NEOCARZINOSTATIN-INDUCED BREAKDOWN OF DEOXYRIBONUCLEIC ACID IN HeLa-S3 CELLS

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Degradation of DNA in HeLa-S3 cells mediated by an acidic antitumor protein, neocarzinostatin (NCS), was examined. The concentration of NCS required for induction of DNA degradation was considerably higher than that which caused inhibition of DNA synthesis. Sedimentation analysis of DNA revealed that HeLa-S3 cell DNA first received single-strand nicks within 60 minutes after exposure to the antibiotic, whereas detectable double-strand scissions eventually gave rise to the accumulation of double-stranded DNA fragments of heterogeneous size. When the cells exposed to NCS were transferred to NCS-free medium at early stages of the degradation, the single-strand nicks caused in DNA were repaired by a process which was sensitive to puromycin.

Neocarzinostatin (NCS) is an acidic antitumor protein with a molecular weight of 11,000 daltons, isolated from a culture of *Streptomyces carzinostaticus*[1~4]. The antibiotic was found to inhibit the synthesis of DNA in *Sarcina lutea* at a very low concentration (0.01 μg/ml) without detectable inhibition of RNA and protein synthesis, and induced degradation of DNA in the same organism at a higher concentration (0.5 μg/ml)[5,6].

The specific inhibition of DNA synthesis was also found in HeLa-S3 cells, as well as the immediate inhibition of mitosis[7,8]. However, the degradation of DNA was not detected in HeLa-S3 cells in previous experiments[5]. We have recently found that the DNA degradation occurred in HeLa-S3 cells, like *S. lutea*[5,6], when the cells were exposed to the concentrations of NCS (>20 μg/ml) much higher than those tested previously.

The present paper summarizes the mode of the DNA degradation induced by NCS in HeLa-S3 cells.

Materials and Methods

Cell culture: Monolayer cultures of HeLa-S3 cells were used throughout the experiments. The cells were grown in a medium consisting of 1 % yeast extract, 0.45 % glucose and 0.5 % lactalbumin hydrolysate in EARLE's solution supplemented with 10 % bovine serum (YLE).

Chemicals: Neocarzinostatin (NCS) was a gift from Kayaku Antibiotic Research Co., Tokyo. 6-^{3}H-Thymidine (14.0 μCi/mmole) was purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo. Puromycin was obtained from American Cyanamide Co., New York.

Determination of DNA degradation: To label the DNA of HeLa-S3 cells, 8 ml of cell suspension (2×10^5 cells/ml) was inoculated into 50 ml prescription bottles. After one day incubation at 37°C, the monolayer culture was washed 3 times with prewarmed HANKS' solution and incubated in a fresh medium containing ^{3}H-thymidine (0.5 μCi/ml) for 8 hours at 37°C. The cells were washed and incubated further in a fresh medium containing a 100-fold concentration of unlabeled thymidine at 37°C for 3 hours. To the labeled HeLa-S3 cell cultures, NCS was added at various concentrations and the cultures were incubated at 37°C for desired

periods of time. After the incubation, the cultures were chilled rapidly, washed with PBS buffer (0.01 M Na-phosphate, 0.15 M NaCl, pH 7.2), and 10% trichloroacetic acid (TCA) was added. TCA-insoluble material was washed sequentially with 5% and 1% TCA, dissolved in 2 ml of 1 N NH_4OH and 0.5 ml aliquots were dried on planchettes. The radioactivity was determined in an Aloka gas flow counter.

Sedimentation analysis of DNA in neutral or alkaline sucrose gradient: The method of McGRATH and WILLIAMS[7] was used for sedimenting DNA in neutral or alkaline sucrose gradient. NCS-treated and untreated control cells were trypsinized, suspended in 2 ml of PBS and received 0.5 ml of 2% sodium dodecyl sulfate (SDS). Four-tenth ml of the SDS lysate was placed on the top of a 4.6 ml 5~20% (w/v) sucrose density gradient of neutral (0.02 M Tris-HCl, 0.01 mM EDTA and 0.15 M NaCl, pH 7.0) or alkaline (0.25 M NaOH, 0.01 mM EDTA and 0.15 M NaCl) buffer. Tubes were allowed to stand at room temperature for 30 minutes in order to facilitate the lysis of the cells with SDS, and then centrifuged in a Hitachi Model 65P ultracentrifuge at 40,000 rpm (Hitachi RPS 50 roter) for 120 minutes at 18°C. After the run, 7 drop fractions were collected from the top of the tube onto glass fiber papers and dried. The paper was immersed in 10 ml of toluene-pop-popop and the radioactivity was determined in an Aloka liquid scintillation spectrometer.

Results

NCS-induced DNA Degradation in HeLa-S3 Cells

In order to examine whether NCS induces degradation of DNA, monolayer cultures of HeLa-S3 cells (5×10^5 cells) prelabeled with ^{3}H-thymidine were exposed to 5, 20 and 50 μg/ml of NCS for the periods indicated in Fig. 1, and the radioactivity remaining in the acid-insoluble fraction was determined. No DNA degradation was detected at a concentration of 5 μg/ml of NCS, the concentration which inhibits completely the synthesis of DNA within 10 minutes after exposure of HeLa-S3 cells to NCS. At 20 μg/ml and 50 μg/ml, the acid-insoluble ^{3}H in the cells gradually decreased after a lag period of about 6 hours. After

Fig. 1. NCS-induced DNA degradation in HeLa-S3 cells.

HeLa-S3 cells prelabeled with ^{3}H-thymidine were exposed to NCS as described in Materials and Methods. The radioactivity remained in the acid-insoluble fraction was determined at the times indicated in the diagram.

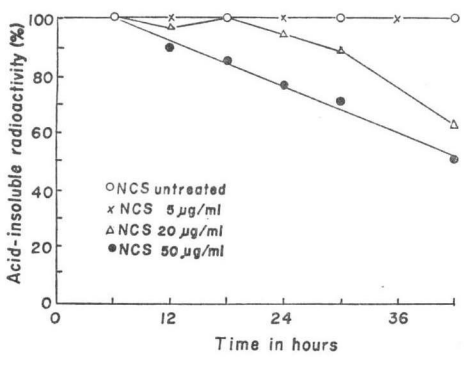
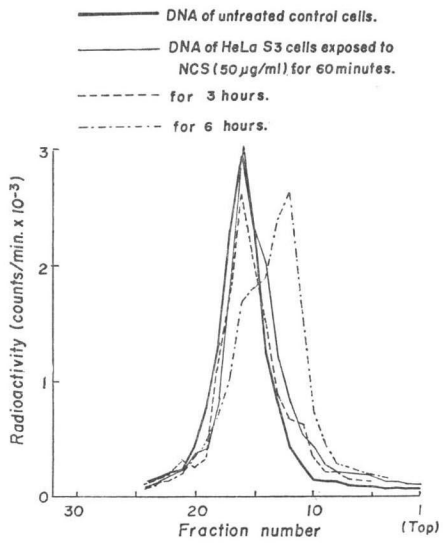


Fig. 2. Neutral sucrose gradient analysis of the DNA from HeLa-S3 cells exposed to NCS.

Sedimentation analysis was performed as described in Materials and Methods.



repeating a series of similar experiments, it was established that the DNA of HeLa-S3 cells degraded into acid-soluble materials when exposed to over 20 μg/ml of NCS.

Endonucleolytic Breakdown of DNA Induced by NCS

The molecular size of DNA after treatment with NCS was analyzed by sedimentation in neutral sucrose gradients. When the untreated cells were analyzed, the DNA banded with a peak at the 16th fraction (Fig. 2). When the cells were exposed to 50 μg/ml of NCS at 37°C, no significant change was detected in the sedimentation profiles after 1 and 3 hours, while after 6 hours the main peak of DNA shifted from the 16th fraction to the 12th fraction, indicating a decrease in molecular weight of DNA.

Fig. 3. Alkaline sucrose gradient analysis of the DNA from HeLa-S3 cells exposed to NCS.
Sedimentation analysis was performed as described in Materials and Methods.

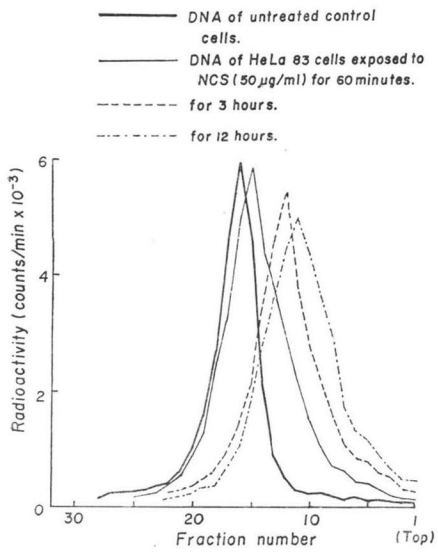
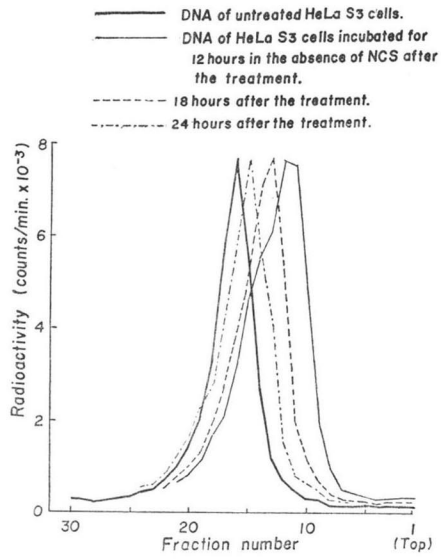


Fig. 4. Repair of the NCS-induced single-strand nicks in DNA.
Sedimentation analysis was carried out in the same way as described in the legend to Fig. 3. HeLa-S3 cells which had been exposed to NCS (50 μg/ml) for 60 minutes were rapidly washed and incubated further in the absence of NCS.



The molecular size of the DNA was also analyzed in an alkaline sucrose gradient under conditions which caused complete strand separation of DNA molecules (Fig. 3). The DNA of control cells banded with a peak at the 16th fraction, while the DNA of the cells exposed to 50 μg/ml of NCS for 1, 3 and 12 hours had peaks at the 15th, 12th and 11th fraction, respectively. These results indicated that single-strand nicks in DNA are detectable within 1 hour after the addition of NCS in contrast to the double-strand scissions of DNA, which were detectable only after 6 hours.

Repair of the NCS-induced Single-strand Nicks in DNA

In order to follow the fate of DNA nicked in single-strand after NCS treatment, the following experiments were carried out. HeLa-S3 cells which had been exposed to 50 μg/ml of NCS for 60 minutes were incubated further in the absence of NCS for 12, 18 and 24 hours,

respectively, and the DNA (in a single-stranded form) was analyzed by sedimentation in the alkaline sucrose gradients (Fig. 4). The main DNA peak of control cells was found at 16th fraction as described above (Fig. 3). When the cells were incubated for 12, 18 and 24 hours in the NCS-free medium following the exposure to NCS for 60 minutes, the main peak of DNA sedimented at the 12th, 13th and 15th fraction, respectively. The results indicated that the pulse treatment with NCS for 60 minutes caused single-strand nicking in DNA to the same extent as that found in continuous treatment (12 hours) with NCS (Fig. 3). Moreover, the sedimentation rate of DNA, which had decreased due to nicking, increased to a value close to that of the untreated cells during further incubation in the absence of NCS. These results suggested that the NCS-induced single-strand nicks in DNA were repaired to intact DNA upon removal of NCS.

Effect of Puromycin on the Repair Process

Since it was previously found that NCS-induced DNA degradation in *Sarcina lutea* was prevented by the presence of puromycin[6], we examined in HeLa-S3 cells the effect of puromycin on the process of DNA nicking and of the repairing of the nicks. For this purpose, HeLa-S3 cells which had been exposed to NCS for 60 minutes were incubated for further 24 hours in NCS-free medium in the presence or absence of puromycin (100 μg/ml) according to the following schedule. (a) Puromycin was present for initial 12 hours after the removal of NCS; (b) for only the later experimental period from 12 hours to 24 hours; and (c) throughout 24 hours. DNA of these cells treated with puromycin in the three different ways was analyzed by sedimentation in the alkaline sucrose gradients.

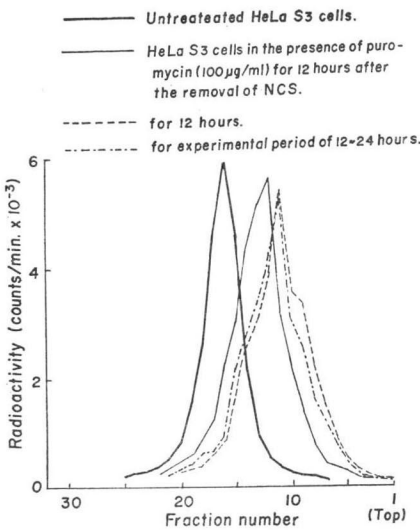


Fig. 5. Effect of puromycin on the single-strand nicking in DNA induced by NCS and the repairing of the nicked DNA.

Sedimentation analysis was carried out in the same way as described in the legend to Figs. 3 and 4. HeLa-S3 cells which had been exposed to NCS (50 μg/ml) for 60 minutes were rapidly washed and incubated further in the absence of NCS.

The results (Fig. 5) indicated that DNA of the cells in all cases, (a) (b) and (c), sedimented slower than that of control cells untreated with both NCS and puromycin, and the sedimentation profiles of DNA in cases (b) and (c) were similar to each other and to that observed when the cells were exposed to NCS for 12 hours (Fig. 3). We concluded from these results that puromycin inhibited the process of repairing the nicked DNA to intact molecules.

Discussion

Degradation of DNA induced by NCS in HeLa-S3 cells was found to proceed in the following three steps. (I) Single-strand nicking in DNA is induced within 60 minutes after

exposure of the cells to NCS, (II) double-strand scissions of DNA occur in about 6 hours, and (III) further breakdown of double-stranded fragments into acid-soluble materials starts at about 6 hours after the addition of NCS. In contrast to the observation with *Sarcina lutea*[5,6], a high concentration (>20 μg/ml) of NCS and long incubation period (>6 hours) were required for the induction of DNA degradation in HeLa-S3 cells. Furthermore, the NCS-induced DNA degradation into acid-soluble materials in HeLa-S3 cells was not prevented by puromycin, which prevented strongly the NCS-induced DNA degradation in *Sarcina lutea*[6]. In these respects, the process of NCS-induced DNA degradation in HeLa-S3 cells resembles DNA breakdown in *E. coli* induced by colicin E_2[10~12] or X-ray radiation[13,14].

Sedimentation analysis of DNA in alkaline sucrose gradient revealed that the first step of NCS-induced DNA degradation in HeLa-S3 cells was endonucleolytic nicking, and that the endonucleolytic breakdown products accumulating in the NCS-treated cells were of heterogeneous size with respect to both single- and double-stranded form (Figs. 4 and 5). The accumulation of such DNA fragments might be due to the specificity of nucleolytic action induced by NCS, or due to partial repairing of DNA. It was found that the DNA nicked in single-strand was repaired to intact DNA when HeLa-S3 cells which had been exposed to NCS for 60 minutes (early stages of the degradation) were incubated further in the NCS-free medium (Fig. 5) and that the repairing process was prevented by puromycin (Fig. 5).

We previously found in *Bacillus subtilis*[15] that upon exposure of the bacteria to NCS, DNA was broken down to double-stranded fragments of a certain size class as was found in HeLa-S3 cells. Simultaneous addition of chloramphenicol together with NCS resulted in a marked stimulation of further breakdown of the DNA fragments into acid-soluble materials. Although it is yet unknown whether repairing of DNA takes place also in the bacterial cells, present results with HeLa-S3 cells strongly suggest that the repairing of nickes in DNA prevents further degradation of the DNA fragments into acid-soluble materials thereby resulting in the accumulation of DNA fragments of various size classes. Presumably, the repairing reaction of single-strand nicks in DNA induced by NCS requires the synthesis of new protein, and the apparent stimulation of NCS-induced degradation into acid-soluble materials by the simultaneous addition of puromycin is due to the inhibition of the repair reaction.

In view of the fact that the first step of NCS-induced DNA degradation is an endonucleolytic reaction, we considered the possibility that, in this initial stage of DNA degradation, a specific endonuclease(s) induced or activated by NCS caused breakdown of the cellular DNA. This possibility is compatible with our unpublished observations that NCS itself has no nucleolytic activity and that high concentrations (>50 μg/ml) of NCS stimulated significantly the activity of pancreatic endonuclease I as tested *in vitro*. From these data, it is conceivable that NCS-induced DNA degradation in HeLa-S3 cells represents a secondary action, which is a consequence of the activation of cellular nucleases.

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