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STUDIES ON THE INTERACTION OF NEOCARZINOSTATIN WITH RAT LIVER DNA *IN VIVO* AND *IN VITRO*

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Abstract Administration of neocarzinostatin (NCS) (10 μg/g) intravenously through the tail vein caused strand breaks in DNA of both resting and regenerating liver as measured by sedimentation in an alkaline sucrose gradient. However, such fragmentation could be largely prevented by perfusing before squashing the liver; the inhibition appears to be more in the resting compared to regenerating liver. Furthermore, when NCS was given intraperitoneally (10 μg/g) either at 1 or 4 hr before killing, it did not cause strand breaks in liver DNA. Rat liver nuclear suspensions, which consist of intact nuclei with little cytoplasm, when incubated with NCS (2 μg/ml of incubation mixture), resulted in extensive strand breakage in liver DNA. These results suggest that NCS may not penetrate the resting liver cell, at least not rapidly, and that the observed DNA strand breaks may be largely due to the interaction of the circulating drug in the portal blood with DNA *in vitro* at one or more steps during the preparation and analysis of the liver cell DNA. Heat denaturation of NCS abolished the property of fragmentation of DNA.

Neocarzinostatin (NCS), an acidic protein isolated from *Streptomyces carzinostaticus* [1,2] is active against some experimental tumors in mice [1,3]. Phase I clinical trials in Japan appear to indicate that NCS is effective in the treatment of certain solid tumors [4]* and leukemia [5]. NCS has been shown to inhibit DNA synthesis in *Sarcina lutea* [6], L1210 [7] and HeLa [8] cells. At high concentrations in *Sarcina lutea*, this drug induces degradation of DNA into acid-soluble nucleotides [9]. Recently, NCS has been shown to cause DNA strand scissions in HeLa [8] and L1210 [7,10] cells and in SV40 [8] and calf thymus [10] DNA *in vitro*.

During the past few years, it has been demonstrated that several carcinogens of low molecular weight induced strand breaks in liver DNA *in vivo* [11–14]. In view of the reactivity of NCS with DNA *in vitro* and its possible importance in clinical therapy, it became of interest to see whether NCS causes liver DNA strand breaks in an intact animal. The results presented in this communication indicate that NCS does not induce liver DNA strand scissions *in vivo*, at least not rapidly, but does cause strand breaks *in vitro* during incubation of the drug with a liver nuclear suspension.

MATERIALS AND METHODS

White male Wistar rats (Carworth Farms) weighing 100 g were partially hepatectomized according to the procedure of Higgins and Anderson [15]. The liver DNA was labeled with thymidine-methyl[^{3}H] (sp. act., 20 Ci/m-mole) (New England Nuclear, Boston, Mass.) during liver cell regeneration [16]. The animals were used after a recovery period of 2 weeks, at which time the liver had returned essentially to a quiescent state [17]. Two-tenths ml of 0·9% NaCl or 0·2 ml of 0·9% NaCl solution of highly purified NCS [18] was administered either intraperitoneally or intravenously through the tail vein. After 30 min the animals were anesthetized with ether, and the livers were excised with or without perfusion *in situ* with about 20 ml of the cold (10°) squash solution (0·024 M EDTA and 0·075 M NaCl, pH 7·5) through the portal vein with a syringe. The inferior vena cava was cut to facilitate the perfusion of the liver.

Preparation of nuclear suspension and analysis of DNA on alkaline sucrose gradient. The liver was quickly removed and dropped into a tube containing cold squash solution. Approximately 1 g of the liver was placed in a cold petri dish along with 2–3 ml of squash solution and gently squashed with a spatula or a fork with bent tines. The suspension was centrifuged for 1 min at 500 rev/min at 4° in an International model PR-6 refrigerated centrifuge. The supernatant, which contained essentially intact nuclei with very little cytoplasm, was used for the analysis. DNA was released by alkaline lysis *in situ* on top of the alkaline sucrose gradient. The alkaline lysing solution consisted of 0·1 M Tris–HCl buffer, pH 12·5, containing 0·03 M EDTA, 0·3 M NaCl and 0·5% SDS. After a lysing period of 30 min at 25°, the gradients were centrifuged for 30 min at 25,000 rev/min at 20° in a

* K. Matsumoto and K. Nakauchi, National Cancer Center, Tokyo, personal communication.

Spinco SW40 rotor. Collection of fractions from the gradients and the determination of acid-precipitable radioactivity in these fractions were performed as described earlier [16].

Sucrose gradients were calibrated using DNA from T_4, T_7 and SV40 nicked circles. Molecular weight values higher than T_4 phage-DNA are extrapolated from the sedimentation pattern of DNA of SV40, T- and T_4 phages [19].

Incubation of rat liver nuclear suspension with NCS. To 0·1 ml of liver nuclear suspension was added an equal volume of squash solution alone or with either NCS, 2 μg, or heat-denatured NCS, 20 μg/ml of incubation mixture. After an incubation period of 30 min at 25°, an aliquot of the incubation mixture was lysed on top of an alkaline sucrose gradient and analyzed for size distribution of the DNA.

Preparation of heat-denatured NCS. NCS (1 mg/ml of squash solution) was heated in a boiling water bath for 10 min and chilled on ice.

RESULTS AND DISCUSSION

The term 'single-strand break' is used operationally and refers to slowly sedimenting DNA in an alkaline sucrose gradient. The effect of NCS on the sedimentation of liver DNA in alkaline sucrose gradient is shown in Fig. 1. The DNA from control perfused liver sedimented mostly into the 2·3 M sucrose cushion at the bottom of the gradients. This DNA was found to be single stranded as judged by physical and electron microscopical examination and by the susceptibility toward S_1 nuclease [19]. In contrast, the liver DNA from the rat that received NCS intravenously (10 μg/g) 30 min before killing exhibited slower sedimentation. However, such slower sedimentation of liver DNA was not observed when the drug was administered intraperitoneally (10 μg/g) either at 1 or 4 hr before killing (data not presented).

These observations, coupled with the relatively large molecular weight of the drug (10,700 daltons), suggested that the hepatic DNA strand breaks observed when the compound was given intravenously might not represent the interaction of NCS with liver DNA *in vivo*. Rather, the strand breaks could well be a consequence of direct interaction of the DNA with the residual drug present in the hepatic blood *in vitro* either during or following the preparation of the nuclear suspension. To study this possibility, the following experiments were carried out. Experiment 1: NCS (10 μg/g) was given intravenously and the rats were killed 30 min later. The liver was perfused 1–2 min before killing. Sedimentation analysis of DNA from such a liver clearly indicated that the DNA behaved like that from a control liver (Fig. 1). The small degree of fragmentation of DNA seen in the perfused liver may be due to the traces of NCS that could not be removed by perfusion. Experiment 2: In contrast to resting liver, dividing cells such as HeLa [8] and L1210 [7,10] cells exhibited DNA fragmentation upon incubation with NCS. This raises the question of whether NCS would cause DNA strand breaks in regenerating rat liver. To study this, rats were partially hepatectomized [15], and 21 hr later (during the S phase) the liver DNA was labeled with tritiated thymidine. After 4 hr, when the newly labeled

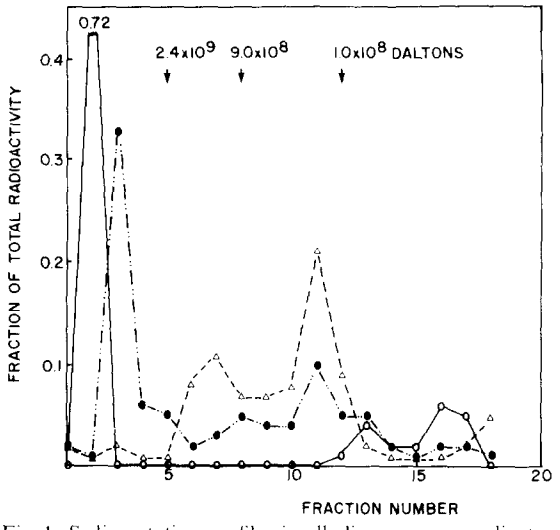

Fig. 1. Sedimentation profiles in alkaline sucrose gradients of hepatic DNA of rats given 0·9% NaCl or 0·9% NaCl containing NCS. NCS (0·2 ml) was given intravenously through the tail vein at a dose of 10 μg/g of body weight. Control rats received equal volumes of 0·9% NaCl intravenously. All the rats were killed 30 min thereafter. One group of rats received NCS and were not perfused (△ ···△). The other two groups received either 0·9% NaCl (○ ——○) or NCS (● ···●), and the livers were perfused 1–2 min prior to killing. Each gradient represents one animal. On the average, the percentage of acid-precipitable radioactivity recovered in fractions 1–5, 6–13 and 14–18 was 73 ± 4, 13 ± 4 and 13 ± 4, respectively (average of five gradients), for the hepatic DNA of control rats; 22 ± 6, 69 ± 6 and 11 ± 1 for the liver DNA of rats given NCS without perfusion (average of four gradients), and 57 ± 7, 29 ± 9 and 14 ± 2 for the DNA of perfused liver of rats given NCS (average of three gradients). The sedimentation is from right to left. Total acid-precipitable radioactivity recovered from each gradient was as follows: control 268 cpm; NCS followed by perfusion, 1069 cpm; and NCS followed by no perfusion, 2256 cpm.

DNA becomes elongated and bands near the bottom of the sucrose gradient [20]. NCS was administered. The results presented in Fig. 2 clearly indicate that: (a) intravenous administration of NCS causes strand breaks in regenerating liver DNA, and (b) perfusion of the liver 1–2 min prior to killing inhibits the fragmentation of liver DNA. However, the extent of inhibition does not appear to be as much as that seen with resting liver, suggesting that regenerating liver may be more permeable than resting liver toward NCS. It is also possible that the residual NCS (left over in the liver after perfusion), if any, may induce more extensive damage in DNA of regenerating liver than in that of resting liver, because the newly made DNA (4-hr pulse) in regenerating liver may be different from the DNA of resting liver (DNA labeled 2–3 weeks earlier) in its size, packaging with histones, nonhistone proteins and so forth. Hence, it will be difficult to draw any conclusion regarding the differential permeability of resting and regenerating liver toward NCS. Further, the possibility that NCS can exert its effect by binding to the membrane without entering the cell cannot be ruled out. Such a mechanism was postulated for the inhibitory action of hepatic cholane on DNA synthesis [21].

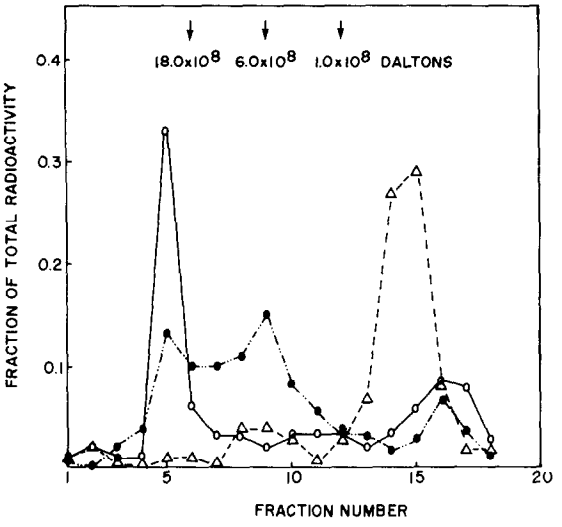

Fig. 2. Sedimentation profiles in alkaline sucrose gradients of DNA of regenerating liver of rats given 0·9% NaCl or 0·9% NaCl containing NCS. Eighteen to 21 hr following partial hepatectomy, rats were given 400 μCi of [^{3}H]thymidine. Four hr later, either 0·2 ml NCS (10 μg/g) or an equal volume of 0·9% NaCl was given intravenously. All the rats were killed 30 min thereafter. One group of rats received NCS and were not perfused (△—△). The other two groups received either 0·9% NaCl (○—○) or NCS (●—●) and the livers were perfused 1–2 min prior to killing. Each gradient represents one animal. On the average, the percentage of acid-precipitable radioactivity recovered in fractions 1–5, 6–13 and 14–18 was 42 ± 4, 30 ± 6 and 29 ± 5, respectively (average of six gradients), for the hepatic DNA of perfused control rats; 12 ± 3, 38 ± 8 and 51 ± 10 for the liver DNA of rats given NCS without perfusion (average of three gradients), and 24 ± 3, 39 ± 9 and 38 ± 7 for the DNA of perfused liver of rats given NCS (average of five gradients). Total acid-precipitable radioactivity recovered from each gradient was as follows: control, 1190 cpm; NCS followed by perfusion, 760 cpm; and NCS followed by no perfusion, 400 cpm.

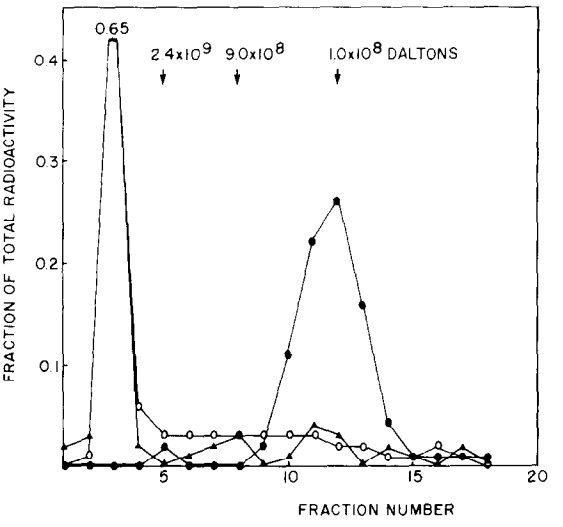

Fig. 3. Effect of addition *in vitro* of NCS and heat-denatured NCS to the rat liver nuclear suspension on the sedimentation profiles of DNA in alkaline sucrose gradients. To 0·1 ml of liver nuclear suspension was added: (1) an equal volume of the squash solution (○—○); (2) the squash solution containing NCS (2 μg/ml of incubation mixture; ●—●); (3) heat-denatured NCS (20 μg/ml; ▲—▲). After an incubation period of 30 min at 25°, an aliquot of the nuclear suspension was lysed on top of an alkaline sucrose gradient. Each gradient represents one incubation. On the average the percentage of acid-precipitable radioactivity recovered in fractions 1–5, 6–13 and 14–18 was 75 ± 6, 20 ± 4 and 4 ± 0·9, respectively (average of six experiments), for (1); 7 ± 3, 81 ± 5 and 12 ± 3 (average of seven experiments) for (2); and 67 ± 7, 25 ± 5 and 8 ± 1 (average of three experiments) for (3). Total acid-precipitable radioactivity recovered from each gradient was as follows: control, 561 cpm; incubated with NCS, 1316 cpm; and incubated with heat-denatured NCS, 233 cpm.

Experiment 3: NCS was incubated *in vitro* with rat liver nuclear suspension. The results presented in Fig. 3 clearly demonstrate that NCS can cause DNA strand breaks under such conditions. In two experiments, the liver nuclear suspension, after incubation for 30 min with NCS, was diluted with 5·0 ml of squash solution and centrifuged at 1000 rev/min in an International model PR-6 refrigerated centrifuge to remove any unreacted NCS. Sedimentation analysis of the DNA of the pelleted nuclei in the alkaline sucrose gradient indicated that removal of the unreacted NCS, if any, from the incubation mixture did not decrease the fragmentation of liver DNA by this agent.

In an attempt to understand the mechanism of action of NCS on the fragmentation of DNA, heat-denatured NCS was incubated with liver nuclear suspension in two experiments. The results in Fig. 3 indicate that even at a concentration of 20 μg/ml, heat-denatured NCS did not cause liver DNA fragmentation. This observation was not unexpected since NCS possesses a very tightly folded conformation, which probably consists to a considerable extent of the β-pleated sheet type of protein [18].

It is interesting to note that incubation of NCS with EDTA-treated *Escherichia coli* cells resulted in fragmentation of the DNA [9]. However, NCS did not cause fragmentation of DNA if the cells were not pretreated with EDTA [9]. These results indicate that *E. coli* cell wall or membrane may not be permeable to NCS. A phenomenon analogous to that observed with NCS was recently reported by Cox *et al.* [22] with the glycopeptide bleomycin. DNA strand damage was not seen in animals given bleomycin if the liver was perfused before preparing the liver for lysis.

Even though the mechanism by which NCS causes DNA strand breaks is not clear, the results presented in this communication suggest that: (1) NCS may not penetrate the resting liver cell, at least not rapidly, and (2) unlike the liver cell membrane, the nuclear membrane under the conditions employed appears to be permeable to NCS. From the chemotherapeutic viewpoint, the inability of NCS to interact *in vivo* with DNA of resting hepatocytes, in contrast to the apparent though limited interaction with proliferating cells such as tumor cells [7,8,10] and regenerating liver, is of considerable interest.

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