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Actinomycin D Amplifies Site-Specific DNA Cleavage Induced by Neocarzinostatin

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Neocarzinostatin (NCS) is an antineoplastic antibiotic causing DNA cleavage. Actinomycin D (ActD) is an antineoplastic antibiotic, which does not cleave DNA strands. We examined the mechanism of ActD-mediated amplification of NCS-induced DNA cleavage using ^{32}P-labeled DNA fragments obtained from the human c-Ha-*ras*-1 proto-oncogene. NCS plus glutathione caused DNA cleavage at thymine and adenine residues in the absence of ActD. The addition of ActD enhanced the DNA cleavage at the sites at which the lesions on opposite strands are staggered 2 bases in a 3′ direction, particularly at the 5′-TCT-3′·3′-AGA-5′, 5′-TGT-3′·3′-ACA-5′ and 5′-ACT-3′·3′-TGA-5′ sequences, suggesting that ActD amplified double-stranded DNA cleavage. The mechanism of ActD-mediated amplification of NCS-induced DNA cleavage can be explained by assuming that binding of ActD to DNA changes the DNA conformation to allow NCS to bind to DNA at the specific sequences. © 1997 Academic Press

Neocarzinostatin (NCS) is an enediyne antitumor antibiotic, which consists of a chromophore (NCS-Chrom) and an apoprotein (1). The chemical structure of NCS-Chrom is shown in Fig. 1. The mechanism by which NCS causes DNA cleavage has been extensively investigated. The naphthoate moiety of NCS-Chrom intercalates between DNA base pairs. The enediyne core is activated in the presence of thiols, such as glutathione (GSH), to form a diradical species causing DNA cleavage (2-6). NCS-Chrom is located in the minor groove of DNA, and abstracts hydrogen atom(s) of the deoxyribose resulting in single- and double-strand breaks of DNA in a site-specific manner. The mechanism and the site specificity of NCS-induced DNA cleavage depend on DNA structure (7-16) and might be changed by the alteration of DNA structure.

Most antitumor drugs cause severe side effects. It is expected that nontoxic amplification of DNA-cleaving activity of antitumor drug enables to treat cancer more effectively and decreases the dose of the drug resulting in reduction of its toxic effects. We have reported that the site-specificity and intensity of DNA cleavage induced by antitumor drugs are changed in the presence of certain DNA-binding drugs, which do not cause DNA damage (17, 18).

Actinomycin D (ActD) is an antitumor antibiotic, which is known as a drug to inhibit mRNA synthesis. ActD consists of a phenoxazone ring and two cyclic pentapeptides (Fig. 1), and does not cause DNA cleavage. The phenoxazone ring of ActD intercalates between two base pairs, particularly at the 5′-GC-3′ sequence, and each pentapeptide is located in the minor groove of the DNA helix to form a hydrogen bond with a deoxyguanosine residue to stabilize the DNA-ActD complex (19, 20). The intercalation of ActD unwinds the DNA helix and widens the minor groove (21). It is interesting to clarify whether the alteration of DNA conformation changes the site specificity of DNA cleavage by NCS.

In the present study, we investigate the effect of ActD on DNA cleavage induced by NCS. We examined the site specificity of DNA cleavage induced by NCS and the changes in the site specificity by the addition of ActD using ^{32}P-5′- and ^{32}P-3′-end-labeled DNA fragments obtained from the human c-Ha-*ras*-1 proto-oncogene.

MATERIALS AND METHODS

Materials. Restriction enzymes (AvaI, XbaI, and PstI) and T_4 polynucleotide kinase were purchased from New England Biolabs. [γ-^{32}P]-ATP and [α-^{32}P]dATP were from New England Nuclear. The Klenow fragment of DNA polymerase I of *Escherichia coli* was from Takara Shuzo Co. (Otsu, Japan). NCS was from Pola Kasei Co. (Japan). GSH was from Nacalai Tesque Inc. (Kyoto, Japan). ActD was from Sigma

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Abbreviations: NCS, neocarzinostatin; NCS-Chrom, neocarzinostatin chromophore; GSH, glutathione; ActD, actinomycin D; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid.

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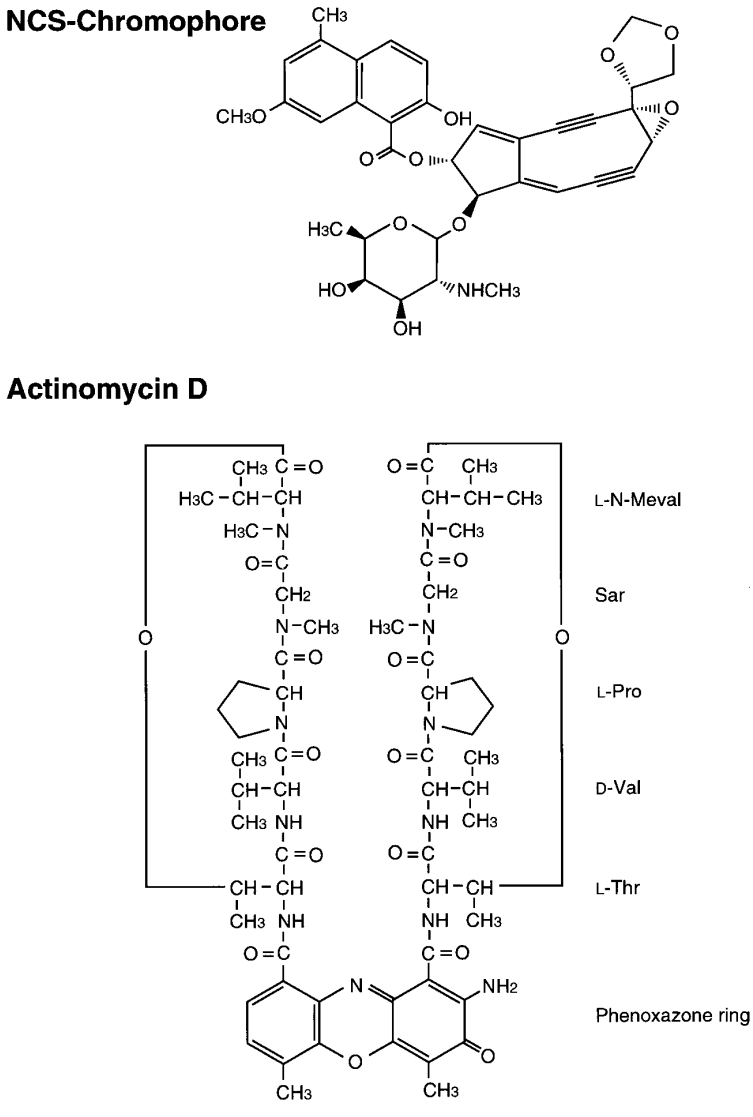

FIG. 1. Chemical structures of NCS-Chrom and ActD.

Chemical Co. Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) was from Dojin Chemicals Co. (Kumamoto, Japan).

Preparation of ^{32}P-labeled DNA fragments from the c-Ha-ras-1 proto-oncogene. DNA fragments were prepared from plasmid pbcNI, which carries a 6.6-kilobase BamHI chromosomal DNA segment containing the human c-Ha-*ras*-1 proto-oncogene (22, 23). The DNA fragments were labeled at 5′ end with [γ-^{32}P]-ATP, and the singly labeled the 341-base pair fragment (*Xba*I 1906-*Ava*I* 2246), 98-base pair fragment (*Ava*I* 2247-*Pst*I 2344) and 337-base pair fragment (*Pst*I 2345-*Ava*I* 2681) were obtained according to the method described previously (22, 23). The asterisk indicates ^{32}P-labeling and nucleotide numbering starts with the *Bam*HI site (24). The 3′-end labeled DNA fragments was obtained by extension of the 3′ termini of the DNA fragments with the Klenow polymerase in the presence of [α-^{32}P]-ATP.

Detection of DNA cleavage induced by NCS in the presence of ActD. The standard reaction mixture in a microtube (1.5-ml Eppendorf) contained 0.5 μM NCS, 1 mM GSH, indicated concentration of ActD, [^{32}P]DNA fragment and 10 μM/base sonicated calf thymus DNA in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37 °C for 30 min, the DNA fragments were treated and electrophoresed as described previously (22, 23). The DNA fragments were not treated with piperidine. The autoradiogram was obtained by exposing an X-ray film to the gel. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure (25) using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 Ultro-Scan XL) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

RESULTS

Effect of ActD on NCS-induced DNA cleavage. Fig. 2 shows the autoradiogram of DNA cleavage induced by NCS and ActD. NCS caused DNA cleavage in the presence of GSH (*Lane 1*). NCS alone did not cause DNA cleavage under the condition used (data not shown). The patterns of DNA cleavage by NCS were

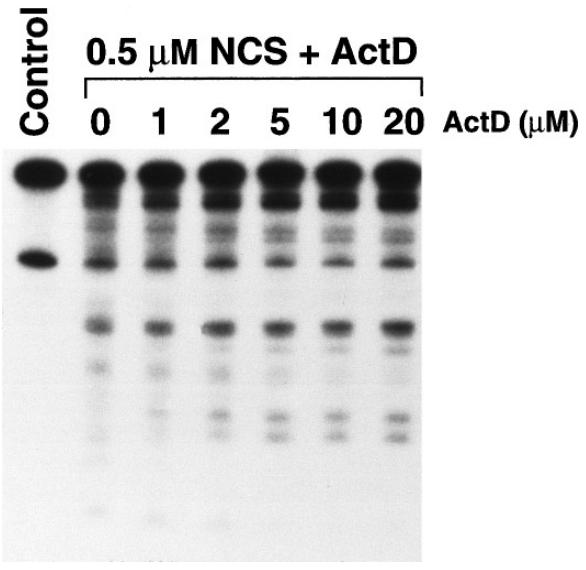

FIG. 2. Autoradiogram of ^{32}P-labeled DNA fragments incubated with NCS plus ActD. The reaction mixture contained the ^{32}P-5′-end-labeled 337-base pair DNA fragment, 10 μM/base of sonicated calf thymus DNA, 0.5 μM NCS, 1 mM GSH and indicated concentration of ActD in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated for 30 min at 37 °C. The DNA fragments were electrophoresed on an 8 % polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing an X-ray film to the gel. Control contained none of NCS, GSH and ActD.

changed slightly by the addition of 1 μM ActD (*Lane 2*), and changed apparently by higher concentrations of ActD (*Lanes 3-6*). DNA cleavage was not caused by 200 μM ActD alone (data not shown).

Alteration in the site specificity of NCS-induced DNA cleavage by ActD. Figs. 3-5 show the relative intensity of the DNA cleavage induced by NCS and ActD, obtained by scanning the autoradiograms with a laser densitometer. NCS plus GSH induced DNA cleavage, particularly at thymine and adenine residues in the absence of ActD (Figs. 3-5). Addition of ActD changed the site specificity of DNA cleavage by NCS. The DNA cleavages at the 5′-TCT-3′·3′-AGA-5′, 5′-TGT-3′·3′-ACA-5′ and 5′-ACT-3′·3′-TGA-5′ sequences were enhanced by the addition of ActD (Figs. 3-5). On the other hand, DNA cleavage at 5′-GCT-3′·3′-CGA-5′ was inhibited by ActD (Figs. 3-5, underscoring shows the cleavage sites).

DISCUSSION

In the present study, we investigated the effect of ActD on NCS-induced cleavage of DNA fragments obtained from the human c-Ha-*ras*-1 proto-oncogene in relation to the amplification of antineoplastic effect of NCS. NCS plus GSH induced DNA cleavage at thymine and adenine residues in the absence of ActD. Addition of ActD changed the patterns of DNA cleavage by NCS and enhanced the cleavages particularly at the 5′-TCT-3′·3′-AGA-5′, 5′-TGT-3′·3′-ACA-5′ and 5′-ACT-3′·3′-TGA-5′ sequences. It has been reported that NCS produces bistranded lesions with cleavage sites on opposite strands staggered 2 base pairs in a 3′ direction in the presence of GSH (2-6). Our results indicate that ActD enhanced double-stranded DNA cleavage induced by NCS. The changes of the site specificity of NCS-induced DNA cleavage would be due to the conformational changes of DNA induced by ActD. Two pentapeptides of ActD are located in the minor groove to form a hydrogen bond

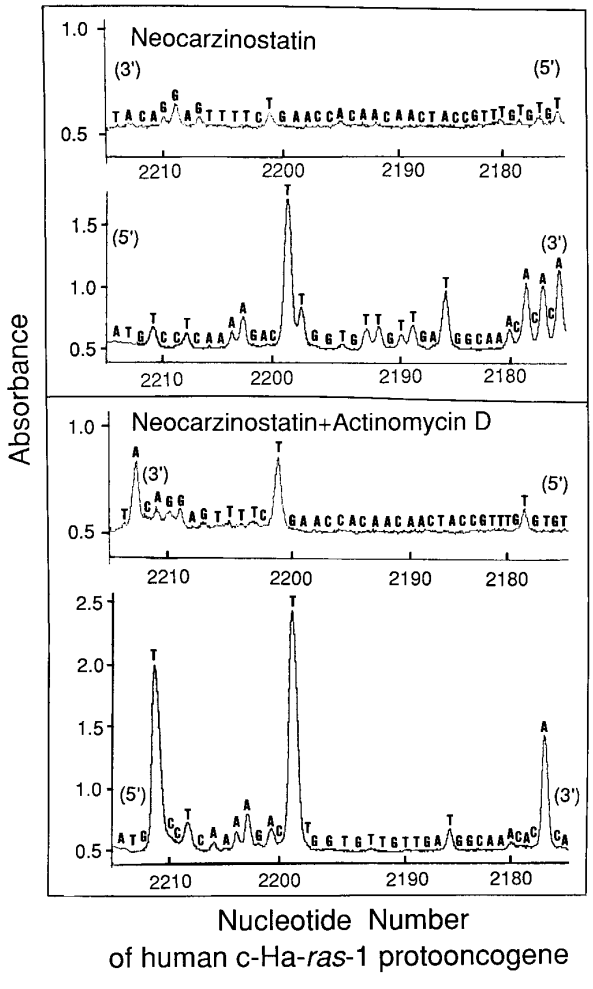

FIG. 3. Site specificity of DNA cleavage induced by NCS plus ActD. The reaction mixture contained the ^{32}P-5′- or ^{32}P-3′-end labeled 341-base pair fragment (*Xba*I 1906-*Ava*I* 2246), 10 μM/base sonicated calf thymus DNA, 0.5 μM NCS, 1 mM GSH and 50 μM ActD in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated for 30 min at 37 °C. The DNA fragments were electrophoresed on an 8 % polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing an X-ray film to the gel. The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL). *Abscissa,* nucleotide number of the human c-Ha-*ras*-1 proto-oncogene starting with the *Bam*HI site (24).

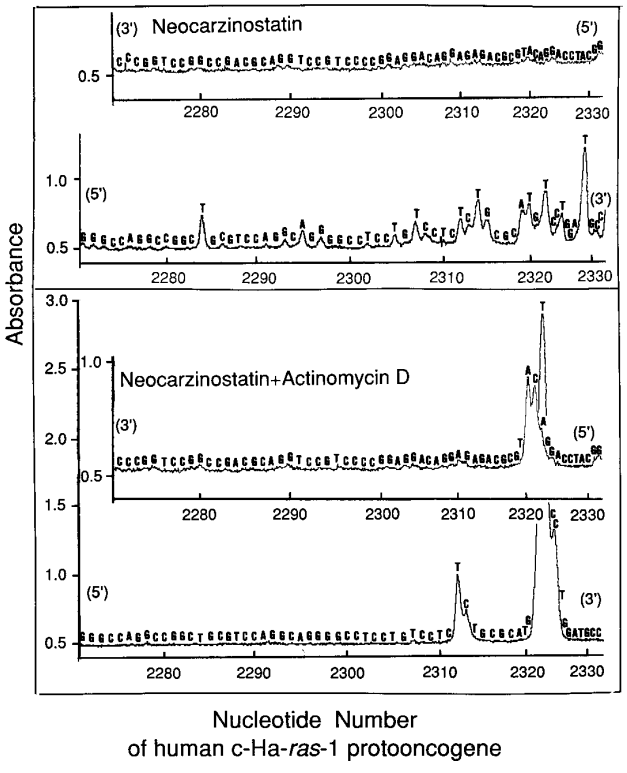

FIG. 4. Site specificity of DNA cleavage induced by NCS plus ActD. The reaction mixture contained the ^{32}P-5′- or ^{32}P-3′-end labeled 98-base pair fragment (*Ava*I* 2247-*Pst*I 2344), 10 μM/base sonicated calf thymus DNA, 0.5 μM NCS, 1 mM GSH and 50 μM ActD in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated for 30 min at 37 °C. The autoradiogram was obtained and analyzed by the method described in the legend to Fig. 3.

with deoxyguanosine residue (19, 20). The phenoxazone ring of ActD intercalates between base pairs particularly at the 5′-GC-3′ sequence to unwind the DNA double helix and widen the minor groove (19-21). NCS-Chrom is located in the minor groove of DNA to cause bistranded lesion particularly at the 5′-AG<u>C</u>-3′·3′-<u>T</u>CG-5′ sequence, consisting of a strand break at the thymine residue and an abasic site at the cytosine residue (2-4). Unwinding DNA helix by ActD would allow NCS-Chrom to bind to DNA at the specific sequences such as 5′-TC<u>T</u>-3′·3′-<u>A</u>GA-5′, 5′-TG<u>T</u>-3′·3′-<u>A</u>CA-5′ and 5′-AC<u>T</u>-3′·3′-<u>T</u>GA-5′ sequences. On the other hand, NCS-induced DNA cleavage at the 5′-AG<u>C</u>-3′·3′-<u>T</u>CG-5′ sequence, the most preferable site of NCS-induced bistranded lesion, was inhibited by ActD. This can be explained by the intercalation of ActD between the 5′-GC-3′ sequence in the 5′-AG<u>C</u>-3′·3′-<u>T</u>CG-5′ sequence, which inhibits the binding of NCS to the DNA helix.

Mechanisms and site specificity of NCS-induced DNA cleavage have been reported in relation to the DNA structure. Mismatch of DNA base pairs resulted in switching of the chemistry of DNA damage induced by NCS-Chrom (7, 8). NCS-Chrom induced bulge-specific cleavage in DNA (9-14). Bulge-specific cleavages have also been observed in HIV-1 transactivation response region RNA and its DNA analogue (15). These studies have suggested that NCS exhibits its unique actions for DNA strand break depending on DNA structure. The present study has demonstrated that the extent and the site specificity of DNA cleavage by NCS can be changed by the addition of ActD, suggesting that DNA structure modulated by DNA binding molecules determines the mechanisms of actions of antitumor drugs causing DNA cleavage.

It is well known that a number of chemotherapeutic drugs cause severe side effects. NCS also causes side effects, such as bone marrow suppression and gastrointestinal symptoms (1). As a method to approach a new chemotherapy to treat cancer more effectively and to

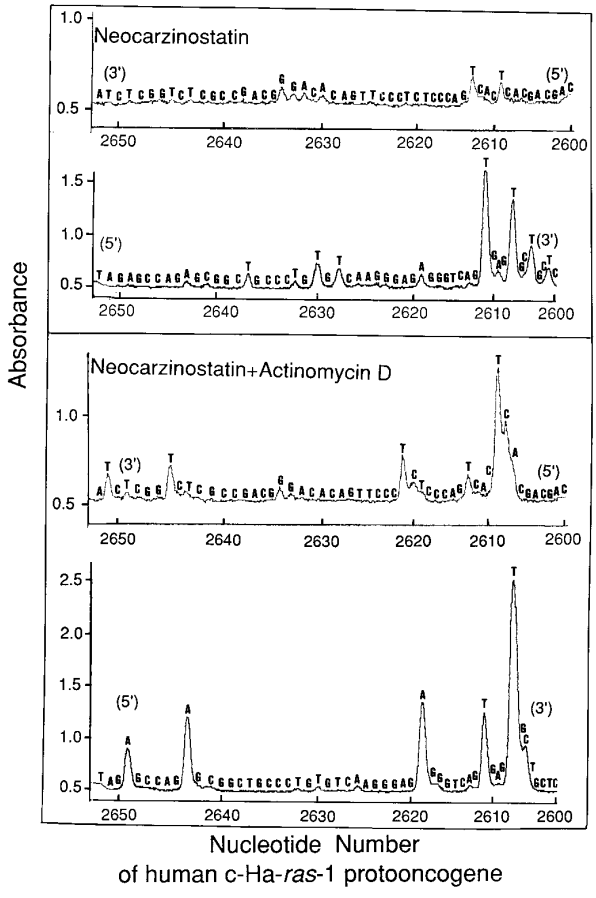

FIG. 5. Site specificity of DNA cleavage induced by NCS plus ActD. The reaction mixture contained the ^{32}P-5′- or ^{32}P-3′-end labeled 337-base pair fragment (*Pst*I 2345-*Ava*I* 2681), 10 μM/base sonicated calf thymus DNA, 0.5 μM NCS, 1 mM GSH and 50 μM ActD in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated for 30 min at 37 °C. The autoradiogram was obtained and analyzed by the method described in the legend to Fig. 3.

reduce toxic side effects, we have demonstrated the DNA cleavages induced by antitumor drugs were enhanced by DNA-binding drugs, which do not cause DNA damage (17, 18). In the present study, we have shown that DNA cleavage by NCS can be enhanced by the addition of ActD, a DNA-binding drug. Further investigation of the combination of chemotherapeutic drugs is needed for the establishment of more effective chemotherapy.

ACKNOWLEDGMENTS

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