Enhancement of X-ray Induced Cytotoxicity by Neocarzinostatin in Asynchronous and Synchronous Rat 3Y1 Fibroblasts

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Enhancement of X-ray Induced Cytotoxicity by Neocarzinostatin in Asynchronous and Synchronous Rat 3Y1 Fibroblasts

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Abstract A polypeptide antibiotic, neocarzinostatin (NCS), has recently been shown to fix potentially lethal damage and inhibit repair of sublethal damage. To extend these observations, the effects of NCS on X-ray induced cytotoxicity at different phases of the cell cycle and the induction of DNA double strand breaks (dsb) were examined using rat 3Y1 fibroblasts. Colony survival assays showed that postirradiation treatment with 0.019μg/ml NCS for 20 min enhanced X-ray cell killing at all phases of the cell cycle. Neutral filter elution analyses showed that NCS increased DNA dsb induction after X-irradiation. Thus, NCS appears to enhance the radiosensitivity by increasing the initial level of radiation-induced DNA dsb.

Introduction

Neocarzinostatin (NCS), isolated from culture filtrates of *Streptomyces carzinostaticus*[6], is a polypeptide antibiotic with a molecular weight of 10700[9]. This compound has radiomimetic properties causing effects such as DNA strand breaks, inhibition of DNA synthesis and G_2 block[4)5)11)]. Therefore, NCS shows some antitumor activities and has been used clinically for the treatment of human cancers.

In previous papers[1)2)], Antoku and Kura have demonstrated that postirradiation treatment with NCS fixes potentially lethal damage (PLD) and inhibits repair of sublethal damage. However, the cellular mechanisms by which NCS sensitizes mammalian cells to ionizing radiation are poorly understood. It is well established that DNA double strand breaks (dsb) are the critical lesions to cell death[10]. Therefore, it would be of interest to know the effects of NCS on the induction of DNA dsb after X-irradiation. The data presented show that NCS enhances the initial dsb formation caused by X-irradiation in rat 3Y1 fibroblasts. It is also shown that NCS enhances the radiosensitivity of 3Y1 cells at all phases of the cell cycle.

Materials and Methods

Cultured Cells

The rat diploid fibroblast line (3Y1) was established by G. Kimura et al[7]. 3Y1 cells were grown in Dulbecco-modified Eagle's minimum essential medium with 100μg/ml streptomycin sulfate and 500 IU/ml penicillin G, supplemented with 10% fetal calf serum (growth medium). The cells were cultured in humid air containing 5% CO_2 at 37°C. The doubling time of growth was 16-17 h.

Irradiation

The radiation used was 200 kVp X-rays with 0.3 mm Cu added filtration at a dose rate of 1.2

Gy/min for colony survival assays. A dose rate of 10 Gy/min without filtration was used for the study of DNA strand breaks.

NCS treatment and colony formation

Purified NCS at a concentration of 1 mg/ml (1000 units/ml) was kindly provided by Kayaku Co., Ltd., Japan. NCS was added to the culture (final concentration of 0.019μg/ml) immediately after X-irradiation. Then the culture was incubated for 20 min at 22°C.

To obtain quantitative dose-survival curves, colony forming ability of single cells was determined by a conventional clonogenic technique. Log phase monolayer cultures at a density of about 2×10^5 cells per 35-mm plastic dish were used in each experiment of irradiation, which were seeded two days earlier at a density of 0.3×10^5 cells per dish. After X-irradiation and treatment with NCS, cells were rinsed, trypsinized, diluted and plated in 60-mm dish containing 5 ml of growth medium. After incubation for 7 days, the colonies were fixed, stained with crystal violet and then counted. The plating efficiency ranged between 70 and 80%. In the figures to follow, the average surviving fractions and standard deviations, which were obtained in at least three experiments, are shown.

Cell synchronization

Confluently grown G_0-resting 3Y1 cells were trypsinized and kept in 100-mm plastic dish containing 10 ml growth medium with 2 mM thymidine for 20 h. Subsequently, the cells, resting in S phase, were incubated in fresh growth medium for 7 h and 40 ng/ml Colcemid was added to the culture. After incubation for 1.5 h, highly synchronous M phase cells were obtained. Mitotic index was about 94%. These cells were diluted, plated in 60-mm dish and irradiated at each phases of the cell cycle.

Neutral filter elution

Cells were labelled with [2-^{14}C] thymidine (1.4 kBq, 1.87 GBq/mmol) for 24 h. At this time, there were approximately 4×10^5 cells per dish in exponentially growing phase. After the medium was changed to fresh medium, the cells on ice bath were irradiated with X-rays. DNA damage was assayed by neutral elution[3] with slight modifications. Immediately after irradiation or after postirradiation treatment with 0.019μg/ml NCS for 20 min, cells were washed at 0°C onto filters and treated as described in detail[12]. The level of DNA dsb induction was calculated as strand scission factor (SSF), which was the negative logarithm of the fraction of DNA retained on the filter after 5 h of elution as a function of X-ray dose.

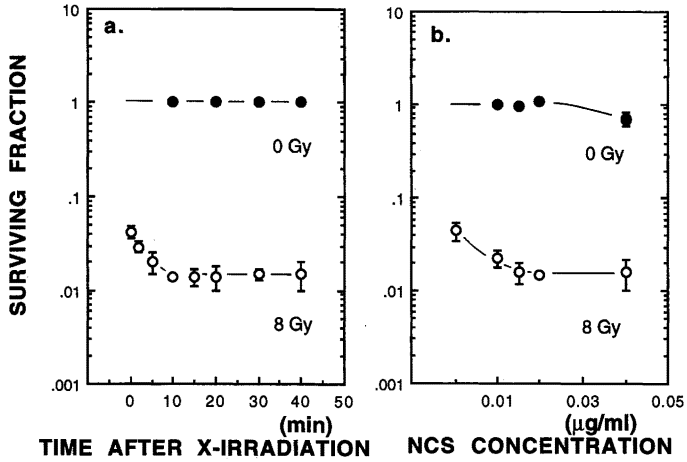

Figure 1 Cytotoxicity and radiosensitizing effects of NCS as a function of treatment period and NCS concentration. (a) NCS 0.019 μg/ml, 22°C. (b) 22°C, 20 min.

Results

The cytotoxicity and radioenhancing effects of NCS for 3Y1 cells as a function of NCS concentration and time in NCS after X-irradiation, respectively, are shown in Figure 1. The fixation of the damage by NCS was very rapid and was completed within 10-20 min at a concentration of 0.019μg/ml NCS. NCS at this concentration afforded a maximum radioenhancement and was non-toxic for 40 min at 22°C. Therefore, treatment with 0.019μg/ml NCS for 20 min at 22°C was used throughout the study.

Figure 2 shows the radioenhancing effects of NCS after X-irradiation in asynchronous 3Y1 cells. Postirradiation incubation with 0.019μg/ml NCS for 20 min decreased the survival. The major effect of the drug appeared to be an increase in the slope of the survival curve. The enhancement ratio of NCS calculated based on the ratio Do values was 1.25.

Figure 3 shows the radiosensitizing effects of NCS at different phases of the cell cycle. Control cells exhibited the fluctuations in radiosensitivity throughout the cell cycle and

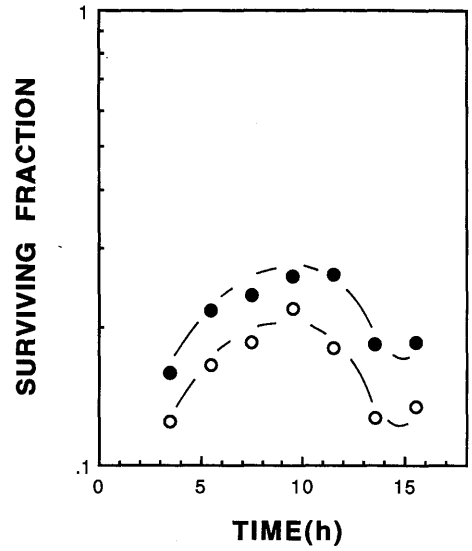

Figure 3 The fluctuations in cell survival throughout the cell cycle after a dose of 6 Gy with or without 0.019 μg/ml NCS (22°C, 20 min). M-phase cells were selected and plated in normal medium at 0 time on the abscissa. (○) After various periods of time, cells were irradiated, kept with 0.019 μg/ml NCS for 20 min and incubated in normal medium again; (●) Control.

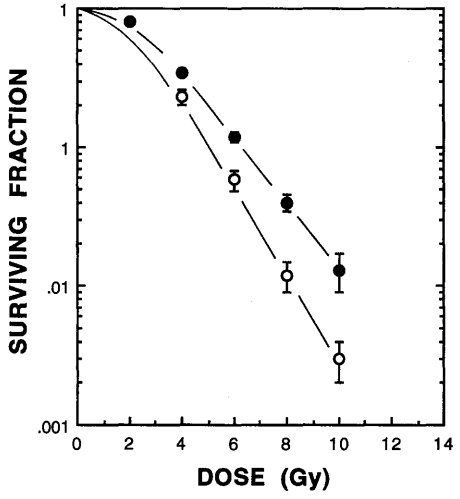

Figure 2 Survival curve of 3Y1 cells treated with NCS. (○) Cells were incubated with 0.019 μg/ml NCS for 20 min after X-irradiation. D_0=1.39 Gy, n=4.5; (●) Control. D_0=1.74 Gy, n=3.3.

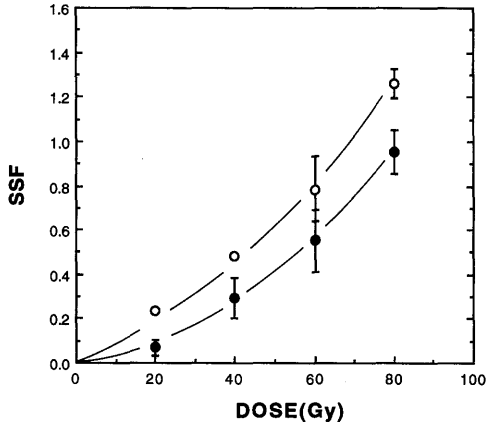

Figure 4 The relationship between DNA dsb frequency and x-ray dose, using neutral filter elution. The elutability of DNA was determined as described in Materials and Methods. (○) NCS 0.019 μg/ml, 22°C; (●) Control.

postirradiation incubation of synchronous cells with 0.019μg/ml NCS for 20 min enhanced cell killing at all phases of the cell cycle.

The induction of DNA dsb by X-irradiation in the presence or absence of NCS was measured in asynchronous 3Y1 cells (Figure 4). 3Y1 cells treated with NCS after X-irradiation demonstrated an increased induction in the frequency of DNA dsb. Without irradiation, no induction of DNA dsb was observed in the cells treated with 0.019μg/ml NCS for 20 min (data not shown).

Discussion

In this paper, it was shown that NCS sensitized 3Y1 cells to ionizing radiation at all phases of the cell cycle. Similar results about fixation of PLD were obtained by Utsumi and Elkind[13], who showed that synchronous cells at different phases of the cell cycle were sensitized to the same extent by hypertonic treatment after X-irradiation. These observations suggest that there are some similarities in the mode of fixation of PLD between NCS and hypertonic treatment.

Neutral filter elution analyses (Figure 4) indicate that the increased level of DNA dsb induction by postirradiation treatment with NCS may be related to enhancement of X-ray cell killing. It is generally accepted that radiation lethality results from damage to genomic DNA[10] and it is possible that NCS somehow augments this damage. NCS by itself also induces DNA strand breaks. However, the low concentration (0.019μg/ml) of NCS, which could not induce DNA dsb by itself, was effective in increasing both X-ray cell killing and DNA dsb. Kuo et al.[8] have suggested that the DNA lesions by NCS are more lethal than those by X-rays, based on the observation that the concentration of NCS required in the DNA strand breaks were much higher than that required for cell killing. It is likely that undetectable DNA lesions induced by NCS may interact with X-irradiation and result in the increase in the induction of DNA strand breaks.

In summary, it was found that NCS enhanced the radiosensitivity at all phases of the cell cycle and increased the induction of DNA dsb after X-irradiation. The characteristics of NCS may be advantageous to a combination therapy with radiation. However, further studies on the differences in response to X-irradiation between tumors and normal tissues are needed in order to avoid the damage of normal tissues by the simultaneous combination of radiation and NCS.

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(和文抄録)

同調および非同調ラット 3Y1 細胞における ネオカルチノスタチンの放射線増感効果

九州大学医学部放射線基礎医学教室（指導：安徳重敏教授）
中　村　和　正

ネオカルチノスタチン（NCS）は放射線による潜在的致死障害を固定し，亜致死障害の回復を阻害する，と報告されている．今回，ラット 3Y1 細胞を用いて，各細胞周期での NCS の放射線増感効果および非同調細胞での放射線照射後の DNA 二重鎖切断に与える影響について検討した．その結果，NCS は各細胞周期でほぼ同様に放射線致死効果を増強し，また，放射線による DNA 二重鎖切断を増加させることがわかった．一般的に，放射線による致死効果は DNA 二重鎖切断によると考えられており，NCS はこの切断を増すことにより増感効果を示すと推測される．