Induction and Repair of DNA and Chromosome Damage by Neocarzinostatin in Quiescent Normal Human Fibroblasts

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ABSTRACT

In an attempt to understand better the molecular basis of chromosome aberration formation, neocarzinostatin (NCS)-induced damage and repair were compared at the chromosome and DNA levels. Chromosome damage and repair in quiescent normal human fibroblasts (PA2 and DET 550) were assayed by the premature chromosome condensation technique in which the Gi, prematurely condensed chromosomes are condensed and easily enumerated. DNA damage was monitored by neutral DNA elution. NCS induced chromosome breaks directly in the Gi cells; thus, its clastogenic action does not require passage of cells through S phase. The dose-response curve for NCS-induced damage suggested a two-hit-type event in the formation of chromosome breaks. The half-time of chromosome repair was approximately 4.5 hr, and all but one of the chromosome breaks were repaired by 46 hr. Neither 1-ß-D-arabinofuranosylcytosine nor cycloheximide blocked the repair of either DNA or chromosome damage induced by NCS in quiescent human cells.

INTRODUCTION

Many chemotherapeutic and radiotherapeutic modalities exert their cell-killing effects through damage to the cellular genome. While this damage is manifest in both DNA and chromosome damage, the exact relationship between the induction and repair of DNA and chromosome lesions is not entirely understood, especially in normal, quiescent cells. The understanding of this relationship has been elusive, because it is difficult to measure DNA and chromosome damage at the same time and dose. However, 2 techniques developed recently, DNA elution and PCC, lend themselves to this comparison. The DNA elution techniques are useful since they are sensitive enough to measure DNA damage at doses that are suitable for chromosome damage studies (41). The PCC technique is useful since it allows the immediate determination of chromosome damage without the need for the treated cells to reach mitosis (29, 33). Thus, DNA and chromosome damage can be measured simultaneously and at similar treatment doses.

Using this approach, we showed recently that, in cycling CHO cells, CHX and streptovitacin A could block the repair of bleomycin-induced chromosome breaks without affecting the repair of DNA single-strand breaks and alkali-labile sites (54, 56). Similarly, CHX could prevent the repair of chromatid breaks but did not prevent the repair of single- and double-strand breaks and alkali-labile sites after γ-irradiation (25). In addition, most of the DNA damage was repaired before any detectable chromosome repair had taken place. These results suggest that only a minority of DNA lesions are associated with the formation of chromosome breaks and that chromosome repair involves more than simply the repair of damaged DNA.

One problem with the above experiments was that the long-term kinetics of DNA and chromosome repair could not be measured due to the cycling nature of the CHO cells. Repair experiments of long duration require noncycling cell populations. Normal human fibroblasts are useful in this regard because they can be brought to a quiescent stage when grown in medium containing low serum concentrations. These quiescent normal cells have been found to arrest in early Gi phase and have condensed Gi PCC suitable for chromosome damage analysis (28). Further, the DNA of these cells can be labeled with radioactive thymidine prior to arrest in low serum and thus is suitable for DNA elution analysis.

The purpose of the experiments reported here was 2-fold: (a) to test the validity of this experimental approach characterizing the induction and repair of chromosome damage induced by NCS in quiescent normal human fibroblasts; and (b) to compare the effects of CHX and ara-C on the repair of damage at both the DNA level (using neutral DNA elution) and chromosome level (using the PCC technique). NCS is an acidic protein antibiotic produced by Streptomyces carzinostaticus variant F-41 (31) which has been shown to induce DNA and chromosome damage (42, 49), slow cellular DNA synthesis (2, 6, 30, 38, 40, 49, 52), cause growth retardation (6, 14, 32), and have some antitumor activity in the clinical setting (22, 43, 51). NCS was useful for these experiments due to its similarity in action to bleomycin and phleomycin in that it can induce DNA double-strand breaks and chromosome breaks directly in cells without the need for the cell to pass through S phase (1, 3, 19, 45, 53, 58). Thus, one can perform long-term experiments on noncycling cell populations.

The studies reported here show that NCS can induce chromosome damage directly in quiescent normal human fibroblasts, and this damage is repaired with a half-time of 4.5 hr. Interestingly, nearly all the chromosome breaks can be repaired within 48 hr. In contrast, bleomycin or γ-irradiation in CHO cells, neither CHX nor ara-C was able to inhibit DNA or chromosome repair after NCS treatment.

MATERIALS AND METHODS

Cells. Normal human fibroblasts (PA2 and DET550) were grown as monolayer cultures in plastic Petri dishes (Flow Laboratories, Inc.,...
Inglewood, Calif.) in Hsu-modified McCoy’s medium (Grand Island Biological Co., Santa Clara, Calif.) supplemented with 15% fetal calf serum and a 1% penicillin-streptomycin mixture (full serum medium). Quiescent cultures were obtained by growing the cells until confluent in full serum medium, followed by incubation in medium containing only 0.5% conditioned serum for at least 48 hr prior to treatment. Low serum conditioned medium was obtained by diluting filtered medium from confluent cultures with medium without serum. HeLa cells were grown as monolayer cultures using the same procedure in Hsu-modified McCoy’s medium containing 10% fetal calf serum and a 1% penicillin-streptomycin mixture.

Drug Treatment. NCS (Kayaku Antibiotics, Tokyo, Japan) was kindly supplied in sodium acetate:acetic acid buffer by the Division of Cancer Treatment of the National Cancer Institute, NIH. Cells were treated with NCS at the appropriate concentration of 30 min at 37° in low serum medium buffered with 10 mM PIPES-N,N',N'-bistr(2-ethanesulfonyl) acid. At the end of the drug treatment, the cell cultures were washed twice in low serum medium and reincubated in low serum medium. This treatment retained cells in a quiescent phase. At the time of harvest, cell cultures were treated with 2 washes of cold 0.05% trypsin:0.02% EDTA until the cells rounded; then the trypsin:EDTA was removed, and the cells were removed from the dishes in Hanks’ BSS (Grand Island Biological Co.).

Cell Fusion and Determination of Aberration Frequency. The procedure for cell fusion and induction of PCC has been described previously (24, 26). Mitotic HeLa cells were obtained by treating exponentially growing cultures with 3 mM thymidine for 24 hr, washing the cells free of excess thymidine, and allowing them to accumulate for 16 hr or more in nitrous oxide (N₂O) at 5 atmospheres pressure (50). After the N₂O treatment, selective detachment of mitotic HeLa cells resulted in populations with greater than 95% mitotic index, and these cells were incubated in Colcemid (0.25 μg/ml) until use in order to further disrupt the mitotic spindle (this results in better chromosome spreads).

The treated cells were trypsinized, washed twice with Hanks’ BSS, and mixed with equal numbers of mitotic HeLa cells. The fusion mixture was then washed twice in Hanks’ BSS and centrifuged, and the cell pellet was resuspended in 0.5 ml Hanks’ BSS without glucose, containing UV-inactivated Sendai virus. The fusion mixture was cooled to 4° for 15 min to promote agglutination, 1 drop each of 20 mM MgCl₂ and Colcemid (5 μg/ml) was added, and then the mixture was incubated at 37° for at least 45 min. Increased incubation time resulted in more condensed PCC (18).

After incubation, the fusion mixture was treated with hypotonic 0.075 M KCI for 10 to 12 min at room temperature, fixed in methanol:glacial acetic acid (3:1, v/v), and dropped on clean, wet slides. After air drying, the slides were stained with Giemsa, allowed to dry, and condensation PCC (18), and the degree of chromosome damage was determined by counting the number of chromosome pieces found in the G₁ PCC following treatment (61) (Fig. 1). Since the centromeres of the G₁ PCC are not readily apparent under these staining conditions, the number of dicentrics following treatment was not enumerated in these studies.

As shown in Chart 1, the number of chromosome breaks per cell was observed to increase in a nonlinear fashion with dose, with 50 μg NCS per ml yielding nearly 20 breaks/cell in PA2 cells. The exact number of breaks per cell varied with the batch of NCS and with the cell line tested. We decided to use a dose of 50 μg/ml for subsequent chromosome repair experiments. While this NCS dose is somewhat high compared to that used in other studies, it facilitated the accurate measurement of the rate of chromosome repair.

Chromosome Repair Kinetics. Quiescent normal cell populations are useful for chromosome repair experiments because they can be treated and maintained in a noncycling state throughout the course of the repair period without having the analysis complicated by cell cycle perturbations. In order to look at the rate and extent of chromosome repair, quiescent cells were treated with NCS (50 μg/ml), rinsed free of drug, and then reincubated in low serum medium for various periods

Chart 1. Dose-response curve for NCS-induced chromosome damage. Quiescent fibroblasts were treated with increasing doses of NCS for 30 min and immediately fused to form G₁ PCC for evaluation of chromosome damage.
of time prior to harvesting for PCC induction.

The result of such a chromosome repair kinetics experiment is shown in Chart 2. As before, chromosome damage could be visualized immediately in the $G_1$ PCC after treatment, and no chromosome repair was apparent within a 0.5-hr interval after the end of NCS treatment. Significant repair of chromosome damage was observed by 3 hr, and half the chromosome breaks were repaired by approximately 4.5 hr. Interestingly, all but one chromosome break per cell were apparently repaired by 46 hr after NCS treatment. While the data are not shown here, similar results were observed in repeat experiments.

**Effect of CHX on DNA and Chromosome Repair.** In previous studies using CHO cells in $G_2$ phase, the treatment of cells with CHX during a 1-hr repair period after bleomycin or $\gamma$-irradiation significantly retarded the repair of chromatid breaks but had no effect on the repair of DNA lesions measured by the alkaline and neutral elution techniques (25, 54, 56). Thus, it was thought that protein synthesis must be necessary in the repair of chromosome aberrations.

We next wanted to test the generality of the above conclusion using NCS in quiescent human cells. Quiescent normal human fibroblasts were treated with 50 $\mu$g NCS per ml for 30 min in the presence or absence of 150 $\mu$g CHX per ml, washed free of drug, and then reincubated in low serum medium in the presence or absence of CHX for 4 hr prior to harvesting for neutral DNA elution analysis.

The results of such an experiment using 25 $\mu$g CHX per ml are shown in Chart 3. CHX at this dose had little effect on the rate of elution of cells not treated with NCS, and it only slightly increased the rate of elution of NCS-treated cells harvested immediately after NCS exposure. At this CHX dose, no effect was also studied using the DNA neutral elution technique, which has been suggested to reflect DNA double-strand breakage (8). While DNA double-strand breaks could be measured at lower NCS doses (19), we found that the use of higher NCS doses facilitated the measurement of DNA repair with neutral elution analysis. The protocol for this experiment was similar to that of the chromosome repair experiment; i.e., quiescent cells were treated with NCS for 30 min in the presence or absence of 25 or 150 $\mu$g CHX per ml, washed free of drug, and reincubated in low serum medium in the presence or absence of CHX for 4 hr prior to harvesting for neutral DNA elution analysis.

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on DNA repair was observed during a 4-hr repair period. Interestingly, DNA repair was apparently not complete by 4 hr. When CHX was used at 150 μg/ml (Chart 4), an apparent retardation of DNA repair was observed (compare Curves E and F). However, it was difficult to distinguish retardation of repair from additional CHX-induced damage, since the CHX-treated control also eluted more rapidly than an untreated control (compare Curves A and B).

Effect of ara-C on DNA and Chromosome Repair. In the final set of experiments, we wanted to determine whether ara-C, an inhibitor of DNA synthesis, could block the repair of NCS-induced damage at either the chromosome or DNA level. Quiescent human fibroblasts were treated with 50 μg NCS per ml in the presence or absence of 5 × 10^-5 M ara-C, washed free of drug, reincubated in low serum medium for 4 hr in the presence or absence of ara-C, and harvested for chromosome breakage analysis. The results are shown in Table 2. Treatment of quiescent cells for 4 hr with ara-C alone resulted in a slight increase in chromosome breaks; however, ara-C did not significantly increase the NCS-induced chromosome damage when cells were treated for 30 min and then evaluated immediately. The presence of ara-C during the repair period did not prevent the repair of NCS-induced damage. The slight increase

in the frequency of breaks observed in cells treated with ara-C during the repair period could be attributed to the action of ara-C alone during the 4-hr repair period.

In order to look at the effect of ara-C on the repair of NCS-induced DNA damage, quiescent cells were treated as described above, only the NCS dose was raised to 250 μg/ml for neutral DNA elution measurements. As shown in Chart 5, while a 4-hr treatment of quiescent cells with 5 × 10^-5 M ara-C induced a small amount of DNA damage, ara-C had little effect on the repair of NCS-induced DNA lesions measured by the neutral DNA elution technique.

**DISCUSSION**

The purpose of these studies was to characterize the action of NCS with regard to the induction of chromosome damage and to correlate the repair of DNA and chromosome damage in the face of the metabolic inhibitors CHX and ara-C. While the action of NCS on cellular DNA has been studied extensively (for review, see Ref. 17), little was known about the effect of NCS on chromosomes except that it induced chromosome aberrations (42, 49).

The results reported here show that NCS can directly induce chromosome breaks in cells without the need for cell cycle traverse. NCS is therefore similar in this regard to ionizing radiation and chemicals, such as bleomycin and phleomycin. Interestingly, NCS differs from bleomycin in the shape of the dose-response curve. In experiments reported previously on the chromosome-breaking action of bleomycin, it was shown that the dose-response relationship yielded a complex curve, with a sensitive component apparent at low doses and a more resistant component observed at higher doses (13, 55). It was postulated that this 2-component curve was the result of differential accessibility within cellular chromatin to bleomycin. Further, the linearity of the 2 components of the dose-response curve suggested that bleomycin resembled ionizing radiations with higher linear energy transfer (i.e., single-hit kinetics). In contrast, the dose-response curve for NCS-induced chromosome damage (Chart 1) suggests that of a compound with a stronger 2-hit component to its action and less regard for

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**Table 2**

Effect of ara-C on the repair of NCS-induced chromosome damage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chromosome damage</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No. of cells scored</td>
</tr>
<tr>
<td>---</td>
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<tr>
<td>— a</td>
<td>42</td>
</tr>
<tr>
<td>NCS</td>
<td>52</td>
</tr>
<tr>
<td>NCS ± ara-C</td>
<td>50</td>
</tr>
<tr>
<td>NCS + ara-C</td>
<td>44</td>
</tr>
<tr>
<td>NCS + ara-C</td>
<td>47</td>
</tr>
<tr>
<td>NCS + ara-C</td>
<td>42</td>
</tr>
</tbody>
</table>

a No treatment. b Mean ± S.D.
chromatin superstructure, at least in highly condensed $G_1$ chromatins.

These conclusions can be supported by the findings of others at the DNA level. NCS has been shown to act directly on DNA to induce strand breakage and alkali-labile bonds (1-3, 6, 45, 52, 58), especially at thymidine and adenine residues (21), resulting in the release of thymine and the generation of both 5'- and 3'-phosphate and 5'-thymidine:5'-aldehyde termini (39, 40, 46). While it is difficult to distinguish true single-strand breaks from alkali-labile bonds in the quantitation of strand breaks using alkaline sucrose gradients, it has been estimated that the ratio of double- to single-DNA strand breaks in vivo is about 1:30 (46) and that, in vivo, it appears to be about 1:5 (19). While double-strand breaks do not appear to be the result of the localization of 2 random single-strand breaks, the fact that thymidine residues are much more sensitive to NCS breaking activity than their complementary base adenine suggests that a double-strand break involves a 2-hit process (11). This might account for the strong 2-hit component to the chromosome dose-response curve shown in Chart 1.

The findings reported here indicate that the repair of NCS-induced chromosome damage in quiescent human cells is slow (half-time, ~4.5 hr), compared to the rate of chromosome repair of $\gamma$-irradiation-induced and bleomycin-induced chromatic breaks in cycling CHO cells (26, 27, 54). The reason for this relatively slow repair rate is not understood. One possibility is that differences in the rate of chromosome repair between cycling and noncycling cells. However, approximately 20 to 25% of the NCS-induced chromosome breaks was repaired within 2 hr (Chart 2), and this is similar to the extent of repair observed in $G_1$, cycling HeLa cells after $X$-irradiation (61).

Another possible explanation for the slow chromosome repair rate observed here is that different cell lines vary in their rate of chromosome repair. For example, Wolff and Luippold found that chromosome lesions that could interact to form exchanges were repaired with a half-time greater than an hr in plant cells (62), while Dewey et al. (12) found this half-time to be around 12 min for CHO cells. While these lesions might not be the same as the chromosome breaks observed in this study, these findings do suggest that chromosome repair rates may differ between cell types.

The slow rate of chromosome repair reflects the comparatively slow rate of DNA repair observed after NCS treatment in both baby hamster kidney cells (using alkaline sucrose gradient analysis) (32) and normal human fibroblasts (using neutral DNA elution) where DNA damage was still not completely repaired at 4 hr (Charts 3 and 4). This slow repair probably reflects the types of DNA lesions produced by NCS (59) rather than the fact that the cells are quiescent, since resting cells show repair rates comparable to those of cycling cells (34-37). For example, it has been shown that NCS produces free DNA ends which are not susceptible to fast repair by DNA polynucleotide ligase (20, 46). Repair of NCS-induced damage must include processing of the DNA termini, and this has been suggested to involve apurinic:apyrimidinic endonuclease activity in association with 5'-3' exonuclease activity prior to the resynthesis step (7, 57). These processes are thought to be relatively slow (60).

The degree of condensation of the $G_1$ PCC has been shown previously to reflect the stage of the cell within $G_1$ phase, with early $G_1$ cells exhibiting highly condensed PCC and late-$G_1$ cells yielding extended $G_1$ PCC (28). Treatment of early $G_1$ cells with agents such as UV and methylnitrosamides has been shown to cause attenuation of $G_1$ PCC, especially when an inhibitor of DNA repair is included in the repair period. On the other hand, $X$-irradiation does not induce this phenomenon (for review, see Ref. 44). In these studies, we found no evidence for attenuation of the $G_1$ PCC after NCS treatment, even when ara-C was included during the repair interval. Thus, the repair of NCS damage probably reflects repair mechanisms similar [e.g., short patch repair (57)] to that after $X$-irradiation.

Since most chromosome repair studies involve cycling cells, it has been difficult to determine whether all chromosome breaks can be repaired if the cell is given sufficient repair time. Nagasawa and Little (44) tried to approach this question by irradiating quiescent mouse 10T 1/2 cells and holding them for various periods of time prior to stimulation and analysis of chromosome damage at the first mitosis. They found that the aberration rate decreased with holding time. In contrast, Evans and Vijayalaxmi (16) found that storage of lymphocytes after mitomycin C treatment enhanced chromosome damage and also resulted in both chromosome-type and chromatid-type lesions appearing in the first mitosis. In this study, we have shown that nearly all the chromosome breaks were repaired by 46 hr after NCS treatment. It is possible that the remaining chromosome break resulted from the formation of a dicentric chromosome and the generation of 2 unrejoined fragments. This possibility is currently being tested using techniques to visualize the centromeres of the $G_1$ PCC. In any event, the observation that nearly all chromosome breaks are repairable, provided the cell is not required to progress through the cell cycle, suggests that noncycling normal tissue might be protected during NCS cancer chemotherapies by virtue of its ability to remain in the quiescent state and repair its chromosome damage.

In previous studies using the PCC technique, it was found that CHX could block the repair of chromosome damage in $G_2$ cells after $\gamma$-irradiation and bleomycin treatment but that this had no effect on the repair of DNA damage (25, 54, 56). In contrast, the present study suggests that CHX cannot block either chromosome or DNA repair after NCS treatment of quiescent human fibroblasts. Thus, it appears that repair of NCS damage in quiescent human fibroblasts does not require ongoing protein synthesis. It is not clear whether this variant result reflects the difference in clastogenic agents, cell types, or cell cycle stage. Studies are in progress to clarify this question.

ara-C has been utilized recently to prevent the resynthesis step in excision repair after a variety of clastogenic agents resulting in an accumulation of single-strand breaks (10, 15, 23). This is especially apparent after agents that induce long patch repair. At the DNA level, accumulation of single-strand breaks might be expected to result in an increase in double-strand breaks (9), and at the chromosome level, this might be expected to result in an increase or change in the types of chromosome breakage observed (4, 47, 48). In the experiment in which ara-C was included in the medium during the repair period (Table 2), no effect on the repair of NCS-induced DNA or chromosome damage was observed. This finding might reflect the fact that NCS repair involves the incorporation of only a few nucleotides into the DNA and thus would not be as
sensitive to the action of ara-C (57). Interestingly, ara-C itself seemed to induce a slight increase in chromosome damage in quiescent cells. While it has been suggested previously that ara-C induces aberrations in non-S-phase cells (5), the possible molecular mechanism for this process is not understood.

Finally, the studies reported here show that the combination of quiescent normal human fibroblasts and the technique of PCC can be useful in the study of the molecular basis of chromosome damage and its repair. The advantages of the system are 3-fold. (a) DNA and chromosome damage can be assayed simultaneously without the need for cells to progress through the cell cycle to mitosis. Thus, correlations can be made without considering DNA alterations that may occur during DNA synthesis. (b) One can maintain the cells in a quiescent state during the repair period and directly measure chromosome damage as a function of time after treatment. This allows one to look at long repair periods, as well as to study the effect of other agents on the repair process. In addition, after various repair intervals, if desired, the cells can then be stimulated to observe the effect of passage through S phase on the remaining DNA lesions. (c) A third advantage of this system is that the treated quiescent cells can be semipermeabilized and further treated with exogenous DNA-modifying enzymes, and then the effect on the chromosomes can be monitored using the PCC technique. This latter approach will be useful in studying the molecular basis of chromosome aberration formation and has potential application for studying the long-term clastogenic effects of chemotherapy and radiotherapy on nondividing normal tissues.

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REFERENCES


Fig. 1. Chromosome damage visualized in the G1 PCC of quiescent normal human fibroblasts. Quiescent cell populations were treated with NCS (50 µg/ml) for 30 min and fused with mitotic HeLa cells, resulting in G1 PCC. A, G1 PCC of untreated cells; B, G1 PCC of NCS-treated cell. Chromosome damage results in an increase in G1 chromosome pieces. The darkly staining mitotic chromosomes are from the mitotic inducer cells. Giemsa, × 1500 (A) and 1400 (B).
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