BIOLOGICAL ACTIVITIES AND PHYSICOCHEMICAL PROPERTIES OF PRE-NEOCARZINOSTATIN AND UV-IRRADIATED NEOCARZINOSTATIN

Sir:

In 1965, an antitumor protein neocarzinostatin (NCS) was isolated from the culture filtrates of *Streptomyces carzinostaticus* var. F-41.[1] NCS was found to have both antibacterial activity (against *Sarcina lutea*) and antitumor activity within the same moiety, which was later reported to be a single polypeptide chain.[2]

More recently, another protein, designated as pre-neocarzinostatin (Pre-NCS), was found to be produced by the same organism.[3] Production of Pre-NCS in the culture broth procedes that of NCS, and Pre-NCS can be separated from NCS by CM-cellulose column chromatography or by isoelectric focusing, since the isoelectric point of Pre-NCS (pI = 3.15) is lower than that of NCS (pI = 3.26).[3] The physicochemical relationship between NCS and Pre-NCS has not yet been clarified, though it has been reported that the dialysis of NCS under weakly acidic pH conditions results in the formation of Pre-NCS.[4]

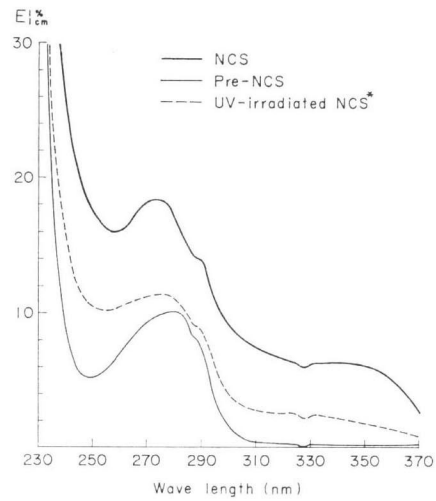
As for the biological properties of Pre-NCS, we previously reported that Pre-NCS has neither antibacterial nor *in vivo* antitumor activities in itself, although it diminishes the NCS-induced inhibition of *S. lutea* growth and DNA synthesis in HeLa-S3 cells if given prior to NCS treatment.[3] In the present work, however, a relatively higher concentration of Pre-NCS (10 ~ 100 μg/ml) was found to inhibit the growth of HeLa-S3 cells (designated as anti-HeLa activity) without any inhibition of bacterial growth. Also, it has been reported that the biological activities of NCS are diminished quantitatively by ultraviolet (UV) irradiation.[5,6] In the present work, we compared the physicochemical and biological alterations of NCS and Pre-NCS induced by UV irradiation in an attempt to elucidate the structural relationship between the two proteins.

Experimentally, 0.5 ml of NCS or Pre-NCS solution (1.0 mg in 1.0 ml of 10 mM phosphate buffered saline, pH 7.2) was irradiated in a Petri dish (35-mm diameter) with a germicidal lamp (two bulbs of Toshiba GL-10 W) at a distance that gave 500 μW/cm^2 energy. After UV irradiation for appropriate times, the dishes were taken out and the remaining antibacterial and anti-HeLa activities were assayed. The antibacterial activity was assayed by the conventional paper-disc diffusion method using *S. lutea*. Also, ^{3}H-thymidine incorporation into *S. lutea* DNA was checked to confirm the complete absence of antibacterial activity. For the assay of anti-HeLa activity, cells were incubated for 48 hours with or without the drugs. The concentration which inhibited the increase in cell number by 50% of control was defined as a 50% inhibitory concentration (IC_{50}). Pre-NCS was purified from the culture filtrates of *S. carzinostaticus* var. F-41 by CM-cellulose column chromatography as described previously.[3]

The UV absorption spectra of NCS, Pre-NCS and UV-irradiated NCS (10-minute irradiation followed by dialysis against distilled water) are compared in Fig. 1. It should be noted that NCS has a UV absorption maximum (λ_{max}) at 274 nm and a definite absorption shoulder at 350 nm. In contrast, Pre-NCS has a λ_{max} of 280 nm and no apparent absorption at 350 nm. The UV absorption spectrum of UV-irradiated NCS is found to be quite similar to that of Pre-NCS as a result of a decrease in the height of both 274 nm λ_{max} and of the 350 nm shoulder. This alteration

Fig. 1. UV absorption spectra of NCS, Pre-NCS and UV-irradiated NCS.

* NCS in 1 mg/ml solution was treated with UV irradiation for 10 minutes at 500 μW/cm^2, followed by dialysis against distilled water for 8 hours in 4°C.



was dependent on the length of irradiation time. In the case of Pre-NCS, no such alteration was detected (data not shown). Gel-filtration through Sephadex G-25 was also carried out with UV-irradiated NCS (Fig. 2). In UV-irradiated NCS, there was a clear absorption peak at fractions 21~23, indicating the release of a low molecular component which passed through the dialysis tube and did not register in the spectrum shown in Fig. 1. Characterization of this low molecular component is yet to be completed, although it has proved to have a λ_{max} of 230~250 nm, to be negative with a ninhydrin reaction and seems to be a non-peptide. As for the protein moiety which remained inside the dialysis tube and was eluted in the void fractions of Sephadex G-25, its characteristics seem to be quite similar to those of Pre-NCS itself. Besides the similarity in the UV absorption spectra (see Fig. 1), they have a common pI of 3.15 on the thin-layer isoelectric focusing and form a common precipitation line with anti-Pre-NCS serum on the Ouchterlony diffusion gel.

The biological activities of NCS, Pre-NCS and UV-irradiated NCS (10-minute irradiation) are compared in Table 1. The minimum inhibitory concentration (MIC) of NCS for the growth of *S. lutea* in nutrient agar was 0.39 μg/ml, while no effect of Pre-NCS or UV-irradiated NCS on the growth of *S. lutea* was detected at the highest concentration of 1,000 μg/ml. Also, the ^{3}H-thymidine incorporation study comfirmed that

Table 1. Biological activities of NCS, Pre-NCS and UV-irradiated NCS.

	Sarcina lutea MIC* (μg/ml)	HeLa-S3 IC_{50}** (μg/ml)
NCS	0.39	0.15
Pre-NCS	>1,000	35
UV-irradiated NCS***	>1,000	34

* MIC: minimum inhibitory concentration in nutrient agar

** IC_{50}: 50% inhibitory concentration for HeLa cell growth at 48 hours.

*** One mg/ml concentration of NCS was treated with UV irradiation at 500 μW/cm^2 energy for 10 minutes.

Fig. 2. Gel filtration of NCS and UV-irradiated NCS.

Half ml samples of NCS and UV-irradiated NCS (10-minute irradiation) were applied on the Sephadex G-25 column (bed volume 10 ml) and eluted with PBS. Half ml fractions were collected from the left to right and measured of the UV absorption at 250 nm, 280 nm and 350 nm.

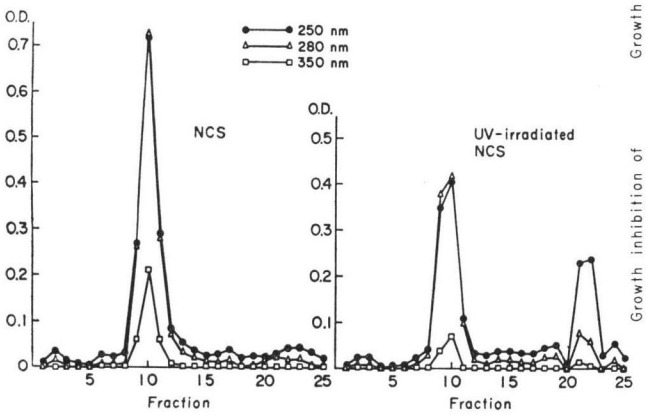
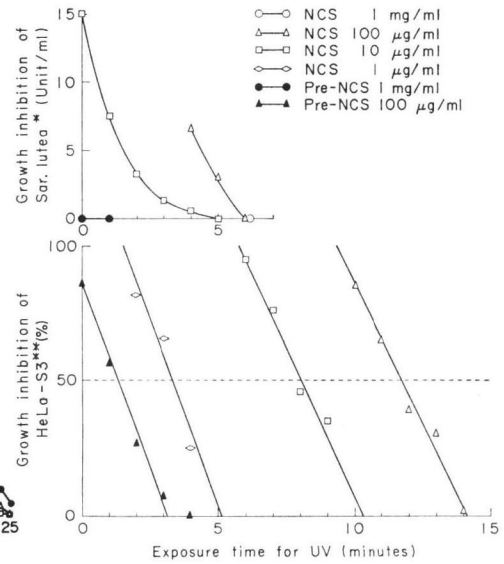


Fig. 3. Biological activities of NCS and Pre-NCS after UV irradiation.

Both NCS and Pre-NCS in 1 mg/ml solution were treated with UV irradiation at 500 μW/cm^2 for indicated times and assayed of their biological activities against *Sarcina lutea* and HeLa-S3 cells, after appropriate dilution.

* Obtained by paper-disc diffusion method (One unit corresponds to the diameter of inhibition zone obtained with 0.67 μg of standard NCS)

** Expressed as % inhibition of HeLa cell growth in 48 hours.

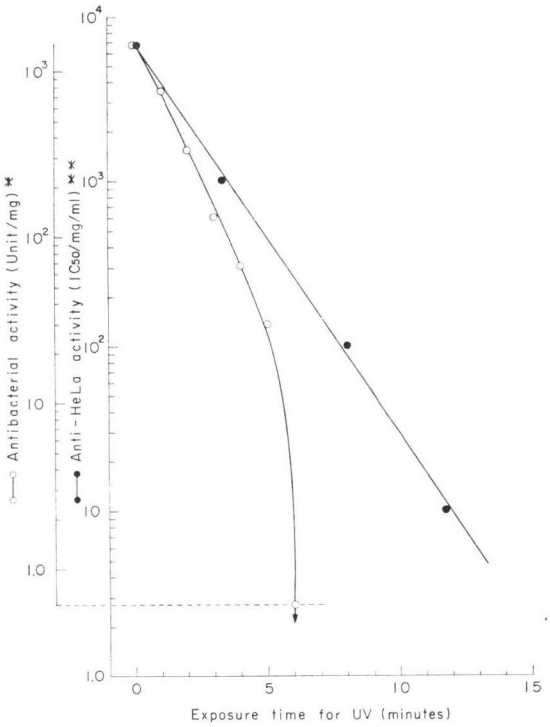


no inhibition of ^{3}H-thymidine incorporation by *S. lutea* in nutrient broth was detected with Pre-NCS or UV-irradiated NCS. However, exponentially growing HeLa-S3 cells were found to be more sensitive to Pre-NCS or UV-irradiated NCS than *S. lutea* was. The IC_{50} of Pre-NCS and UV-irradiated NCS was 35 μg/ml and 34 μg/ml, respectively, whereas that of untreated NCS was 0.15 μg/ml. When the irradiation time was extended to 20 minutes, however, the anti-HeLa activity of NCS was no longer detected (Fig. 3). Also, the anti-HeLa activity of Pre-NCS (assayed at 100 μg/ml) decreased linearly depending on the length of the irradiation time and was no longer detected after 4 minutes (Fig. 3). The dissociation of antibacterial activity and anti-HeLa activity of NCS upon UV irradiation was clearly shown in Fig. 4. After 6 minutes of UV irradiation, although the antibacterial activity was completely lost, the anti-HeLa activity remained at almost one-twentieth that of untreated NCS. The biological properties of UV-irradiated NCS (6-minute to 10-minute irradiation) were found, therefore, to be quite similar to those observed with Pre-NCS, although the exact mode of their anti-HeLa activity remains to be elucidated.

Taken together, these results suggest that some UV-sensitive component may be associated with the NCS molecule. This component, which seems to be non-protein in nature from its physicochemical properties, may be denatured and dissociated from a NCS molecule by UV irradiation, as it may be after dialysis under weakly acidic pH conditions.[4] Pre-NCS and UV-irradiated NCS seem to be lacking in this component and also lacking in most, if not all, of the biological activities of NCS. Therefore, this component may play an essential role in the biological activities of NCS.

It was also shown that there is a difference in sensitivity to UV irradiation between the antibacterial activity and the anti-HeLa activity of NCS. UV-irradiated NCS, like Pre-NCS, specifically inhibits mammalian cell growth, but not bacterial growth (Table 1). This observation may suggest that there are different mechanisms which are responsible for the inhibition of mammalian cell growth and of bacterial growth. In the former, a certain substance or structure other than DNA may be one of the targets, while in the latter DNA itself may be the exclusive one. Possibly, the specific anti-HeLa activity of Pre-NCS and UV-irradiated NCS might be explained by the effect on the cytoplasmic microtubules which is observed in a relatively higher concentration of NCS[7,8].

Fig. 4. Inactivation of NCS by UV irradiation.
The results of Fig. 3 were rearranged so that the remaining antibacterial (○—○) and anti-HeLa (●—●) activities per 1 mg/ml of NCS were plotted against the UV exposure time.
* Same as Fig. 3.
** Expressed as multiplicity of IC_{50} for HeLa cell growth in 48 hours.



The existence of a non-protein component has also been postulated for macromomycin, an antitumor polypeptide produced by *Streptomyces macromomyceticus*.[9] Recently, YAMASHITA *et al*[10] reported that a new antibiotic, named auromomycin, is also produced in the culture filtrates of *S. macromomyceticus* and ascribed the difference between macromomycin and auromomycin in their biological activities to the quantitative difference of chromophore present in the molecule that has a λ_{max} of 350~360 nm in the UV absorption spectrum. It is possible that the non-protein component of NCS which dissociates from the protein moiety as observed in the present report may correspond

to such a chromophore.

While this report was in preparation, NAPIER *et al*[11] reported the separation of a non-protein chromophore from NCS using a methanol extraction. Finding the relation between the methanol-extracted chromophore and the UV-released non-protein component will require further work.

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