Binding to and Internalization by Cultured Cells of Neocarzinostatin and Enhancement of Its Actions by Conjunction with Lipophilic Styrene-Maleic Acid Copolymer

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ABSTRACT

The binding of a copoly(styrene-maleic acid)-conjugated neocarzinostatin (NCS) designated as smancs (M, 16,000), and parental NCS (M, 12,000) to cultured cells was investigated. These drugs were labeled with fluorescein isothiocyanate which retained biological activity and were used for binding studies. The binding of these drugs to HeLa cells was dependent on time and temperature, with 2 times more drug being bound at 37°C than at 0°C. In the presence of a 100-fold molar excess of unlabeled NCS, the binding of smancs or NCS to HeLa cells was inhibited similarly. Therefore, it was suggested that smancs binds to NCS-binding sites (receptor) of the cell surface. However, the amount of cell-bound smancs was increased about 20-fold compared with that of NCS. Scatchard plot analyses of the binding of these drugs to HeLa and WISH cells indicated that this increase was due to alterations in affinity resulting from polymer conjugation of the drug to receptor rather than to an increase in the number of drug-binding sites at the cell surface. Furthermore, when the cytotoxicity of these drugs to HeLa cells was compared, smancs needed only 5 min to achieve 50% inhibition of the control. In contrast, the same dose level of NCS required more than 90 min to achieve the same toxic effect. More rapid internalization of smancs than NCS was also elucidated under fluorescence microscopy at 37°C. There was no intracellular incorporation of these drugs below 20°C.

These results indicated that an increased lipophilicity of smancs appears to be responsible for its increased cell surface affinity, internalization rate, and toxic effect. Concordant to this interpretation was that among various copoly(styrene-maleic acid) esters, a more hydrophobic derivative showed more internalization (butyl > ethyl > carboxylate). These results suggest the possibility of utilizing protein tailoring to augment the subcellular activity of functional proteins.

INTRODUCTION

NCS3 is a proteinaceous antitumor antibiotic (M, ~11,700) obtained from the culture filtrate of Streptomyces carzinostaticus. Its primary structure has been reported previously (1-5). It is an acidic single-chain polypeptide with 113 amino acid residues, but with no carbohydrate. The molecular mechanism of action of NCS in bacteria and mammalian tumor cells is known to be the arrest of DNA synthesis by direct DNA strand scission involving its prosthetic group (6). In addition, Maeda et al. (7-10) have reported the subcellular fate of NCS and clarified its penetration into the cytosol and the nucleus of cancer cells by utilizing fluorescent or 14C-labeled NCS.

A lipophilic high-molecular-weight derivative of NCS designated as smancs was prepared by chemical conjugation of SMA and NCS (11-13). The derivative retains the original DNA-damaging properties of NCS in vitro (14). There are also major pharmacological improvements by this chemical modification: extension of biological half-life; enhanced tropism to solid tumor and lymphatics; and increased hydrophobicity (12-15). Those improvements in several pharmacological properties led to a pronounced antitumor effect against animal and human tumors (12-18).

However, the details of smancs action at the subcellular level remain to be clarified. The present study was undertaken to correlate the improved pharmacological characteristics of smancs and its cytotoxic action on cells. We therefore studied the binding and cytotoxicity of NCS, smancs, and various derivatives of SMA copolymers to HeLa cells and WISH cells in detail and to correlate the role of the SMA copolymer portion of smancs in its binding to the cell surface and the internalization process.

MATERIALS AND METHODS

Materials. NCS was obtained from Kayaku Co., Ltd., Tokyo, Japan. SMA anhydride (mean molecular weight, approximately 1600) was a generous gift from Kuraray Co., Ltd., Osaka, Japan; it is a water-soluble copolymer containing an average of 25-35% styrene residues with a few anhydride groups per repeating unit. SMA used for conjugation with NCS was a butyl ester derivative in which 50% (mol/mol) of the carboxyl groups of maleic acid were half-esterified with n-butyl alcohol unless specified. Both SMA and smancs were prepared in this laboratory or at Kuraray Co., Ltd. The details have been described elsewhere (12). Briefly, butyl-SMA was added to NCS in 0.5 M sodium bicarbonate at pH 8.5 intermittently, so that two amino groups of NCS reacted with anhydride. At least two SMA groups were conjugated through an amide linkage, one at Ala 1 and the other at Lys 20; thus, the drug used in the present study had an approximate molecular weight of 16,000 as estimated by polyacrylamide gel electrophoresis with sodium dodecyl sulfate and by elemental analysis (see Ref. 12). Absorption and fluorescence spectroscopy showed that smancs also possessed specific absorption of the nonprotein chromophore at about 340 nm and fluorescence at 425 and 490 nm with an excitation wavelength of 340 nm, similar to NCS (Fig. 1). Quantification of the chromophore content of smancs by spectroscopy showed that it has almost the same level of chromophore as that in parental NCS. These spectroscopic characteristics were not observed in pre-NCS, which was known to be an apoprotein. Smancs is soluble in both water and some organic solvents (11-13).

Newborn calf serum and MEM were obtained from Gibco, Grand Island, NY. Both FITC isomer I and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, a water-soluble carbodiimide, were purchased from Dojin Chemical Laboratories, Kumamoto, Japan. All other chemicals were from commerical sources.

Cells. HeLa and WISH cells were maintained in monolayer cultures at 37°C in MEM containing 10% heat-inactivated newborn calf serum. The cells were removed from culture bottles 1 day before use by treatment with 0.1% trypsin-0.05% EDTA in PBS and seeded onto plastic wells (Falcon) at a suitable concentration.

FITC Labeling. F-NCS, which possessed original antitumor activity,
was prepared by essentially the same method as described previously (19). In brief, 50 mg of NCS were added to 10 ml of 0.5 M sodium bicarbonate buffer, pH 8.3, containing 10 mg of FITC. After stirring for 4 h at 4°C, the reaction mixture was applied to a column (3 x 70 cm) of Sephadex G-25 previously equilibrated with distilled water, followed by lyophilization. During these processes, loss of biological activity of NCS in F-NCS did not occur. Inasmuch as smancs has no free amino groups to utilize to introduce the fluorescence probe, smancs was conjugated with L-lysine by using water-soluble carbodiimide and L-lysine (20). Fifty mg of smancs were added to 50 ml of a solution containing 100 mg of carbodiimide adjusted to pH 6.0 with 0.1 M HCl. To the mixture were added 200 mg of L-lysine, with stirring, at 4°C. During the reaction, the pH was maintained at 6.0 with 0.1 M HCl solution. After 1 h, the reaction mixture was applied to a column (3 x 70 cm) of Sephadex G-25 previously equilibrated with distilled water, after which it was lyophilized. The smancs-lysine conjugate was also labeled with FITC by the method used with NCS. Two types of SMA were similarly subjected for labeling with FITC. For H-SMA, all maleic anhydride in SMA was hydrolyzed to a carboxyl group after reaction with L-lysine. Butyl-SMA, which is the butyl ester derivative, is reacted with L-lysine in a manner similar to the reaction of SMA with NCS. Then, both H-SMA-Lys and butyl-SMA-Lys were labeled with FITC as described above.

Binding Studies. HeLa or WISH cells plated at a density of 5 x 10⁴ cells/well (wells were 16 mm in diameter) (No. 3047 Falcon 24-well plate) were washed twice with PBS, and medium was replaced with Krebs-Ringer phosphate buffer, pH 7.4. This medium is routinely utilized in all binding assays. A final volume of 0.5 ml containing a constant amount of F-NCS or F-smancs was used for binding studies at 0°C or 37°C for different times. At the end of the incubation period, supernatant was discarded and unbound material was rapidly removed by washing the plates three times with PBS. Cells were lysed in 1 ml of 20 mM Tris-HCl buffer, pH 8.5, containing 0.2% sodium dodecyl sulfate. The fluorescence intensity of the solubilized cell solution was measured with a fluorescence spectrophotometer (Hitachi Model 650-40) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. In this condition no quenching of fluorescence due to binding to cells was observed. The protein content was then quantified by the method of Lowry et al. (21) with bovine serum albumin as a standard. The value of cell-bound fluorescent drug (F-NCS or F-smancs) was determined from the relationship between the concentration and fluorescent intensity of each FITC-labeled drug.

For binding inhibition studies, a 100-fold molar excess of unlabeled NCS was added to FITC-labeled drug, and the total volume was kept at 0.5 ml. After the indicated period of incubation, the amount of cell-bound FITC-labeled drug was determined as described above.

The number of apparent binding sites per cell and the apparent association constants were determined from Scatchard plot analysis (22). The value of cell-associated fluorescent intensity at 0 time was subtracted from all groups as nonspecific binding. The subtracted values were usually less than 2% of the maximal fluorescent intensity of cell-associated labeled drug (e.g., 2 h, at 37°C). The data were expressed as mol bound per mg of cell protein.

Fluorescence Microscopy. For fluorescence microscopy HeLa cells were plated on 24-well Falcon No. 3047 plate and were used 2 days after plating. Cells were incubated with 100 µg/ml of FITC-labeled butyl-SMA or H-SMA in Krebs-Ringer phosphate buffer, pH 7.4. After 60 min of incubation at 37°C, cells were washed three times with PBS and then observed by fluorescence microscopy (inverted fluorescence microscope equipped with phase contrast; Model IMT-2; Olympus Co., Ltd., Tokyo, Japan) at x600.

RESULTS

Time Course of Binding and Internalization of F-NCS and F-smancs to HeLa Cells. The time course of F-NCS and F-smancs binding to HeLa cells was examined at drug concentrations of 8.3 and 6.3 nmol/ml, respectively (Fig. 2). The binding of both Fig. 2. Kinetics of binding of F-NCS (A) and F-smancs (B) to HeLa cells. Cell monolayers (5 x 10⁴ cells/well) were incubated with 100 µg/ml F-NCS (A) or F-smancs (B) at 0°C (C, Δ) or 37°C (Φ, △) for various times in the absence (C, Φ) or in the presence (Δ, △) of a 100-fold molar excess of unlabeled NCS. After incubation, the amounts of cell-bound FITC-labeled drugs were determined as described in the text. Each point represents the average of duplicate determinations.
F-NCS and F-smancs to HeLa cells was time dependent; it proceeded rapidly for the initial 30 min and then appeared to reach an equilibrium after about 2 h.

With respect to the effect of temperature on binding, the amounts of FITC-labeled drug bound to cells at 37°C were greater than those at 0°C. In general, endocytosis of cell-bound ligands was almost completely prevented at 0°C. Therefore, the increase in the amount of cell-bound drug at 37°C might be due to temperature-dependent internalization of the drugs. In the presence of a 100-fold molar excess of unlabeled NCS, binding of F-NCS and F-smancs was inhibited. These results suggested that the association of F-NCS with HeLa cells was mediated by a specific receptor (or binding site) on the cell surface and that F-smancs also bound to the same binding sites. However, the amounts of F-NCS and F-smancs bound to HeLa cells were different. After 2 h, the amount of F-smancs bound to HeLa cells was about 20 times greater than that of F-NCS.

Determination of Apparent Association Constants and the Number of Binding Sites. The increase in binding activity of smancs could be accounted for either by increasing the number of binding sites on the cell surface or by increasing the affinity for the binding sites and/or rapid internalization. To discriminate among these three possibilities, we measured the number of binding sites and the apparent association constant (K_a) by using HeLa and WISH cells. HeLa and WISH cells were incubated for 2 h with various concentrations of FITC-labeled drugs, and the amount of labeled drugs bound to each cell type was measured as described in the text. Scatchard plots of these binding data (Fig. 3) gave straight lines under the conditions used, which indicates that the binding sites possess a single affinity. The results are summarized in Table 1. The association constants of F-smancs for both HeLa and WISH cells were 10–26 times greater than that of F-NCS at 0°C, whereas the number of binding sites of these drugs was slightly greater for NCS than for smancs in WISH cells contrary to the data in HeLa cells. The interpretation of this difference in these drugs is difficult; WISH cells are of benign origin while HeLa cells are of malignant origin.

Role of Alky] Groups in the Ester of SMA Polymers on Cell Binding. SMA used in above experiments was a butyl ester derivative (butyl-SMA) in which 50% (mol/mol) of maleic acid carboxyl group were half-esterified with n-butyl alcohol. To investigate the role of the alkyl ester group of SMA itself on cell binding, we examined the binding of the FITC-labeled butyl-SMA, nonesterified carboxylate form of SMA (H-SMA), together with the respective conjugates with NCS (butyl- and H-smancs) similarly. Fig. 4 shows the time course of binding of these SMA derivatives. The results indicated that the amount of butyl-SMA bound to HeLa cells was 3–5-fold greater than that of H-SMA. Furthermore, butyl-smancs also showed stronger binding activity than ethyl-smancs or H-smancs. Since the longer alkyl chains possess a higher hydrophobicity, these results suggested that enhanced cell binding of butyl-SMA derivatives is partly due to interactions of cell membrane and the hydrophobic alkyl ester groups of the SMA polymer.

After exposure of HeLa cells to these FITC-labeled SMA derivatives, fluorescent microscopic examination was carried out and the result showed a strong fluorescence in the cell treated with butyl-SMA derivative (Fig. 5).
the increased binding affinity, we compared the cytotoxicity of NCS and smancs by assaying their cell-killing activity by the colony formation method. HeLa cells were exposed to a fixed concentration (30 nm) of each drug for various times (from 5 to 120 min) at 0°C or 37°C. Cells were then allowed to grow in a drug-free growth medium for colony formation. As shown in Fig. 6, at 37°C the surviving fractions exposed to smancs were immediately decreased, and about 80% of exposed cells were killed after only 5 min. In contrast, in the case of NCS, a much longer exposure period (more than 90 min) was required to kill 50% of the cells. On the other hand, at 0°C, after a prolonged exposure period (up to 2 h) no significant cytotoxicity of either NCS or smancs was observed. These results suggest that smancs is more rapidly internalized into the target cells than NCS and that its toxic action is a temperature-dependent phenomenon as was reported previously (10, 23).

DISCUSSION

The binding of F-smancs and F-NCS to cultured cells was examined in detail. The binding of the two FITC-labeled drugs to HeLa cells was dependent on temperature. In fact, the amounts of drugs bound to HeLa cells were about 2-fold greater at 37°C than at 0°C (Fig. 2). Transmembrane internalization may be attributed for this higher value at 37°C than that at 0°C. In the presence of a 100-fold molar excess of unlabeled NCS, binding of F-NCS and F-smancs was inhibited (Fig. 2). About 20-fold more association and perhaps internalization of smancs than NCS was evidenced by fluorescence intensity (Fig. 2, A versus B) and fluorescence microscopy after incubation with these drugs in which fluorescence was visible in the cells (Fig. 5). These results suggested that both NCS and smancs were internalized by a common receptor-mediated endocytosis similar to those of bacterial toxins such as of diphtheria, botulinum, and tetanus (24–26).

It is interesting that the amount of F-smancs bound to cells was about 20-fold greater than that of F-NCS at both cell lines (Table 1) and also both 37°C and 4°C (compare Fig. 2A and Fig. 2B). From Scatchard plot analyses (Fig. 3), it was found that the increase in binding activity of smancs could be attributed more to an increase in drug-receptor affinity rather than to an increase in the number of drug-binding sites (Table 1). Kᵣ values of smancs for HeLa and WISH cells were found to be 1.34–2.9 × 10⁵ M⁻¹ (Table 1), which were 15–20-fold greater than those of NCS (0.05–0.2 × 10⁵ M⁻¹). The association constant of 10⁴–10⁵ M⁻¹ appears relatively weak when compared with other toxins (10⁷–10⁹ M⁻¹).

These results were consistent with the more rapid toxic action of smancs compared with that of NCS (Fig. 6). At the same dose (30 nm), smancs achieved 50% inhibition of colony formation of control (no drug) with less than 5 min of exposure; in contrast, NCS needed more than 90 min to achieve same toxic effect. In our previous finding a correlation between the internalization of NCS into cells and its cytotoxicity and DNA-damaging effects was elucidated (7, 9, 10). The present finding of enhanced binding of smancs to cells and its rapid cell killing (Fig. 6) appears to indicate that the process of internalization is also rapid for smancs when compared with NCS.

A role of the hydrophobic component for cell binding and subsequent internalization was tested by the butyl ester, ethyl...
ester, and carboxylate forms of SMA copolymer and also for their corresponding conjugate to NCS (smancs). The results showed concordantly that, in both SMA copolymer and corresponding smancs, more binding to cells and internalization were observed in the butyl-SMA derivative than the carboxylate form of SMA (H-SMA). Furthermore, SMA with plain carboxylate form-conjugated NCS exhibited much greater cell binding and internalization than parental NCS. Therefore, these results clearly demonstrated that cell surface affinity and uptake velocity of NCS by cultured cells were enhanced by chemical conjugation with the hydrophobic polymer (SMA). It is likely that this enhanced affinity of smancs is due to interactions of the cell membrane and the hydrophobic SMA region of smancs.

On the other hand, it is now well known that cytotoxic, antitumor, and DNA-damaging activities of NCS were elicited by its nonprotein chromophore (6, 27, 28). Since smancs also possess a nonprotein chromophore of at least 95% parental NCS (Fig. 1), its cytotoxicity might be elicited by mechanisms similar to that for NCS. Namely, the A340/A235 ratio was 0.296 for NCS and 0.299 for smancs, similar to the reported value of 0.308 (29). However, the detail mechanisms of internalization of NCS or its chromophore remained to be clarified. Since the chromophore is tightly bound to apoprotein and its release does not easily occur under physiological conditions, the mechanism of chromophore release from NCS is not adequately elucidated at cellular level. Therefore, it is difficult at this point to comment on the different rate of release of chromophore from NCS and smancs which could result in different kinetics in cytotoxicity. In addition, SMA residue is also slowly liberated from smancs thus yielding native NCS.* The process is similar to demaleylation from a maleylated amino group. Thus the situation is very complicated. Studies are now in progress to clarify the mechanism of chromophore release.

Recently, we found that cell-bound NCS or smancs translocated from the cell surface, which had a slightly alkaline external medium, to the acidic microenvironment, which indicates the pH of the endosome or the lysosome. Furthermore, lysosomotropic agents such as NH4Cl and chloroquine protected HeLa cells against the cytotoxicity of NCS and smancs. These finding suggested that NCS and smancs with chromophore was internalized by endocytosis and acidic environment was important for the action of these drugs at the cellular level.

Inasmuch as both NCS and smancs act on DNA (7, 10, 14)* as described above, it is clear that these molecules must enter the cytoplasm through the plasma membrane or endocytotic vesicles (Fig. 2). A similar situation applies to diphtheria (24) and cholera (30) toxins. These findings on transmembrane movement of these bacterial toxins may have relevance for protein translocation across membranes in general. A well-known model proposed for this process is the signal hypothesis of Blobel and Dobberstein (31) in which the hydrophobic leader peptide or signal sequence forms a transmembrane channel through which a protein can penetrate the endoplasmic reticulum membrane. Another model is the direct transfer model of Von Heijne and Blomberg (32) and Wickner (33). In these models, the hydrophobic contributions of the leader sequence and subsequent amino acids lead to direct disruption of secreted proteins through the lipid bilayer.

In the case of smancs, the hydrophobic SMA portion appears to play an important role in the penetration of the lipid bilayer, similar to the hydrophobic leader sequence or signal peptide described above. In fact, smancs can be fully dissolved in some organic solvents such as pyridine and acetone or partially dissolved in the lipid contrast medium Lipiodol (11, 12, 16–18).

The anionic portion may also be important for internalization. Many polycations may bind to the cell surface but are not internalized. Carboxyl groups became hydrophobic in the acidic environment of the endosome by protonation; thus, they may facilitate the binding of anionic agents, including smancs, to the hydrophobic milieu of cell and cell membrane.

In conclusion, the findings obtained in this study exemplify that protein tailoring may be applied to toxic proteins or protein drugs to enhance the uptake velocity into the cells or to potentiate biological activity at the cellular or subcellular level.

REFERENCES


* Unpublished observations.


Conjugation with Lipophilic Styrene-Maleic Acid Copolymer Neocarzinostatin and Enhancement of Its Actions by Binding to and Internalization by Cultured Cells of Neocarzinostatin

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