S., Krplick, K. A. and Uhr, J. W. Neoplastic B cells as targets for antibody-ricin A chain immunotoxins. *Immunol. Rev.*, **62**, 159–183 (1982).
20) Raso, V., Ritz, J., Basala, M. and Schlossman, S. F. Monoclonal antibody-ricin A chain conjugate selecting cytotoxic for cells bearing the common acute lymphoblastic leukemia antigen. *Cancer Res.*, **42**, 457–464 (1982).
21) Shimizu, N., Shimizu, Y. and Miskimins, W. K. EGF-ricin A conjugate, kinetic profiles of cytotoxic effect and resistant cell variants. *Cell Struct. Funct.*, **9**, 203–212 (1984).
22) Miskimins, W. K. and Shimizu, N. Synthesis of a cytotoxic insulin crosslinked to diphtheria toxin fragment A capable of recognizing insulin receptors. *Biochem. Biophys. Res. Commun.*, **91**, 143–151 (1979).
23) Simpson, D. L., Cawley, D. B. and Herschman, H. R. Killing of cultured hepatocytes by conjugates of asialofetuin and EGF linked to the A chains of ricin or diphtheria toxin. *Cell*, **29**, 469–473 (1982).
24) Gibson, B. W., Herlihy, W. C., Anantha Samy, T. S., Hahm, K-S., Maeda, H., Meienhofer, J. and Biemann, K. A revised primary structure for neocarzinostatin based on fast atom bombardment and gas chromatographic-mass spectrometry. *J. Biol. Chem.*, **259**, 10801–10806 (1984).
25) Ashwell, G. and Harford, J. Carbohydrate specific receptors of liver. *Ann. Rev. Biochem.*, **51**, 531–554 (1982).
26) King, A. C., Hernaez-Davis, L. and Cuatrecasas, P. Lysomotropic amines cause intracellular accumulation of receptors for epidermal growth factor. *Proc. Natl. Acad. Sci. USA*, **77**, 3283–3287 (1980).