Vol. 87, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1979 Pages 575-580

APHIDICOLIN DOES INHIBIT REPAIR REPLICATION IN HELA CELLS

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Received February 23,1979

SUMMARY

Aphidicolin was shown to be a specific inhibitor of eukaryotic DNA polymerase α. We have examined the effect of aphidicolin on repair synthesis as well as replication of HeLa cell DNA, and found that it inhibits not only DNA replication but also UV-induced DNA repair in hydroxyurea-arabinosyl cytosine treated cells.

INTRODUCTION

Mammalian cells and other higher eukaryotic cells contain at least three kinds of DNA polymerases (DNA nucleotidyltransferases), α, β and γ. There are many lines of circumstantial evidence that DNA polymerase α plays an important role in DNA replication of mammalian cells, however, those are all indirect ones. Recently, it was reported that aphidicolin prevents DNA replication in several systems including the growth of herpes simplex virus both in cultured human embryonic lung cells and in the rabbit eyes (1), the DNA replication of uninfected cultured cells (1), and the mitotic division of sea urchin embryos (2), and that it is a specific inhibitor of DNA polymerase α (2, 3). Those studies gave us strong supports about the functional role for α polymerase in DNA replication. It is interesting to know whether the compound inhibits DNA repair or not. We report here the evidence that aphidicolin inhibits not only DNA replication but also UV-induced DNA repair replication in HeLa cells.

0006-291X/79/060575-06$01.00/0

METERIALS AND METHODS

Chemicals: Deoxyribonucleoside triphosphates, ATP, hydroxyurea and arabinosyl cytosine were purchased from Sigma Chemical Co. Tritiated dTTP (30 Ci/mmol), [^{3}H]thymidine (27Ci/mmol), [^{3}H]uridine (5Ci/mmol) and [^{3}H]leucine (53Ci/mmol) were obtained from The Radiochemical Centre, Amersham, U. K. Aphidicolin was obtained from the culture filtrate of the fungus, *Harziella entomophila*, as described elsewhere (4).

Cell culture, synchronization and UV irradiation: HeLa S3 cells were maintained in a monolayer in Eagle's minimal essential medium supplemented with 10% calf serum. Logarithmically growing cells were synchronized with hydroxyurea (1mM) for a period of 16h. Cells referred to as S phase were harvested at 3h after removal of hydroxyurea. UV irradiation was performed on the cells which mainly in G2 phase. Ten hours after removal of hydroxyurea, cells were incubated for 1h with 3mM hydroxyurea and 10μM arabinosyl cytosine to inhibit the residual DNA replication. Then cells were exposed to 315ergs/mm^2 of UV (as determined by potassium ferrioxalate actinometry). UV-irradiated cells were incubated for another 1h in the presence of hydroxyurea and arabinosyl cytosine.

Assay of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine incorporation: "Coverslip technique" of Baltimore and Franklin (5) was used. The final concentrations of the radioactive precursors were 0.5μCi/ml. In the case of the incorporation of [^{3}H]uridine, 10μM (final concentration) of thymidine was added to minimize the incorporation of [^{3}H]uridine to the DNA.

Preparation of cell homogenate: Cells were collected by trypsinization and centrifugation. The methods used for cell disruption were essentially those described by Friedman and Mueller (6), which were slightly modified as follows: Cells were homogenized in hypotonic buffer (10mM Tris-HCl, pH7.5, 2mM $MgCl_2$), and rehomogenized after addition of the equal volume of the buffer containing 120mM Tris-HCl, pH8.5, 160mM NaCl, 22mM glucose, 0.1%(v/v) Triton X-100, 1mM EDTA, 2mM 2-mercaptoethanol. The resulting homogenate, which contained both nuclei and cytoplasm, was used for the assay of *in vitro* DNA synthesis.

Assay of [^{3}H]dTTP incorporation: The reaction mixture for the incorporation of [^{3}H]dTTP into S phase nuclei contained, in a final volume of 0.3ml, 50mM Tris-HCl, pH8.5, 40mM NaCl, 5mM $MgCl_2$, 5mM 2-mercaptoethanol, 0.025mM EDTA, 0.025%(v/v) Triton X-100, 5.5mM glucose, 3.3mM ATP, 33μM each of dATP, dCTP and dGTP, 3.3μM [^{3}H]dTTP (1Ci/mmol), and cell homogenate equivalent to 1.25x10^6 cells. After incubation at 37°C for 10min with shaking, the reaction tubes were chilled in an ice-water bath, and acid-insoluble radioactivity was measured as described (7), except the addition of carrier DNA was omitted. The incorporation of [^{3}H]dTTP into UV-irradiated G2 phase nuclei was detected in the reaction mixture (0.3ml) as described above, except 50mM Tris-HCl, pH7.8 was used in place of pH8.5, and ATP was omitted. Under these conditions, 10^{4}cpm corresponds to 45pmoles of dTMP incorporated.

RESULTS AND DISCUSSION

Effect of aphidicolin on DNA, RNA and protein synthesis in HeLa cells. As shown in Fig. 1, the incorporation of [^{3}H]thymidine into DNA of HeLa cells was inhibited by 95% by the treatment with aphidicolin at a concentration of 2μg/ml.

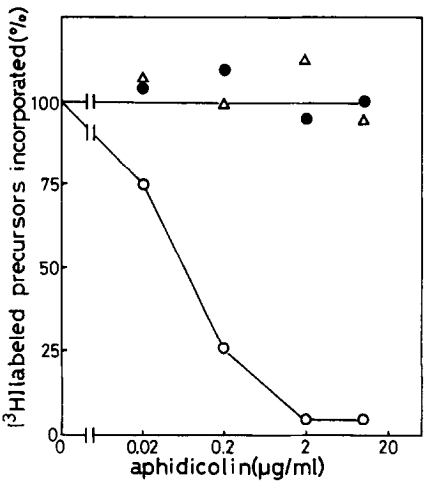
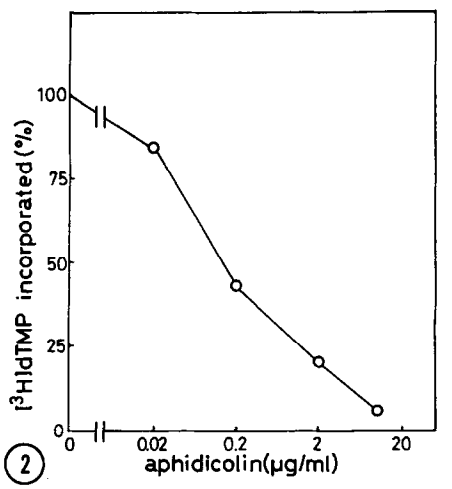


Fig. 1. *Effect of aphidicolin on DNA, RNA and protein synthesis in HeLa cells.* Tritiated thymidine (○), [^{3}H]uridine (●) or [^{3}H]leucine (△) was added to the medium with various concentrations of aphidicolin, and the cells were fixed after 1h. The radioactivities in the absence of aphidicolin (control values) were as follows: 2.7x10^{3}cpm ([^{3}H]thymidine), 2.2x10^{3}cpm ([^{3}H]uridine) and 6.3x10^{2}cpm ([^{3}H]leucine). Aphidicolin was dissolved in DMSO and then dispersed in the growth medium to give a final DMSO concentration of less than 0.1%(v/v). At these concentrations, DMSO did not affect DNA, RNA or protein synthesis in HeLa cells.

On the other hand, the incorporation of [^{3}H]uridine and [^{3}H]leucine into the acid-insoluble material was not affected by the exposure to aphidicolin at concentrations from 0.02 to 10μg/ml. Thus the effect of the compound is specific for DNA synthesis in HeLa cells.

Effect of aphidicolin on [^{3}H]dTTP incorporation into S-phase HeLa nuclei. In order to investigate further the inhibitory effect of aphidicolin on cellular DNA synthesis, we used an *in vitro* DNA replication system (6, 8). Analysis of the DNA product revealed that this system reflects at least in part the DNA replication *in vivo* (9, 10). When the *in vitro* DNA replication system was exposed to aphidicolin (2μg/ml) in the presence of [^{3}H]dTTP and the other necessary components, the incorporation of [^{3}H]dTTP was inhibited by 80% (Fig. 2). This demonstrates that aphidicolin inhibits DNA replication *in vitro* as well as DNA replication *in vivo*. Since we preliminarily observed the selective inhibition of DNA polymerase α in HeLa cells as same as in sea urchin embryo (2), the above result indicates the involvement of DNA polymerase α in DNA replication *in vitro*.



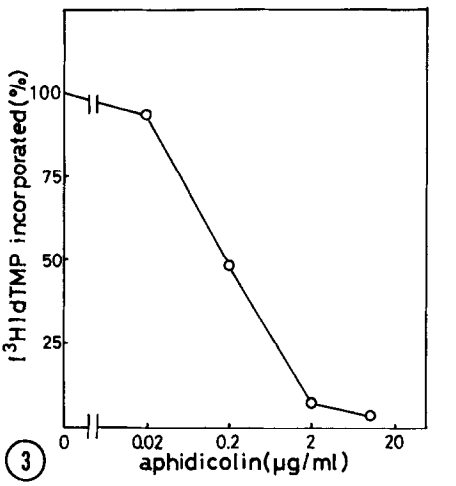


Fig. 2. *Inhibitory effect of aphidicolin on [^{3}H]dTTP incorporation into S phase HeLa nuclei.* Incubations were performed as described in *Materials and Methods.* The control value was 8.0×10^2cpm. Aphidicolin was dissolved in DMSO and dispersed in the reaction mixture as described in Fig. 1. At these concentrations, DMSO did not affect the activity of the *in vitro* DNA replication system.

Fig. 3. *Inhibition of [^{3}H]dTTP incorporation into UV-irradiated G2 phase HeLa nuclei by aphidicolin.* Conditions for the assay of [^{3}H]dTTP incorporation are described in *Materials and Methods.* The control value of this experiment was 1.5×10^3cpm. Aphidicolin was used as described in the legend of Fig. 2.

Effect of aphidicolin on [^{3}H]dTTP incorporation into UV-irradiated G2-G1 phase HeLa nuclei. It is tempting to know whether aphidicolin inhibits DNA replication only, or DNA repair as well as the replication. To compare DNA repair with DNA replication at the same level, we developed the *in vitro* DNA repair system with very high sensitivity (manuscript in preparation). In brief, the key of the system is as follows: i) Use of G2-G1 phase cells (which minimizes the replicative DNA synthesis). ii) Incubation of UV-irradiated cells in the presence of hydroxyurea and arabinosyl cytosine (which inhibis the residual DNA replication, and possibly decreases the endogenous dTTP level). iii) Omission of ATP from the assay mixture (ATP is the essential component for the *in vitro* DNA replication). Incorporation of [^{3}H]dTTP in this system is substantially dependent on the dose of UV irradiated to the cells. Fig. 3 shows that aphidicolin (2μg/ml) inhibited the incorporation of [^{3}H]dTTP by 90%.

Thus the degree of the inhibition by aphidicolin of the UV-induced DNA repair replication was nearly the same as that observed for DNA synthesis in the intact cells and in the *in vitro* replication system. We also have data that UV-induced [^{3}H]thymidine incorporation into G2-G1 phase cells is inhibited by the compound (although the radioactivity of this type of experiment is much less than that of Fig. 3).

These results suggest that DNA polymerase α plays an essential role in UV-induced DNA repair as well as DNA replication in HeLa cells. As we reported previously, HeLa cells have at least two forms of DNA polymerase α with different degree of affinity to DNA (7). We also found that the variation in the activity of one of the α polymerase, termed P-I, which possesses a higher binding affinity to DNA, is correlated with the rate of DNA synthesis *in vivo* (submitted for publication). There is a possibility that two forms of DNA polymerase α share their role in DNA metabolism, that is, replication and repair. We are at present carrying out further studies designed to test this possibility.

Finally, there is an observation that DNA polymerase β level correlates with DNA repair capacity in the lymphocyte culture (11). We cannot exclude the possibility that β polymerase works on DNA repair process. Since we used hydroxyurea and arabinosyl cytosine to facilitate the detection of DNA repair by reducing the background level of replicative DNA synthesis, these drugs might themselves damage DNA and induce a kind of abnormal repair. Although our repair system is perfectly dependent on UV irradiation, it is possible that the combined action of hydroxyurea plus arabinosyl cytosine sensitized the cells to UV light, and caused increased damage on DNA. In fact, the accumulation of single-strand breakes and the change of chromosome decondensation in hydroxyurea and/or arabinosyl cytosine treated, UV-irradiated cells are reported (12, 13). In this context, we can say so far that DNA poltmerase α seems essential for UV-induced DNA repair replication in hydroxyurea-arabinosyl cytosine treated HeLa cells.

ACKNOWLEDGEMENTS

This work was supported in part by Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

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