Effect of Poly(Adenosine Diphosphate-Ribose) Polymerase Inhibitors on Neocarzinostatin-induced G₂ Delay in HeLa-S₃ Cells

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ABSTRACT

The antitumor antibiotic neocarzinostatin (NCS), which produces single-strand breaks in mammalian cell DNA in vivo, stimulated the activity of chromatin bound enzyme, poly(ADP-ribose) polymerase in HeLa-S₃ cells. Because of the possible causal relationship between the poly ADP-ribosylation of chromatin protein and NCS-induced temporary G₂ arrest in the cell cycle, several classes of inhibitors of poly(ADP-ribose) polymerase were examined to evaluate the effect on NCS-induced polymerase activity as well as on progression in the cell cycle of synchronized HeLa cells which had been treated with NCS in G₂. Compared at the same concentration of 2 mM, the polymerase-inhibiting activity was larger in the order of thymidine, 3-aminobenzamide, nicotinamide, theophylline, and caffeine. Among these agents, caffeine, theophylline, and thymidine caused a reduction in the G₂ delay in this order by stimulating the cells to undergo mitosis after NCS treatment. However, 3-aminobenzamide and nicotinamide were poor reducers, if any, of NCS-induced G₂ delay. These results suggest that there is not a direct involvement of poly ADP-ribosylation of chromatin protein in the mechanism of NCS-induced G₂ delay. The effect of caffeine on G₂ delay will probably be independent of its activity as a poly(ADP-ribose) polymerase inhibitor.

INTRODUCTION

A variety of DNA-damaging agents, including ionizing radiation (1), alkylating agents (2), bleomycin (3), and NCS (4, 5), are known to cause mammalian cells to arrest temporarily at late G₂ resulting in the delayed initiation of mitosis. Although the relationship of DNA strand breakage by these agents to G₂ delay is unknown, posttreatment with xanthine derivatives such as caffeine or theophylline prevents or reduces G₂ delay following the DNA damage (6–9). Recently, many DNA-damaging agents including all of the above mentioned have been found to cause an increase in the activity of poly(ADP-ribose) polymerase and a concomitant decrease in cellular NAD⁺ content (10–13). Such an increase of polymerase activity is inhibited by several classes of inhibitors of poly(ADP-ribose) polymerase-inhibiting activity was larger in the order of thymidine, 3-aminobenzamide, nicotinamide, theophylline, and caffeine. Among these agents, caffeine, theophylline, and thymidine caused a reduction in the G₂ delay in this order by stimulating the cells to undergo mitosis after NCS treatment. However, 3-aminobenzamide and nicotinamide were poor reducers, if any, of NCS-induced G₂ delay. These results suggest that there is not a direct involvement of poly ADP-ribosylation of chromatin protein in the mechanism of NCS-induced G₂ delay. The effect of caffeine on G₂ delay will probably be independent of its activity as a poly(ADP-ribose) polymerase inhibitor.

MATERIALS AND METHODS

Chemicals. NCS was a gift from Kayaku Antibiotics Research Co., Ltd. (Tokyo, Japan). Caffeine, theophylline, nicotinamide, and nicotinic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), hydroxyurea, thymidine, deoxyycytidine, and 3-aminobenzamide were from Sigma Chemical Co. (St. Louis, MO); propidium iodide was from Calbiochem (La Jolla, CA); [methyl-⁴H]thymidine and [adenine-¹⁴C]NAD⁺ were from Amersham International, Plc. (Amersham, United Kingdom).

Cell Culture. HeLa-S₃ cells were grown in Eagle’s minimum essential medium supplemented with 10% fetal calf serum (M. A. Bioproducts, Walkersville, MD), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified, 5% CO₂ atmosphere. This cell line was determined to be free of mycoplasma contamination by the direct culture method. The doubling time was approximately 21 h. Cell synchronization at the G₁–S boundary was performed in two steps with the combination of an excess thymidine (17) and hydroxyurea (18). The cells were first placed in a medium containing 2 mM thymidine for 24 h, then returned to a fresh medium containing 10⁻³ M deoxyycytidine and left for 12 h, followed by another 12 h in a medium with 1 mM hydroxyurea. The cells were released from the G₁-S block by replacing the medium with fresh normal medium.

Drug Treatment. A stock solution of NCS in 0.015 M sodium acetate buffer, pH 5.0, was kept in the dark at 4°C and was diluted with the medium just prior to the experiment. After the culture medium was removed, fresh medium containing NCS was added to the culture and was maintained for 30 min. At the end of the treatment the medium containing NCS was removed and the culture was rinsed once before fresh medium was added. Concentration of the poly(ADP-ribose)-polymerase inhibitors in the posttreatment was 2 mM throughout this study.

Enzyme Assay. Poly(ADP-ribose) polymerase activity was measured in permeabilized cells according to the method of Berger et al. (19). The cells were collected by trypsinization, washed once with phosphate-buffered saline, and were rendered permeable to exogenously supplied nucleotides by an incubation for 20 min at 4°C in a hypotonic buffer composed of 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 4 mM MgCl₂, 20 mM 2-mercaptoethanol, 25 μM of [¹⁴C]NAD⁺ (specific activity, 298 mCi/μmol), and 1 x 10⁶ permeable cells. After incubation for 10 min at 30°C the reaction was terminated with an excess of cold 20% TCA. Inhibitors of the enzyme dissolved in 10 mM Tris-HCl (pH 7.8) were added to the system and were present throughout the hypotonic treatment and the enzyme reaction. The TCA-insoluble fraction was collected on a Whatman GF/C disc filter, washed extensively with 10% TCA and then with ethanol, and measured for radioactivity with a scintillation solution of 0.4% PPO and 0.01% POPOP in toluene.

Determination of Mitotic Index. The cells grown on a coverslip (18 x 18 mm) placed in a 35-mm Petri dish were fixed with methanol for 10 min at room temperature, stained with Giemsa solution, and mounted on a slide. The percentage of the cells in mitosis was determined by counting 1000 cells/slide. At fixation, the medium was removed gently and without rinsing the cells methanol was poured from the edge of the Petri dish so as to cover the cell surface very slowly. With this procedure produces a direct cause for the delayed initiation of chromosome condensation, i.e., G₂ delay.
the loss of mitotic cells from the cover slip was negligible.

**Determination of Cells in DNA Synthesis.** To determine the fraction of cells synthesizing DNA at each time, the cells were pulsed for 30 min with [3H]thymidine (25 Ci/mmol; 2 μCi/ml). At the end of the labeling, the cells were rinsed twice with ice-cold phosphate-buffered saline and fixed in methanol for 30 min at -20°C. After drying the coverslip was mounted on a slide and dipped in an emulsion (Sakura NR-M2) for autoradiography. The slide was developed after 2 weeks of exposure and the fraction of labeled nuclei was determined by counting 1000 cells/slide.

**Cell Cycle Analysis.** To analyze progression in the cell cycle, the cells were prepared for flow cytometry by the method of Krishan (20). The cells were collected by trypsinization and aliquots of 1 to 3 x 10^6 cells were washed with phosphate-buffered saline containing glucose and fixed with cold 70% ethanol. They were then treated with RNase A, 1 mg/ml (Worthington Biochemical Corp., Freehold, NJ) for 30 min at 37°C. After washing the cells were stained for DNA with a solution of propidium iodide (50 μg/ml in 1.12% sodium citrate and 0.1% Nonidet P-40) for 30 min at 4°C. Flow cytometry was performed with a cell sorter, Model CS-20 (Showa Denko Co., Ltd., Tokyo, Japan), to obtain a DNA histogram of approximately 3 x 10^6 cells. The fraction of cells in various phases of the cell cycle was calculated from areas under the peaks (21).

**RESULTS**

**Stimulation of Poly(ADP-Ribose) Polymerase Activity by NCS.** It has been established that lowering of the cellular NAD* content observed following DNA damage by such agents as ionizing radiation and alkylating agents is due to the stimulation of nuclear poly(ADP-ribose) polymerase activity (12, 15) which can be measured by the uptake of radioactive NAD* into the permeabilized cell system (15, 19). Since NCS had already been reported to cause a rapid lowering of NAD* concentration in L1210 cells (11), we first attempted to confirm that NCS treatment of HeLa cells caused an increase in nuclear poly(ADP-ribose) polymerase activity as measured in the permeable cell system.

Chart 1 shows the activity of poly(ADP-ribose) polymerase in exponentially growing HeLa cells following the 30-min treatment with NCS. A few min of exposure to NCS is known to be sufficient to exert the maximum DNA damage in vivo (22). With NCS, 10 µg/ml, the enzyme activity increased to approximately 4 times that of the control within the initial 30 min and then declined slowly (Chart 1A). When different concentrations of NCS were used the increase in the enzyme activity at 30 min was dependent on the NCS concentration (Chart 1B). However, the concentration of NCS required for a detectable increase in the enzyme activity was much higher compared with that required for the cytotoxicity, since the 37% survival dose is 0.01–0.03 µg/ml in the colony formation experiments following 30 min exposure to NCS (7, 23).

**Inhibition of Poly(ADP-Ribose) Polymerase.** Table 1 shows the effect of several different classes of known inhibitors of poly(ADP-ribose) polymerase on the enzyme activity induced by NCS. After treating exponentially growing cells with NCS for 30 min the cells were put into ice-cold hypotonic buffer containing one of these agents. The agent at a concentration of 2 mM was present throughout the 20 min of permeabilization and 10 min of enzyme assay. Thymidine, 3-aminobenzamide, and nicotinamide were found to be the most potent inhibitors of the enzyme, giving more than 95% inhibition. Among xantine derivatives theophylline was highly effective and caffeine was a less effective inhibitor of the enzyme. Nicotinic acid, which was used as a control of nicotinamide, was a poor inhibitor of the enzyme. These results are basically in agreement with previous reports on the activity of polymerase inhibitors using other systems (14, 15).

**G2 Delay Induced by NCS.** HeLa cells were synchronized at the G1-S boundary. After release from the G1-S block the percentage of the cells in DNA synthesis as revealed by autoradiography began to increase immediately, reaching a peak at 3-5 h. The next cycle of DNA synthesis occurred at about 19 h (Chart 2A). The mitotic figures first appeared at 9-10 h, reaching a peak of mitotic index at 11 h. Thus we regarded 8 h after release as the G2 of the cell cycle and treated the cells with NCS at 8 h in the subsequent experiments. Treatment for 30 min with NCS, 0.02 µg/ml, was sufficient to cause a pronounced G2 delay, shifting the peak of mitosis to 20 h. However, when caffeine was present in the medium after the removal of NCS the G2 delay was markedly shortened, resulting in the mitotic peak at 14 h. Treatment with caffeine alone caused a slightly earlier rise of normal mitosis.

We also analyzed the cell cycle progression of the cells under these conditions by flow cytometry (Chart 2B). Between 8 and 16 h most of the cells accomplished progression from G2 to G1 as indicated by the increase in the fraction of the cells with G1.

<table>
<thead>
<tr>
<th>Agent (2 mM)</th>
<th>[14]NAD* incorporation (cpm)</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>18,632</td>
<td>71</td>
</tr>
<tr>
<td>Theophylline</td>
<td>5,480</td>
<td>94</td>
</tr>
<tr>
<td>Thymidine</td>
<td>972</td>
<td>97</td>
</tr>
<tr>
<td>3-Aminobenzamide</td>
<td>479</td>
<td>97</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>522</td>
<td>97</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>17,446</td>
<td>6</td>
</tr>
</tbody>
</table>

*Radioactivity incorporated into 10^6 permeable cells in 10 min at 30°C, mean of duplicate assays.

Table 1

**Effect of chemical agents on poly(ADP-ribose) polymerase activity in NCS-treated cells**

Cells treated with NCS (10 µg/ml) for 30 min were measured for enzyme activity as in Chart 1. The indicated agent was added immediately after removal of NCS. The amount incorporated by NCS-untreated control cells was 4,200 cpm.

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DNA content to that with G₂ + M DNA content. In the cells treated with NCS at 8 h, cell cycle progression was completely blocked in G₂. With posttreatment with caffeine, however, a considerable fraction of the cells had DNA content of G₁ at 16 h, indicating that caffeine not only induced initiation of mitosis but also caused the normal accomplishment of mitosis.

**Effect of Poly(ADP-Ribose) Polymerase Inhibitors on NCS-induced G₂ Delay.** The agents in Table 1 which were examined for activity to inhibit poly(ADP-ribosyl) polymerase were also examined for the effect on cell cycle progression of NCS-treated cells. After the synchronized cells were treated at 8 h with NCS, 0.02 µg/ml, for 30 min, one of the agents was added to the medium and maintained continuously. The subsequent cell cycle progression was monitored both by counting mitotic index and by performing a flow cytometric analysis every h (Table 2). As expected from Chart 2B the G₁:G₂ + M ratio in the DNA histogram gave an estimation of cell cycle progression from G₂ to G₁. When added without NCS treatment (experiment 1) the agents did not cause a significant change in the normal cell cycle progression, with the exception of thymidine (2 µM), which increased the S fraction at 16 h. This is reasonable when we consider the suppression by thymidine of S-phase cells which were present in the population at 8 h. Neither immediate cytotoxicity, as judged by dye exclusion, nor cell detachment was seen with any agent at the concentration of 2 µM. After NCS treatment (experiment 2) caffeine was the most effective agent in overcoming NCS-induced G₂ block, as shown by an increase in both the mitotic index at 14 h and in the G₁:G₂ + M ratio at 16 h. Since no other agent combined with NCS brought about an earlier peak of mitosis than did caffeine, the mitotic index and the G₁:G₂ + M ratio are shown in Table 2 only at 14 and 16 h, respectively, to compare the modification of NCS-induced G₂ delay by these agents. Theophylline, another xanthine derivative, was also effective in ameliorating G₂ delay, although less so than was caffeine. Thymidine was effective to some extent as far as judging by the two parameters. However, nicotinamide, which was shown to be a potent inhibitor of poly(ADP-ribose) polymerase (Table 1), was a poor if at all inhibitor of NCS-induced G₂ block. Furthermore, 3-aminobenzamide had no significant effect on the cell cycle progression of NCS-treated cells. Nicotinic acid was less effective than nicotinamide but not less than 3-aminobenzamide. Comparing the results of Table 1 and Table 2 it can be concluded that there is no clear correlation between the ability of the agents as poly(ADP-ribose) polymerase inhibitors and their ability to overcome NCS-induced G₂ block.

**DISCUSSION**

Increasing evidence suggests that DNA strand breakage in mammalian cell, whether formed directly by cytotoxic agents or secondarily through the cellular DNA repairing mechanism, gives rise to several common biological phenomena, i.e., inhibition of DNA synthesis, G₂ delay in the cell cycle, and increase in the nuclear poly(ADP-ribose) polymerase activity. Although G₂ delay following the DNA damage tends to attract less attention than does inhibition of DNA synthesis, it should be noted that the former occurs with much lower doses of the DNA-damaging agent. For example, in the case of X-irradiation a nontoxic dose of only 9 rads can cause G₂ delay in Chinese hamster ovary cells (1), while at least several hundred rads are required to cause a significant decrease in DNA synthesis (9). A similar dose-effect discrepancy is obtained with bleomycin (3, 7) and NCS (7).

The role of G₂ delay following DNA damage is not well understood, but there is evidence that providing cells with the time necessary for DNA repair before onset of mitosis has a role in...
increasing cell survival. Thus arrest of the cell cycle at G2 was postulated by Tobey (5) as a "surveillance mechanism" for cells with altered DNA. Caffeine is known to enhance the cytotoxicity of a variety of DNA-damaging agents (23-26). Although this phenomenon is usually attributed to an inhibition by caffeine of postreplication repair of DNA damage (25, 27), a recent report by Lau and Pardee (8) showed that the posttreatment of mammalian cells by caffeine following exposure to an alkylating agent resulted in an increase of chromosome fragmentation in the subsequent mitosis suggesting that the reduced length of the G2 delay might be responsible for the caffeine-induced enhancement of cytotoxicity. A similar suggestion was made for X-irradiation (28). On the other hand there is growing evidence that poly(ADP) ribosylation of chromat protein participates in the cellular DNA repair pathway (29) by, as one possibility, activating DNA ligase (30). Posttreatment with enzyme inhibitors has been reported to inhibit the rejoining of single-strand breaks and cause increased cell death (29, 31).

The aim of this study was to test by using several different classes of enzyme inhibitors the hypothesis that poly(ADP) ribosylation of chromat protein is the direct cause of G2 delay. As shown in the text a 30-min exposure to NCS as low as 0.02 μg/ml caused a pronounced G2 delay, while hundreds of times as much concentration were necessary to stimulate polymerase activity as judged by [3H]NAD+ incorporation. This discrepancy is in itself unfavorable for the hypothesis, although a possibility cannot be ruled out that a slight change in the polymerase activity which is technically indetectable might be critical in the cell progression in G2. Furthermore, 3-aminobenzamide and nicotinamide, which were the most potent inhibitors of the enzyme, had a poor ability to ameliorate NCS-induced G2 delay, contrary to the hypothesis. Since the assay of these agents in the enzyme inhibition was carried out in permeabilized cells, there remains a possibility that in the assay of the effect on the cell cycle, 3-aminobenzamide and nicotinamide are taken up by intact HeLa cells much less or inactivated in the cells much faster than is caffeine. We have not tested this possibility but it has never been suggested in any of the previous reports using these inhibitors on intact cells (14, 15). The xanthine derivatives caffeine and theophylline were effective in reducing the G2 delay in this order, which is the reverse of that of the activity of these agents as polymerase inhibitors. Since theophylline is known to have more cytotoxicity than caffeine when given to intact HeLa cells much less or inactivated in the cells much faster than is caffeine. Although their result was questioned recently by other investigators (40) who showed that this is not the case in all AT cells, it is attractive that AT cells seem to be impaired somehow in recognizing DNA damage and thus are very similar to caffeine-treated cells. It is possible that caffeine interferes with a certain cellular mechanism which recognizes DNA damage and which is intrinsically defective in AT cells. Research along these lines will contribute to further elucidate cellular response to DNA damage.

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Effect of Poly(Adenosine Diphosphate-Ribose) Polymerase Inhibitors on Neocarzinostatin-induced G2 Delay in HeLa-S3 Cells

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