AN ELECTRON SPIN RESONANCE STUDY OF A SPIN ADDUCT OF THE NON-PROTEIN COMPONENT (NPC) OF NEOCARZINOSTATIN

Sir:

The antitumor antibiotic neocarzinostatin (NCS) produced by *Streptomyces carzinostaticus* var. F-41 had been believed to be a single acidic polypeptide chain of molecular weight 10,700 with two disulfide bonds[1,2]. Recently, we[3] and others[4] separated a non-protein component (NPC), or so called "chromophore", from the protein component (PC) of NCS. In addition, we have isolated a 1-naphthalenecarboxylic acid derivative as a partial component of NPC[5]. In the course of our studies on these two components of NCS, we have found that NPC, and not PC, plays the principal role in the biological activities of NCS, such as growth inhibition of bacteria and tumor cells[3], inhibition of DNA synthesis in cultured mammalian cells as well as strand scission of DNA *in vivo* and *in vitro*.[6] Important in the understanding to these biological responses are the results of investigations of the effects of 2-mercaptoethanol (2-ME) and several organic solvents on the structural conformation and biological activities of NCS[7,8] and the suggestions[9,10] of the role of a free radical mechanism for NPC-induced DNA strand scission. Therefore, we have attempted to clarify the generation of a radical(s) from NCS and NPC and its involvement in DNA strand scission induced by NCS or NPC. In this communication, the generation of a free radical from NPC in the presence of 2-ME is demonstrated on the basis of results of an electron spin resonance (ESR) study of the specific spin adduct of N-*tert*-α-butyl-phenylnitrone (BPN). The signal intensity of this radical parallels the extent of DNA degradation induced by NPC.

Purified NCS, NPC, and PC were gifts from Kayaku Antibiotics Research Laboratory Co. Ltd. ^{3}H-Thymidine-labeled *E. coli* DNA was prepared by the SDS-phenol method[11]. Degradation of ^{3}H-thymidine-labeled *E. coli* DNA by drugs was quantified by counting the radioactivity released into the 5% trichloroacetic acid-soluble fraction. ESR spectra were recorded on a Japan Electron Optics Laboratory JES-FE-9 ESR spectrometer equipped with 100 KHz field modulation. The g-value was determined with Mn^{2+} in MgO powder as a field marker, with an accuracy of $\pm$0.0005. Hyperfine coupling was obtained by comparison with peroxylamine disulfide [spacing 13.0G]. The reaction mixture for spin trapping consisted of 500 μg/ml or 1,000 μg/ml of drugs and 50% saturated BPN in 0.5 M phosphate buffered solution (pH 7.4).

Initially, the effects of various compounds on the DNA-cleaving activity of NCS and NPC in terms of release of acid-soluble radioactivity from ^{3}H-thymidine-labeled *E. coli* DNA were determined (Table 1). The enhancement by 2-ME of DNA-cleaving activity of NCS has been established previously[7,8]. In the present work, 2-ME was found to enhance NPC-induced DNA degradation as much that induced by NCS. Thus, NPC itself requires 2-ME for exerting its DNA-cleaving activity. It has been reported that several organic solvents including isopropanol (ISOP), in spite of their known role as free radical scavengers are reported to enhance the DNA-cleaving activity of NCS in the presence of 2-ME[8]. In this work, we have confirmed that 1 M of ISOP enhanced 2-ME-dependent DNA degradation by NCS by more than three times. However, 2-ME-dependent DNA degradation by NPC was unaffected, or slightly reduced in the presence of ISOP.

Next, the effects of several specific radical scavengers on the NDA-cleaving activity of NPC were examined. As shown in Table 1, superoxide dismutase, an enzyme known to remove superoxide anions, and α-D-mannitol, a scavenger of certain free radicals including hydroxyl radical ($\cdot$OH), did little inhibit NPC-induced DNA degradation. However, 0.5 mM of α-D-tocopherol was found to reduce NPC-induced DNA degradation by approximately 40%. These effects of radical scavengers are in agreement with those reported on the DNA-cleaving activity of NCS[9,10].

In the last several years several groups[12,13] have suggested that certain anticancer drugs such as bleomycin and adriamycin induce DNA strand scission through mechanisms involving the generation of free radicals such as superoxide anion ($\cdot O_2^-$) and $\cdot$OH, which can be analyzed by ESR spectra. Consequently, ESR studies on NCS and its components were performed. Fig. 1 shows the ESR spectrum of the radical formed by NPC (500 μg/ml) in the presence of 2-ME (5 mM). This spectrum has the parameters:

Table 1. The effect of various agents on the DNA degradation by NPC and NCS.

Drugs	Acid-soluble radioactivity	
	cpm	%
NPC	<25	<2
NPC+2-ME	1,165	100
NPC+ISOP	<25	<2
NPC+2-ME+ISOP	980	84
NPC+2-ME+SOD	974	84
NPC+2-ME+α-D-mannitol	970	83
NPC+2-ME+α-D-tocopherol	666	57
NCS	<25	<4
NCS+2-ME	700	100
NCS+ISOP	<25	<4
NCS+2-ME+ISOP	2,130	300

NPC: non-protein component (100 μg/ml), NCS: neocarzinostatin (100 μg/ml), 2-ME: 2-mercaptoethanol (5 mM), ISOP: isopropanol (1 M), SOD: superoxide dismutase (10 units), α-D-mannitol (5 mM), α-D-tocopherol (0.5 mM).

^{3}H-Labeled *E. coli* DNA (total 25,000 cpm/20 μg) was incubated with the agents indicated for 3 hours in 37°C and the radioactivity solublized in 5% TCA was counted.

g=2.006, a^N=15.6G and a^H_β=4.1G. These values are different from those known for the ·OH or $\cdot O_2^-$ spin adduct of BPN (Table 2)[14]. NCS produced the same kind of radical as NPC as indicated by the same signal pattern in the ESR spectrum but no radical was detected for PC (data not shown). For the quantification of the radical formation by NCS and NPC, the intensity of the ESR signal was measured as the maximum height of the first derivative curve. Using this parameter, the effects of various compounds on the radical formation by NCS and NPC were examined (Table 3).

2-ME enhanced the generation of radicals by NCS and NPC by several times. On the other hand, ISOP slightly enhanced 2-ME-dependent radical formation by NCS but slightly reduced that by NPC. Of the free radical scavengers, SOD and α-D-mannitol were little effective but α-D-tocopherol was effective in reducing NPC-induced radical formation. These effects of various agents on radical formation induced by NCS or NPC parallel with those on the DNA degradation induced by the two compounds.

Fig. 1. ESR spectrum from NPC (500 μg/ml) in the presence of 2-mercaptoethanol (5 mM) and saturated N-*tert*-butyl-α-phenylnitrone (BPN).

The conditions of ESR spectroscopy: microwave power, 20 mW; modulation amplitude, 1G; time constant, 1 second; scan time, 8 minutes.

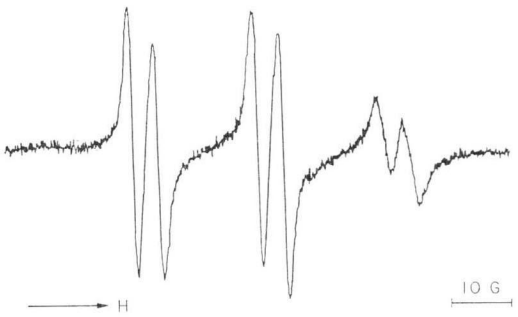

Table 2. ESR parameters for spin adducts of NCS, NPC, ·OH and $\cdot O_2^-$ radicals.

	a^H*	a^H_β*	g
NCS radical	15.6	4.1	2.006
NPC radical	15.6	4.1	2.006
·OH radical**	15.3	2.75	2.0057
$\cdot O_2^-$ radical**	14.8	2.75	2.0057

* Gauss.

** Reference in 14.

Therefore, the data strongly suggest that the generation of a radical plays an important role in the DNA-cleaving activity of NCS and its active component, NPC.

The analysis of the ESR spectra (Table 2) and the effects of several free radical scavengers (Table 3), suggest that the radical formed by NCS or NPC is neither $\cdot O_2^-$ nor ·OH, but presumably a peroxyl radical, which is known to be removed by α-D-tocopherol[9,10]. In this regard, NCS is different from several other agents such as bleomycin and adriamycin, which as proposed generate many kinds of free radicals following the initial formation of $\cdot O_2^-$ followed by oxidation of reductive moieties of these drugs. Although the elucidation of the mechanism of radical formation by NCS and its relationship with the biological activities of NCS requires further investigation, the following possible mechanism of NCS-induced DNA strand scission *in vitro* may by suggested. NCS is a loose complex of PC and NPC, a molecular

Table 3. The effect of various agents on the radical generation by NPC and NCS.

Drugs	Signal intensity*	%
NPC	54	25
NPC+2-ME	220	100
NPC+ISOP	124	56
NPC+2-ME+ISOP	154	70
NPC+2-ME+SOD	200	91
NPC+2-ME+α-D-mannitol	176	80
NPC+2-ME+α-D-tocopherol	116	53
NCS	33	37
NCS+2-ME	89	100
NCS+ISOP	30	34
NCS+2-ME+ISOP	92	103

NPC: non-protein component (500 μg/ml), NCS: neocarzinostatin (1,000 μg/ml), 2-ME: 2-mercaptoethanol (5 mM), ISOP: isopropanol (0.2 M), SOD: superoxide dismutase (10 μg), α-D-mannitol (5 mM), α-D-tocopherol (0.1 mM).

* The signal intensities were measured by peak-to-peak height of first derivative curve.

assembly not linked through a covalent bond which can be dissociated in the presence of organic solvents such as ISOP. 2-ME may also have a dissociating effect. The free NPC is then converted from its inactive form to an active form in the presence of reducing agents such as 2-ME. This active form of NPC reacts with oxygen to produce a free peroxyl radical, which leads directly or indirectly to single strand scission of DNA. The role of PC may be protection of NPC from conversion to the active form.

One important question is: What components *in vivo* have the roles of ISOP or 2-ME found in the *in vitro* experiments? We have to elucidate the mechanism of activation of NCS in living organisms which gives rise to the generation of free radicals and subsequent DNA damage. Another question is: Is DNA damage an exclusive aspect of the biological activities of NCS? If the generation of a free radical is the first step in the action of NCS, it may then cause a variety of cell damage involving not only DNA strand scission but also damage to membrane lipids or various enzymes and structural proteins. At least a part of the biological effects of NCS might be explained by the action of a radical on the cell components other than nucleic acids. We are now engaged in active study on these respects of the action of NCS and its components on living organisms.

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References

1) ISHIDA, N.; K. MIYAZAKI, K. KUMAGAI & M. RIKIMARU: Neocarzinostatin, an antitumor antibiotic of high molecular weight. J. Antibiotics, Ser. A 18: 68~76, 1965
2) MEIENHOFER, J.; H. MAEDA, C. B. GLASER, J. CZOMOS & K. KUROMIZU: Primary structure of neocarzinostatin, an antitumor protein. Science 178: 875~876, 1972
3) KOIDE, Y.; F. ISHII, K. HASUDA, Y. KOYAMA, K. EDO, S. KATAMINE, F. KITAME & N. ISHIDA: Isolation of a non-protein component and a protein component from neocarzinostatin (NCS) and their biological activities. J. Antibiotics 33: 342~346, 1980
4) NAPIER, M. A.; L. S. KAPPEN & I. H. GOLDBERG: Effect of nonprotein chromophore removal on neocarzinostatin action. Biochemistry 19: 1767~1773, 1980
5) EDO, K.; S. KATAMINE, F. KITAME, N. ISHIDA, Y. KOIDE, G. KUSANO & S. NOZOE: Naphthalenecarboxylic acid from neocarzinostatin (NCS). J. Antibiotics 33: 347~351, 1980
6) OHTSUKI, K. & N. ISHIDA: The biological effect of a non-protein component removed from neocarzinostatin (NCS). J. Antibiotics 33: 744~750, 1980
7) ISHIDA, R. & T. TAKAHASHI: Role of mercaptoethanol in *in vitro* DNA degradation by neocarzinostatin. Cancer Res. 38: 2617~2626, 1978
8) KAPPEN, L. S. & I. H. GOLDBERG: Mechanism of the effect of organic solvents and other protein denaturants on neocarzinostatin activity.

Biochemistry 18: 5647~5653, 1979

9) SIM, S. K. & J. W. LOWN: The mechanism of the neocarzinostatin-induced cleavage of DNA. Biochem. Biophys. Res. Commun. 81: 99~105, 1978

10) KAPPEN, L. S. & I. H. GOLDBERG: Activation and inactivation of neocarzinostatin-induced cleavage of DNA. Nucleic Acids Res. 5: 2959~2967, 1978

11) SAITO, H. & K. MIURA: Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta 72: 619~629, 1963

12) SUGIURA, Y. & T. KIKUCHI: Formation of superoxide and hydroxyl radicals in iron (II)-bleomycin-oxygen system: Electron spin resonance detection by spin trapping. J. Antibiotics 31: 1310~1312, 1978

13) SATO, S.; M. IWAIZUMI, K. HANDA & Y. TAMURA: Electron spin resonance study on the mode of generation of free radicals of daunomycin, adriamycin and carboquone in NAD (P)H-microsome system. Gann 68: 603~608, 1977

14) HARBOUR, J. R.; V. CHOW & J. R. BOLTON: An electron spin resonance study of spin adducts of OH and HO_2 radicals with nitrones in the ultraviolet photolysis of aqueous hydrogen peroxide solutions. Canad. J. Chem. 52: 3549~3553, 1974