THE BIOLOGICAL EFFECT OF A NONPROTEIN COMPONENT REMOVED FROM NEOCARZINOSTATIN (NCS)

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(Received for publication March 10, 1980)

The biological effects of both nonprotein component (NPC) and PC (protein component) from NCS have been studied *in vivo* and *in vitro*. NPC was found to not only inhibit DNA synthesis in growing cells but also induce DNA degradation *in vivo* and *in vitro*. However, neither these two biological activities of PC were detected even at a 100-times higher concentration of NPC (0.2 μg/ml) which inhibited 50% DNA synthesis in growing cells. NPC-induced DNA degradation *in vitro* was stimulated by 2-mercaptoethanol as has been reported for NCS. These results show that the NPC removed from NCS is responsible for the biological activities such as the inhibition of DNA synthesis in growing cells and the induction of DNA degradation *in vivo* and *in vitro*.

Neocarzinostatin (NCS), an antitumor antibiotic, is a single polypeptide with a molecular weight of 10,700 with two disulfide bonds[1,2]. Low levels of NCS selectively inhibit DNA synthesis in sensitive bacteria and mammalian cells[3,4] and also induce DNA degradation *in vitro* and *in vivo*[4~11]. A correlation appears to exist between the ability of NCS to induce DNA degradaiton in HeLa cells and its inhibition of DNA synthesis and cell growth[12]. However, both the mechanism of DNA degradation and the inhibitory action of DNA synthesis induced by NCS are still not completely understood. Recently, we and others have reported separately that NCS possesses a nonprotein component (designated as NPC) that can be removed by methanol extraction[13~15]. We determined that a part of the chemical structure of a nonprotein component from NCS is a derivative of naphthalenecarboxylic acid[14]. In addition, it has been postulated that a nonprotein component (nonprotein chromophore) of NCS may be responsible for the inhibition of DNA synthesis and DNA degradation induced by NCS[17].

The present study was undertaken to examine the biological activities, *i.e.*, inhibition of DNA synthesis in growing cells and induction of DNA degradation *in vivo* and *in vitro*, by NPC. Available evidence suggests that NPC directly cleaves DNA molecules *in vitro* and the stimulating effect of 2-mercaptoethanol on NPC-induced DNA degradation *in vitro* was also observed as it has been reported for NCS[4~11].

Materials and Methods

Chemicals

NCS was kindly supplied by Dr. Y. KOYAMA (Kayaku Antibiotic Res. Corp., Tokyo). [^{3}H]-Thymine (30 Ci/mmol) and [^{3}H]thymidine (TdR) (50 Ci/mmol) were obtained from New England Nuclear Corp., calf thymus DNA from Sigma Chemical Corp., poly dA, poly dT, poly dG, poly dC,

Abbreviations used are: NCS, neocarzinostatin; NPC, a nonprotein component from NCS; PC, a protein component from NCS; TdR, thymidine; SDS, sodium dodecyl sulfate.

poly dA: poly dT, poly dG: poly dC and poly rI: poly rC from Miles Laboratories, Sephadex LH-20 from Pharmacia.

NPC and PC preparation

All preparation procedures were carefully carried out in a dark room. NCS powder (65 g) was suspended in a liter of 0.1 N HCl - methanol (1: 10) and stirred for 2 hours at 4°C. After centrifugation (5,000×*g* for 20 minutes), the supernatant (crude NPC preparation) was concentrated *in vacuo* to dryness. The resulting precipitate was relyophilized and used as a crude PC preparation (about 50 g). The crude NPC (about 13 g) was dissolved in 200 ml of 0.1 N HCl - methanol (1: 10) and applied to a Sephadex LH-20 column (3.0×110 cm) as previously described[14,15]. The column was eluted with 0.1 N HCl - methanol (1: 10). The NPC fraction which exhibited inhibitory activity against the growth of *Sarcina lutea* PCI 1001 was obtained. The active fractions were pooled and evaporated to dryness to brownish crystalline NPC (170 mg). On the other hand, the crude PC was further purified through Sephadex G-25 and CM-cellulose columns, successively, as previously reported[14]. PC was purified from NCS with a protein yield of less than 20%.

[^{3}H]T_4 phage DNA preparation

For [^{3}H]T_4 phage DNA preparation, *Escherichia coli* B3 was grown in a medium containing 10 mM potassium phosphate (pH 7.4), 10 mM $(NH_4)_2SO_4$, 2 μM $Fe(NH_4)_2(SO_4)_2$, 1% glucose and 5 μg/ml of thymine for 6 hours at 37°C. The bacteria (5×10^8 cells/ml) were infected with T_4 phage and then reincubated for 3 hours at 37°C in the presence of [^{3}H]thymine (2.0 μCi/ml). After harvesting, the bacteria were suspended in 20% (v/v) CCl_4 and centrifuged at 5,000×*g* for 20 minutes. DNase (2 μg) and RNase (2 μg) were added to the supernatant and incubated for 40 minutes at 37°C. After centrifugation (35,000× *g* for 25 minutes), the [^{3}H]T_4 DNA was extracted from the phage by the SDS phenol method[17]. Finally, [^{3}H]T_4 phage DNA was purified by $CsCl_2$ density gradient centrifugation.

Sedimentation analysis of degraded DNA

The degraded DNA by NPC was analyzed by a 5~25% (w/v) alkaline sucrose density gradient centrifugation. After [^{3}H]T_4 phage DNA was incubated with NPC under the indicated conditions, the reaction mixture (0.2 ml) was directly layered on a 4.8 ml of 5~25% alkaline sucrose density gradient containing 0.5 M NaOH, 0.15 M NaCl and 1 mM EDTA. After centrifugation (105,000× *g* for 4.5 hours at 4°C), the gradient was divided into 10 drop fractions by dripping from the bottom of the tube. The [^{3}H] radioactivity of the indicated fractions were determined in a liquid scintillation spectrometer.

Results

Effect of NPC on DNA Synthesis in HeLa S_3 Cells

The effect of NPC on the incorporation of [^{3}H]TdR into the acid-insoluble fractions of HeLa S_3 cells was examined and its inhibitory activity was compared with that of PC and NCS. Table 1 shows that low levels of NPC (0.2 μg/ml) highly inhibit the incorporation of [^{3}H]TdR into the acid-insoluble fractions as compared with that of NCS, whereas no effect of PC on the [^{3}H]TdR incorporation into DNA synthesis was detected at even a 100-times higher concentration of NPC. PC inhibited 9.9% DNA synthesis in HeLa S_3 cells when 200 μg/ml of PC was added to the culture medium. These results showed that NPC inhibits DNA synthesis in the cells at 0.1 the concentration than that of NCS and also suggested that NPC of NCS is responsible for the inhibition of DNA synthesis in the cells.

NPC-induced DNA Degradation

After DNA of HeLa S_3 cells was labelled with [^{3}H]TdR, the cells were exposed to NPC or PC, separately, for 3 hours at 37°C. The [^{3}H] radioactivity released from the cells treated with NPC into the culture medium was detected and increased in proportion to the incubation period and NPC con-

Table 1. Effect of NPC, PC and NCS on the incorporation of [^{3}H]TdR into the acid insoluble fractions.

Addition	Concentration (μg/ml)	[^{3}H]TdR incorporation (cpm)	Inhibition (%)
None (control)	—	11,032	0
NPC	0.2	5,455	50.6
	2.0	1,025	90.7
	20.0	283	97.4
PC	2.0	11,023	<0.1
	20.0	11,005	<0.3
	200.0	9,939	9.9
NCS	0.2	8,052	23.5
	2.0	3,280	70.3
	20.0	473	95.7

HeLa S_3 cells (2.5×10^5 cells) were incubated with either NPC, PC or NCS at the indicated concentrations in MEM medium containing 2.0 μCi/ml of [^{3}H]TdR (50 Ci/mmol) for 2 hours at 37°C. The incorporation was stopped by the addition of 1.0 ml of 20% trichloroacetic acid (TCA) to the cell cultures. The [^{3}H] radioactivity of the acid-insoluble fractions was determined after a pass through glass-filter paper as previously reported[6,7].

Fig. 1. DNA cleaving activity of NPC and PC in growing cells.

HeLa S_3 cells (1.2×10^5 cells) were previously cultured in MEM medium containing 10% calf serum and [^{3}H]TdR (2.0 μCi/ml) for 6 hours at 37°C. After the cells were washed with fresh MEM medium and further cultured for 12 hours at 37°C. The labelled cells (2.5×10^5 cells) were recultured in 1 ml of MEM medium in the presence of either NPC (○—○) or PC (●—●) at the indicated concentrations. After 12-hours incubation at 37°C, the [^{3}H] radioactivity released from the cells into the culture medium (0.1 ml) was determined in a liquid scintillation spectrometer.

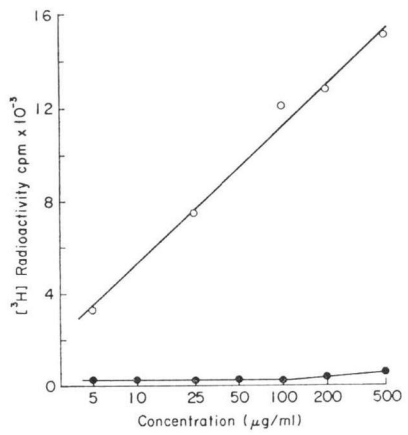
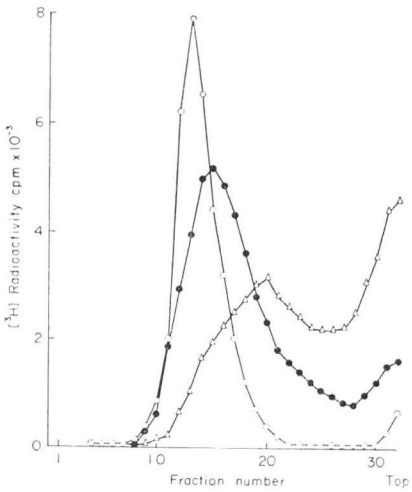


Fig. 2. NPC-induced DNA degradation *in vitro*.

[^{3}H]T_4 phage DNA ($A_{260}=1.2$) was incubated with NPC (5 μg/ml) in the presence or absence of 2-mercaptoethanol (2 mM) for 20 minutes at 37°C. The reaction mixture (0.2 ml) was layered on a 4.8 ml of 5~25% alkaline sucrose density gradient containing 0.5 M NaOH, 0.15 M NaCl and 1 mM EDTA, then centrifuged at 105,000× g for 4.5 hours at 4°C. After centrifugation, the gradients were divided into 32 fractions. The ^{3}H radioactivity of the indicated fractions were determined. ○—○, control; ●—●, NPC (5 μg/ml); △—△, NPC (5 μg/ml) + 2-mercaptoethanol (2 mM).



centrations, but no DNA degradation was detected in PC-treated cells (Fig. 1). The ability of NPC to induce DNA degradation *in vitro* as well as to inhibit DNA synthesis in growing cells was detectable at 0.1 the concentration than in the case of NCS.

NPC-induced DNA degradation in HeLa S_3 cells was confirmed by *in vitro* experiments using [^{3}H]T_4 phage DNA. The [^{3}H]T_4 phage DNA was incubated with NPC in the presence or absence of 2-mercaptoethanol which stimulates DNA degradation induced by NCS *in vitro*[10]. Fig. 2 shows that NPC degradates [^{3}H]T_4 phage DNA *in vitro* and the DNA degradation is stimulated greatly by 2-mercaptoethanol (2 mM). The ability of NPC to induce DNA degradation in the presence of 2-mercaptoethanol was detected at 0.1 the concentration than that of NCS (2 μg/ml), as described above. However, no DNA degradation was observed when the DNA was incubated with PC even at a concentration of 200 μg/ml in the presence or absence of 2-mercaptoethanol (2 mM) (data not shown).

Sequence Specificity in DNA Scission by NPC

The above experiments clearly showed that NPC not only inhibits DNA synthesis in growing cells but also induces DNA degradation *in vivo* and *in vitro*. In addition, it has shown that NCS creates single strand breaks in DNA at the position of adenine and thymine in the presence of 2-mercaptoethanol[8,10]. Therefore, to determine the sequence specificity in the DNA which is preferentially degraded by NPC, the [^{3}H]T_4 phage DNA was incubated with NPC (5 μg/ml) in the presence or absence of homopolymers such as poly dA, poly dT, poly dG, poly dC, poly dA: poly dT, poly dG: poly dC and poly rI: poly rC.

The effect of homopolymers on NPC-induced DNA degradation *in vitro* is summarized in Table 2. NPC-induced DNA degradation was reduced significantly when poly dT or poly dA: poly dT was added to the reaction mixture, whereas no significant effect of other homopolymers on the DNA degradation was observed. These results show that NPC cleaves preferentially the high AT sequence in the DNA as has been reported for NCS[8,10].

Table 2. Effect of homopolymers on NPC-induced DNA degradation *in vitro*.

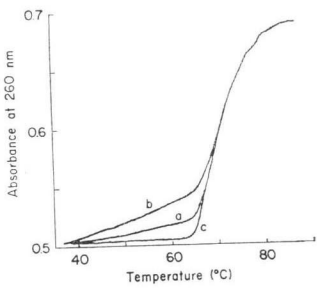
Addition	Radioactivity of acid-insoluble fraction (cpm)
None	11,000
NPC	4,805
NPC+poly dA	7,733
NPC+poly dT	9,155
NPC+poly dG	5,320
NPC+poly dC	5,498
NPC+poly dA: poly dT	9,715
NPC+poly dG: poly dC	5,221
NPC+poly rI: poly rC	5,008

The reaction mixture (0.1 ml) contained 15 mM NaCl, 1.5 mM sodium citrate, 5 μg of [^{3}H]T_4 phage DNA (2,200 cpm/μg) and 2 mM 2-mercaptoethanol. The mixture was incubated for 3 hours at 37°C in the presence of a different homopolymer (5 μg). The reaction was stopped by the addition of 0.5 ml of 0.2 M sodium pyrophosphate containing 1 mg/ml of bovine serum albumin and 0.1 mg/ml of heat denatured calf thymus DNA and 0.5 ml of 20% trichloroacetic acid (TCA). The ^{3}H radioactivity of TCA precipitate fraction was determined as previously reported[6,7].

Fig. 3. Effect of NPC on the thermal melting of DNA.

Calf thymus DNA dissolved in 1/10 SSC (0.15 M NaCl and 0.015 M sodium citrate) was incubated with NPC (5 μg/ml) in the presence or absence of 2-mercaptoethanol (2 mM) for 30 minutes at 37°C. The thermal melting of the DNA (A_{260}=0.5) was determined by photospectrometer (Beckman model Acta III) at the temperature increase rate of 0.7°C/min.

c, control; a, NPC (5 μg/ml); b, NPC (5 μg/ml)+2-mercaptoethanol (2 mM).



Effect of NPC on the Thermal Melting of DNA

Since the above experiments suggested that NPC cleaves preferentially the high AT sequence in the DNA, a preliminary experiment to compare the thermal melting profile of DNA incubated or unincubated with NPC (5 μg/ml) in the presence or absence of 2-mercaptoethanol (2 mM) was carried out. Fig. 3 shows that the thermal melting of DNA remarkably increased at low temperatures (below 60°C) when the DNA was incubated with NPC for 30 minutes at 37°C. 2-Mercaptoethanol (2 mM), which greatly stimulated DNA degradation induced by NPC or NCS, highly enhanced an increase of thermal melting of DNA at low temperature levels. Although an increase of DNA thermal melting induced by NPC was reproduced by NCS under the same conditions, the similar increase of DNA melting at low temperatures by NCS required a 10-times higher concentration of NPC (data not shown). No difference between Tm (71.4°C) of control DNA and that of DNA incubated with NPC in the presence of 2-mercaptoethanol was observed (Fig. 3). Also, no effect of PC (200 μg/ml) on the thermal melting of DNA was observed (data not shown).

Discussion

We presented evidence which shows that a nonprotein component (NPC) removed from NCS not only inhibited DNA synthesis in growing cells but also induced DNA degradation *in vitro* and *in vivo* at low levels (0.2 μg/ml). The 50% inhibition of DNA synthesis in growing cells was at about 0.1 the concentration than that observed for NCS on a weight basis (Table 1). In addition, 2-mercaptoethanol greatly enhanced NPC-induced DNA degradation *in vitro* in a manner similar to that reported for NCS[8,10]. However, neither of these two biological activities of PC (protein component from NCS) were detected even at a 100-times higher concentration than that of NPC so far tested. These results strongly suggested that NPC from NCS is responsible for two biological activities, *i.e.*, the inhibition of DNA synthesis in growing cells and the induction of DNA degradation *in vitro* and *in vivo*. Since it has been reported that NCS blocks the G_2 phase of cell cycle[18], there is a possibility that PC may cause a suppression of G_2 phase through alteration of membrane-cytoskeletal systems by the protein[19]. However, the inhibitory mechanism of the G_2 block by PC and NCS is unknown in the present time.

Recently, two research groups have reported separately that NCS creates single breaks in DNA at the position of adenine and thymine in the presence of 2-mercaptoethanol[8,10]. In this study, we found that the thermal melting of DNA increases at low temperature levels when incubated with NPC, and also found that 2-mercaptoethanol significantly stimulates the DNA melting induced by NPC (Fig. 3). This finding suggests that NPC-induced DNA breakage may begin at the high AT base pair in the DNA. This possibility was supported by the following evidence: (a) unwinding of the DNA chain begins at the region of the high AT base pair and proceeds to the region of a progressively higher GC content; (b) NPC-induced DNA breakage *in vitro* is significantly reduced by the addition of poly dT or poly dA: poly dT (Table 2); and (c) NCS breaks DNA at the position of adenine and thymine[8,10]. Moreover, there is another possible explanation in that NPC-induced increase of DNA melting at low temperature levels may result in the restrictive nick at the position of adenine or thymine, because there is no difference between Tm of control DNA and that of DNA incubated with NPC (Fig. 3). Taken together, provided evidence suggests that both NPC-induced DNA degradation *in vitro* and NPC-induced inhibition of DNA synthesis in growing cells may be due to the same mechanisms as those have been reported for NCS[4~8]. Also, the stimulating effect of 2-mercaptoethanol on these two biological activities of NCS was observed with the activities of NPC at 0.1 the concentration than that of NCS.

As it has been shown previously that the *in vitro* DNA cleaving activity of NCS is rapidly inactivated by light or UV-irradiation[13,20], the ability of NPC to induce DNA degradation *in vitro* was more rapidly inactivated by UV-irradiation as compared with that of NCS under the same conditions.

A possibility that the biological activity of NPC may be stabilized by its binding to PC was rejected since no effect of PC on the biological effect of NPC was observed (data not shown). Although we reported that PC has no biological effect against the growth of NPC-sensitive bacteria, the biological activity of NPC on bacterial growth is greatly enhanced when PC is reconstituted with NPC[15]. This observation shows that there is still the possibility that PC can stabilize the biological activity of NPC under the some suitable conditions.

The mechanisms of both DNA degradation and inhibition of DNA synthesis induced by NCS are very similar to those reported for bleomycin[21,22]. Macromomycin, a macromolecular antibiotic, inhibits DNA synthesis and also induces DNA degradation in growing cells[23]. Auromomycin is converted to macromomycin after passing through Amberlite XAD-7 column chromatography[24]. Auromomycin has a nonprotein component (chromophore) which induces DNA degradation *in vitro*[25] in a manner analogous to that shown here in NPC. The complete chemical structure of NPC and the binding site of NPC on the NCS molecule are now under active study.

Acknowledgments

We are grateful to Drs. T. EBINA and S. ISEKI for valuable discussions and to Dr. Y. KOYAMA for a generous gift of NCS and to Dr. Y. KOIDE for technical help of NPC and PC preparations. This work was supported in part by grants from the Ministry of Health and Welfare of Japan and the Sendai Institute of Microbiology.

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