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The Acidic Groups of the Neocarzinostatin Protein Play an Important Role in Its Biological Activity

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SUMMARY

Despite the extensive literature on the mechanism of action of the anticancer agent neocarzinostatin (NCS), the role of the protein moiety is still not clear. The model involving endocytosis of intact holo-NCS has been dismissed in favor of a theory proposing entry of free dissociated chromophore. However, the fact that the NCS protein has a certain affinity for cell membranes cannot be disregarded. In the present work the protein moiety has been modified by transformation of the carboxyl groups into isopropylamide groups. This modification resulted in a broad shift of the protein pI towards higher values, accompanied by a marked reduction of toxicity *in vitro* and reduced binding of protein to cells. Coincubation of modified NCS with inactive native apo-NCS led to restoration of the biological activity of native holo-NCS. It has been shown chromatographically that the modified NCS is capable of transferring active chromophore to native apo-NCS. The inactive form (apoprotein) of the charge-modified NCS, however, is capable of inhibiting the toxicity of the active form, as has been described for the corresponding native pair. Binding experiments with tritium-labeled proteins revealed that the modified protein has diminished affinity for membranes, in comparison with native NCS.

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NCS (molecular mass, 11,400 Da) is an acidic protein antitumor drug isolated from *Streptomyces carzinostaticus* culture medium (1). NCS consists of a polypeptide component (apo-NCS) and a tightly ($K_d \approx 1 \times 10^{-10}$ M) (2) but noncovalently bound chromophore. The chemical structure of the chromophore was elucidated by Edo *et al.* (3) and bears a novel bicyclo[7,3,0]dodecadienediyne system, α-D-*N*-methylfucosamine, 2-hydroxy-7-methoxy-5-methyl-1-naphthalenecarboxylic acid, an ethylene cyclic carbonate, and a highly strained ether epoxide. NCS, like similar compounds such as esperamicin, calichemicin, and dynemicin (4–6), produces its antibiotic and antitumor activity by conversion into an active biradical intermediate that abstracts hydrogen atoms from the sugar-phosphate backbone of DNA (7, 8). The mechanism of this process is associated with the Bergmann cycloaromatization reaction (9).

Whereas the mechanism of DNA cleavage is well understood, the role of the protein moiety, apart from stabilizing the labile chromophore, is still unknown. Earlier studies using high doses of fluorescently labeled and radiolabeled NCS suggested that the intact holo-NCS permeates into the interior of cells and cell nuclei (10–12). Receptor-mediated uptake of NCS was assumed, despite the very low binding affinity of NCS, in the range of 10^{-4} to 10^{-5} M^{-1} (13). On the other hand, it was found that agarose-immobilized NCS retained its activity (14). More recent experiments strongly suggest that the majority of the cytotoxicity of NCS results from the direct entry of dissociated chromophore into the cell (15, 16). The current model for the action of intact holo-NCS proposes a carrier or transport function for the protein moiety, stabilizing the labile chromophore and perhaps supporting its extrusion across the cell membrane into the cytoplasm.

To examine whether the protein moiety assists the chromophore in penetrating cell membranes, we altered the surface charge by chemical modification of the abundant acidic groups, to obtain CM-NCS, and we determined the effect of this modification on the biological activity of the drug.

Materials and Methods

Modification of carboxyl groups in NCS. Clinical-grade NCS generously provided by Kayaku Antibiotics (Tokyo, Japan) was used. The apo-form of NCS was prepared as described by Povirk and Goldberg (2). The procedure for modification was essentially that of Hoare and Koshland (17). Isopropylamine (0.8 M) was coupled to 10 mg of holo-NCS (3.6 mg/ml) or apo-NCS with the activating agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (80 mM), in 3

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ABBREVIATIONS: NCS, neocarzinostatin; CM-NCS, charge-modified neocarzinostatin; PBS, phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumin.

ml of 0.2 M NaCl, pH 4.0, for 4 hr at room temperature. The pH of the reaction mixture was adjusted to pH 4.0 by addition of traces of 200 mM NaOH during the course of the reaction as necessary. CM-NCS was then separated from excess reagents and the resulting urea by gel filtration on Sephadex G25 and was stored in 50 mM PBS, pH 7.2. The concentration of active CM-NCS was determined using the fluorescence assay for the NCS chromophore reported by Gottschalk *et al.* (18).

Preparation of ^{3}H-labeled NCS. Both native apo-NCS and the corresponding CM-NCS (apo-form) were labeled following the method of Kummer (19). Briefly, 200 μg of protein were incubated with 2×10^{-8} mol (0.74 MBq) of *N*-succinimidyl [2,3-^{3}H]propionate (Amersham) for 3 hr at room temperature. Excess radiolabel and unwanted reaction products were removed by gel filtration. The two radiolabeled products were diluted with the corresponding unlabeled protein to give preparations of equal specific activity (2.4×10^{12} Bq/mol).

Biological assays. The germination of *Bacillus subtilis* spores on agar plates was used to determine the biological activity of the drug preparations. Inhibitory zones were evaluated using a calibration curve under standardized conditions (agar diffusion test).

Osteogenic sarcoma 791T cells (1×10^4/well) were incubated with the corresponding drug preparation in Dulbecco's modified Eagle's medium (GIBCO/BRL) in a microtest plate for 1 hr, followed by a 46-hr recovery phase in medium with 10% FCS. The cells were then incubated with 3.7 kBq of [^{3}H]leucine (specific activity, 2.11 TBq/mmol; Amersham) for an additional 6 hr. Finally, the cells were harvested onto glass filter membranes and the radioactivity was counted in a Beckmann LS300 scintillation counter.

Binding studies. Eppendorf vials were pretreated with a 1% solution of BSA for 30 min at 4°. Each vial was then loaded with 1×10^5 791T cells in 50 mM PBS plus 1% BSA, pH 7.2, and treated with suitable dilutions of ^{3}H-labeled protein (specific activity, 2.40×10^{12} Bq/mol) in 50 mM PBS, pH 7.2, supplemented with 1% BSA, at 4° for 1 hr (all samples were in triplicate, in a volume of 200 μl). The resultant cell-bound radioactivity was determined after two successive washing and centrifugation steps.

Results

Characterization of CM-NCS. Transforming the carboxyl groups into isopropylamide groups led to a shift of the protein pI towards higher values. Isoelectric focusing of the modified NCS revealed a mixture of several distinct components with pI values of 3.5, 3.7, 3.9, and 4.1, as well as some minor fractions including residual native NCS at pI 3.3 (Fig. 1).

The substitution of carboxyl groups in NCS was determined by reflector-type time of flight mass spectrometry using a UV-matrix-assisted laser desorption ionization mass spectrometry method. The results showed a distribution with a maximum at 11,283 mass units, compared with native NCS at 11,100, representing an average substitution of 4.5 carboxyl groups. No NCS dimers were observed either by matrix-assisted laser desorption ionization or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PhastSystem; Pharmacia), where only a single band was seen (data not shown).

Fluorescence measurements proved that the chemical modification did not lead to a significant inactivation of the chromophore. It could be shown by repeated assays of the same batch, over a period of 3 months, that the modified NCS was as stable as native NCS in PBS, pH 7.2. The chemical modification, as observed by the isoelectric focusing pattern, proved to be stable as well.

The transfer of active chromophore between CM-NCS and native apo-NCS was also investigated, to determine whether the modification of the protein moiety affects the chromophore

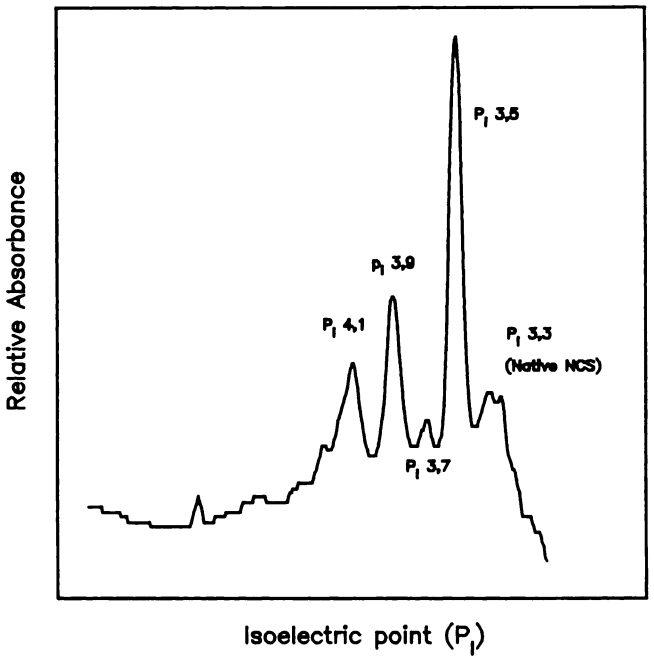

Fig. 1. Densitrometric scan of an isoelectric focusing gel of CM-NCS (see text for details). The masked NCS consists of a mixture of four proteins with pI values of 3.5, 3.7, 3.9, and 4.1, with little residual unmodified NCS, as determined with a calibration standard.

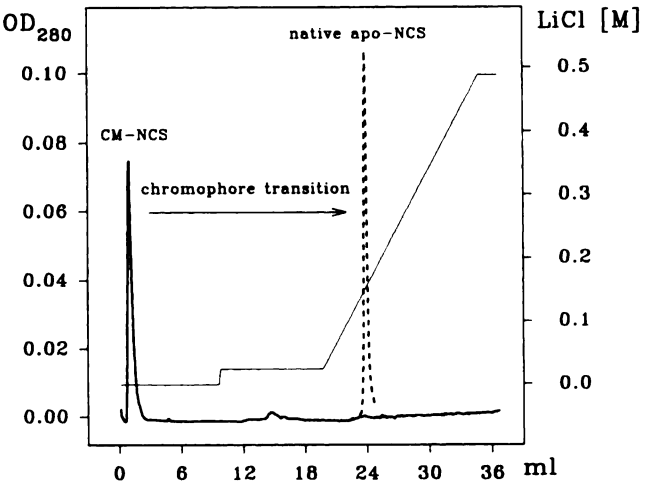

Fig. 2. Ion exchange chromatography of CM-NCS using a Mono-Q column. Buffer A was 20 mM piperazine, pH 5.0, and buffer B was buffer A plus 0.5 M LiCl. Additionally, the peak of the corresponding chromatogram of pure native apo-NCS (*dashed line*) has been overlaid to demonstrate the chromophore transition.

binding affinity. As shown earlier, holo-NCS and apo-NCS can be separated by ion exchange chromatography using a Mono-Q column (Pharmacia) (20). Under those conditions the modified NCS did not bind to the column, in either the active or the inactive form (Fig. 2).

When apo-NCS is mixed with modified NCS and a transfer of chromophore occurs, native holo-NCS should be detectable. Both proteins were prepared in buffer A (20 mM piperazine, pH 5.0). Equimolar amounts were mixed, incubated for various times at room temperature, and analyzed on a Mono-Q column. Fig. 3 shows the resulting chromatogram after a reaction time of only 1 min. Longer incubation times did not increase the concentration of holo-NCS, indicating that equilibrium had

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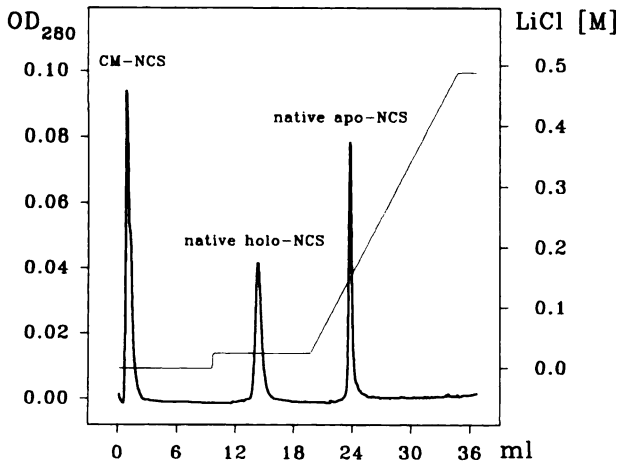

Fig. 3. CM-NCS (as chromophore donor) and native apo-NCS (as chromophore acceptor) were mixed and allowed to interchange chromophore for 1 min at room temperature (in 20 mM piperazine, pH 5.0) before separation with a Mono-Q column. The resulting peak of native holo-NCS is clearly visible.

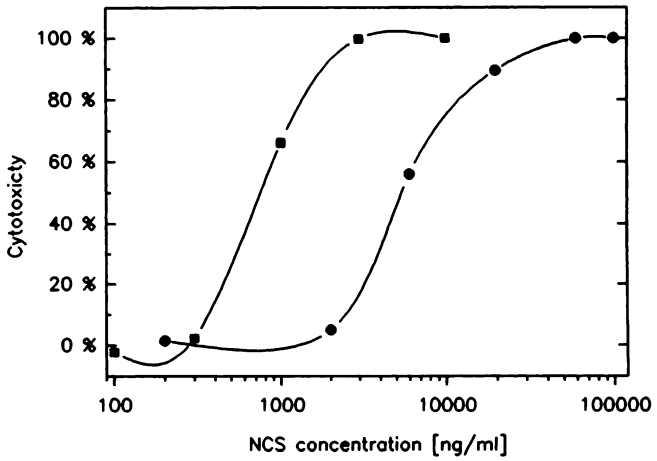

Fig. 4. Cytotoxicity assay using 791T cells. The cells were incubated for 1 hr with CM-NCS (●) or native NCS (■) in medium without serum, followed by a 46-hr recovery period in medium with 10% FCS (see also text).

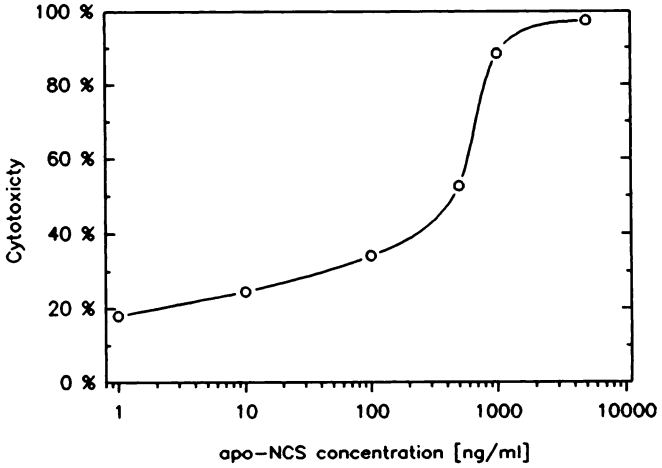

Fig. 5. Cytotoxicity assay using 791T cells. The cells were incubated for 1 hr with a constant amount of 4 μg/ml CM-NCS and additional native apo-NCS in medium without serum, followed by a 46-hr recovery period in medium with 10% FCS.

been attained in this short reaction time. From the integrated peak areas and taking into account the extinction coefficients of the reactive partners, we calculated that comparable concentrations of CM-NCS and native apo- and holo-NCS were obtained. These results strongly suggested that the chromophore affinities of CM-NCS and native NCS were comparable.

Agar diffusion assay. The biological activity of the modified NCS, as determined by the agar diffusion test, was reduced by a factor of 20, compared with native NCS, in the concentration range up to 200 μg/ml (data not shown).

An additional experiment was conducted to determine the presence of active chromophore in the modified protein. A constant amount of 200 μg/ml CM-NCS was mixed with different quantities of native apo-NCS, incubated for 1 hr at room temperature, and then applied to an agar diffusion test plate. Because native NCS and CM-NCS have the same binding affinity for the chromophore, the latter should be transferred to apo-NCS until the equilibrium has formed, restoring native holo-NCS. Because native NCS exhibits greater toxicity than does the corresponding modified drug, one would expect an increase of biological activity, depending on the amount of native apo-NCS added. A continuous increase of toxicity up to a factor of 11, with a molar ratio of apo-NCS to CM-NCS of 2.5, has been observed (data not shown). Although the chromophore-binding properties of CM-NCS and native NCS are similar, the biological activities at comparable chromophore concentrations are considerably different. This result supports our assumption that the protein moiety of the drug influences the biological activity of the chromophore. We have therefore extended our investigations to human cell lines.

Cytotoxicity assay. A cytotoxicity assay using the osteosarcoma cell line 791T revealed that CM-NCS was about 8 times less toxic than native NCS (Fig. 4). The IC_{50} value for CM-NCS was 6000 ng/ml, and the corresponding value for native NCS was 700 ng/ml.

An experiment analogous to the one described above was performed to allow the expression of the cytotoxic activity of CM-NCS as determined by fluorometric estimation of the active chromophore concentration. A constant amount of 4 μg/ml CM-NCS was coincubated with various quantities of native apo-NCS. The mixtures were kept overnight at 4°, allowing the interchange of chromophore to occur. Again, an increase in toxicity was obtained, showing the presence of active chromophore (Fig. 5).

Furthermore, we investigated whether the apo-form of the modified NCS is able to inhibit the toxicity of the active form, as described for the corresponding native pair. The cells were incubated with a constant amount of 10 μg/ml CM-NCS and various ratios of apo-CM-NCS, with BSA as a control. As shown in Fig. 6, the apo-form of CM-NCS indeed reduced the activity of native holo-NCS.

Binding studies. Both native and modified apo-NCS were tritium labeled with a specific activity of 2.40×10^{12} Bq/mol. As shown in Fig. 7, the surface-modified NCS (masked with isopropylamide groups) has a significantly lower affinity for cell membranes than does native NCS. It is noteworthy that the proportion of native NCS remaining cell bound after two washing steps was very small, approximately 2% of input activity. This reflects the low affinity reported for binding of NCS to cells (13).

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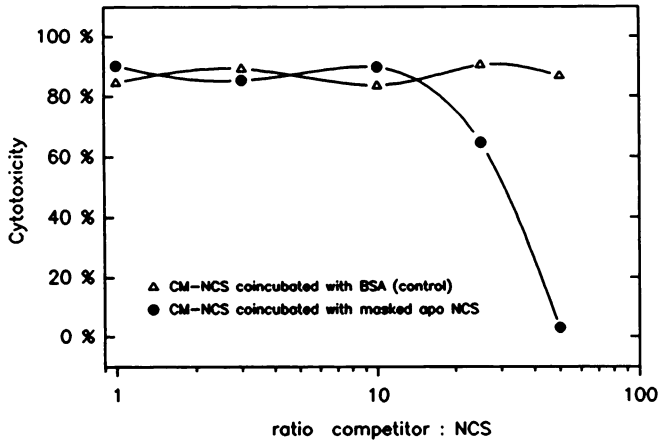

Fig. 6. Inhibition toxicity assay. The 791T cells were coincubated for 1 hr with a constant amount of 10 μg/ml CM-NCS and various amounts of apo-CM-NCS (●) or BSA (control) (△) in medium without serum, followed by a 46-hr recovery period in medium with 10% FCS.

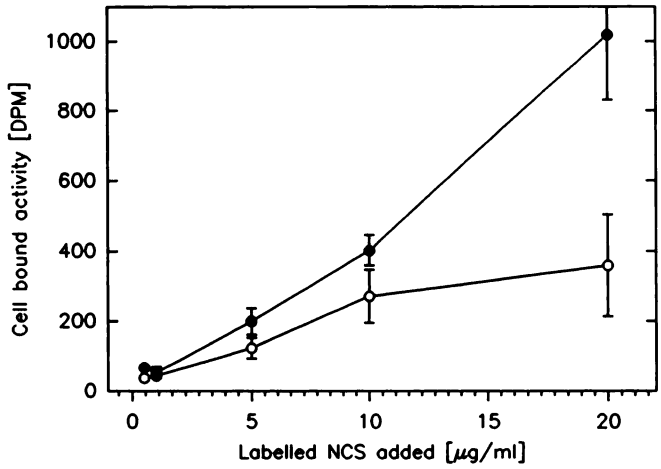

Fig. 7. Binding studies. Incubation of 10^5 791T cells with tritium-labeled native apo-NCS (●) and apo-CM-NCS (○) (both with the same specific activity of 2.396×10^{12} Bq/mol) for 2 hr at 4°. After two washing steps the cell-bound activity was counted.

Discussion

NCS is a single-chain polypeptide with 113 amino acid residues, 11 of them possessing carboxyl groups (21). The acidic groups have been masked by chemical modification with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and isopropylamine, resulting in isopropylamide bonds. Isoelectric focusing demonstrated that the modification was successful and stable. Fluorescence measurements revealed that the chromophore stability was not affected by the reaction and that the preparation was stable in PBS at 4° in the dark for several months, thus resembling the behavior of native NCS.

The highly reduced toxicity of CM-NCS in the agar diffusion test (20-fold), compared with the cytotoxicity test (where a factor of only 8 was observed), cannot be attributed to reduced mobility of CM-NCS, because the molecular weight difference was only 2% and dimers were not found. We therefore conclude that CM-NCS was less effectively bound to germinating *B. subtilis* spores.

CM-NCS, however, exhibited markedly reduced biological activity not only in the agar diffusion test but also in cytotoxicity assays with the osteogenic sarcoma cell line 791T. Incubation of the modified protein with native apo-NCS led to an increase of toxicity, due to chromophore interchanging and equilibrating between the proteins, restoring native holo-NCS. It could be shown by ion exchange chromatograpy that the interchange indeed occurred and that the equilibrium was formed in <1 min at room temperature (at pH 5.0). The inactive form of CM-NCS was able to inhibit the toxicity of the active form, exactly as described for the corresponding native pair (22). This experiment corroborates the assumption that the apo-form inhibits the activity of NCS by scavenging free dissociated chromophore, following the mass action law, rather than by occupying a putative binding site.

Binding studies with tritium-labeled proteins revealed that CM-NCS had a diminished ability to bind to cell surfaces. From these experiments it can be concluded that the carboxyl groups of the protein are involved only in enhancing the uptake of NCS-chromophore by increasing the interaction between the protein and the cell surface. It is unlikely that the acidic groups affect the affinity of the protein for the chromophore, because the latter is readily transferred to apo-NCS until an equilibrium, with equimolar peak fractions, has formed (Fig. 3).

Apart from protecting the chromophore from degradation, the protein apparently functions to transport the chromophore and maintain it near the cell membrane, where it is enriched in comparison with the surrounding liquid. The dissociated chromophore then enters the cell on its own and subsequently exhibits its DNA strand-breaking activity. The decreased toxicity of CM-NCS, in comparison with native NCS, is due to the statistical distribution between the cell surface and the solution. However, these results do not exclude the possibility that the protein plays an active role in transport of the chromophore across the cell membrane; preliminary results with a CM-NCS immunoconjugate indicate that an accumulation of chromophore near the cell membrane is sufficient to increase the toxicity even beyond that of native NCS. With respect to the application of NCS for immunotherapy of cancer, the modified protein appears to be a promising choice to increase selectivity.

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