Delayed Cytotoxicity and Selective Loss of Mitochondrial DNA in Cells Treated with the Anti-human Immunodeficiency Virus Compound 2’,3’-Dideoxycytidine*

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The compound 2’,3’-dideoxycytidine (ddC) is a potent inhibitor of human immunodeficiency virus replication in vitro and is currently in clinical trials for treatment of acquired immunodeficiency syndrome. The compound was found to exert delayed cytotoxicity against Molt-4F cells, a human T lymphoblastic cell line. At a concentration as low as 0.1 μM, the doubling time of the cells was increased after 8 days of ddC treatment. This concentration is 5-fold lower than plasma levels reached in clinical trials. The cells finally died after a 2-week exposure to 0.1 or 0.2 μM ddC. The delayed cytotoxicity was not due to a greater accumulation of 2’,3’-dideoxycytidine triphosphate in cells with longer exposure to the compound. The cellular content of mitochondrial DNA was found to decrease and the rate of glycosylation was found to increase with continuous exposure of cells to ddC. The mitochondrial toxicity and cell growth inhibition were reversed when ddC was removed. The reduction in cellular content of mitochondrial DNA caused by ddC may partially explain the delayed toxicity observed in acquired immunodeficiency syndrome patients treated with the drug.

Several 2’,3’-dideoxynucleoside analogs have been used as potential antiretroviral agents (1, 2). Among the dideoxynucleosides, ddC has been shown to be the most potent inhibitor of human immunodeficiency virus (HIV) replication in cell culture. HIV is the etiologic agent of acquired immunodeficiency syndrome. The antiviral effect of ddC is thought to result from DNA chain termination and/or inhibition of HIV reverse transcriptase activity (3, 4). To exert its inhibitory effect, ddC is first phosphorylated by deoxycytidine kinase and further anabolized to the active metabolite 2’,3’-dideoxycytidine triphosphate (ddCTP) (5, 6). Although both cytoplasmic and mitochondrial deoxycytidine kinases are able to phosphorylate ddC to 2’,3’-dideoxycytidine monophosphate, ddC has a slightly higher affinity for the mitochondrial enzyme than its cytoplasmic counterpart (4).

Results from initial clinical trials (7) indicate that ddC markedly reduces HIV replication and improves immune functions. After 8–12 weeks of ddC treatment, however, some patients develop a reversible peripheral neuropathy (7). This adverse reaction implies that in addition to the effect on reverse transcriptase and viral DNA synthesis, ddC interacts at other sites in vivo. Several lines of evidence suggest that ddC may affect mitochondria. Since ddCTP is a nucleotide derivative, it may interact with the cellular DNA polymerases, including DNA polymerases α, β, γ, and δ. Of the polymerases examined, DNA polymerase γ was shown to be very sensitive to ddCTP (4, 8). This enzyme is believed to be responsible for mitochondrial DNA synthesis. Furthermore, ddCTP inhibits DNA replication in isolated mitochondria of HeLa cells, a human cervical carcinoma cell line (8). In addition, mitochondrial defects have been related to peripheral neuropathy (9). These observations suggest that mitochondria may be the primary target for the adverse effects of ddC. Since elimination of mitochondrial DNA by bifunctional DNA intercalators is characterized by a delayed arrest of cell growth (10), we investigated whether ddC induces delayed cytotoxicity and affects mitochondria in Molt-4F cells, a human T lymphoblastic cell line.

Previous reports indicate that exposure of cells to ddC for 2 or 3 days results in inhibition of cell growth (4, 11, 12). The concentration of ddC required to inhibit the growth of Molt-4F cells by 50% ranges from 5 to 25 μM (4, 11, 12). Incorporation of ddC into cellular DNA may be related to the cytotoxicity observed (4, 13). Since this concentration range is well above the concentration of ddC required to inhibit HIV, the cytotoxicity at therapeutic concentrations was examined. In this report, a delayed cytotoxicity and a dramatic reduction in the mitochondrial DNA content of Molt-4F cells induced by clinically relevant concentrations of ddC are described.

MATERIALS AND METHODS

Chemicals and Reagents—2’,3’-Dideoxycytidine was purchased from Pharmacia LKB Biotechnology Inc. Phenol and BanH1 were obtained from Bethesda Research Laboratories. RNase A was obtained from Sigma. Proteinase K, lactic acid assay kit, and random primer labeling kit were from Boehringer Mannheim. [3H]Dideoxycytidine (5.2 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Topoisomerase I cDNA was isolated from a Agt11 cDNA library of HeLa cells in this laboratory.² This cDNA fragment contained 1.8 kilobases of the 3’ end of the topoisomerase I cDNA.

Cell Culture—Molt-4F cells (ATCC) were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum. To determine the effect of ddC on cell growth, the cells (2 x 10⁶ cells/ml) were treated with various concentrations of ddC. The culture medium and ddC were changed every 2 days. The cell number was determined by using a hemacytometer. The cells grew exponentially until the cell doubling time was affected by ddC. The estimation of doubling time was based on the increase in cell number determined at 2-day intervals.

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‡ The abbreviations used are: ddC, 2’,3’-dideoxycytidine; HIV, human immunodeficiency virus; ddCTP, 2’,3’-dideoxycytidine triphosphate; HPLC, high performance liquid chromatography.

² B. S. Zhou and Y-C. Cheng, unpublished results.

11934
Determination of ddCTP Content—To determine intracellular ddCTP content, a perchloric acid extraction method (14) was used to prepare acid-soluble cell extracts from [3H]ddC-treated cells. The extract was analyzed by anion exchange HPLC with a Partisil 10 SAX column (Whatman). The elution buffer used for this analysis was 0.15 M potassium phosphate, pH 7.5, and the flow rate was 1 ml/min (4). The amount of [3H]ddCTP in the collected HPLC fractions was measured by liquid scintillation counting.

Lactic Acid Determination in Molt-4F Cells—Molt-4F cells (4 × 10^6 cells/ml) were treated with ddC for 5 days. The cell number was determined, and the culture medium was collected every 24 h. The medium was filtered using Acrodisc (Gelman Sciences, Ann Arbor, MI) and stored at −20 °C. The lactic acid content of the medium was measured by using the Boehringer lactic acid assay kit.

Extraction of Total Cellular DNA from Molt-4F Cells—Cells were washed twice with phosphate-buffered saline. The cells were suspended in phosphate-buffered saline (10^6 cells/ml) and stored at −70 °C until used. Ten volumes of lysing buffer (containing 0.5 M EDTA, pH 8.0, 0.5% Sarkosyl, and 100 µg/ml proteinase K) was added. The cell lysate was incubated at 50 °C for 3 h and deproteinized by extraction with phenol. The DNA extract was then dialyzed against a solution containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 10 mM NaCl. The sample was treated with DNase-free RNase at 37 °C for 3 h. After deproteinization by extraction with phenol, the DNA was precipitated overnight at −20 °C with 1 volume of isopropyl alcohol. The precipitate was then dissolved in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

Measurement of Mitochondrial DNA—Total cellular DNA (15 µg) was digested with the restriction enzyme BamHI and analyzed on a 0.8% agarose gel. DNA in the gel was transferred to nitrocellulose paper following the procedure described previously (15). To detect the mitochondrial DNA on the nitrocellulose paper, a 0.88-kilobase mitochondrial DNA fragment labeled with 32P was used as a probe. The mitochondrial DNA probe spans from nucleotide 13,370 to 14,258 of the mitochondrial genome, a circular DNA with 16,569 base pairs (16). After BamHI treatment, the DNA was analyzed by agarose gel electrophoresis. The DNA in the gel was transferred to nitrocellulose paper and then monitored by hybridization either with mitochondrial or nuclear DNA probes. Fig. 3A shows that the mitochondrial DNA content dropped markedly after ddC treatment. After a 2-day exposure to 0.2 µM ddC, there was an approximately 5-fold decrease in the mitochondrial DNA content of Molt-4F cells. During the first 6 days of ddC treatment, the mitochondrial DNA content continuously diminished in spite of normal cell growth. The topoisomerase I gene was chosen as a control for the amount of genomic DNA.

RESULTS

Delayed Cytotoxicity and Intracellular Content of ddCTP—Molt-4F cells grew normally in the presence of 0.1 or 0.2 µM ddC for the first 6 days (Fig. 1). Afterward, cell growth was inhibited, and the cells died in 2 weeks. The ddC concentrations required for observation of this delayed cytotoxicity were within the range required for inhibition of HIV replication in cell culture (1) and were less than the plasma concentration reached in clinical trials (0.5 µM) (7). The delayed cytotoxicity could result from the cellular accumulation of the active metabolite ddCTP during the long period of exposure to ddC, however HPLC analysis showed that ddCTP levels did not increase from day 2 to day 14 of ddC treatment (Fig. 1). Therefore, the delayed cytotoxicity in Molt-4F cells after exposure to ddC did not result from continued accumulation of ddCTP in the cells.

Effect of ddC on Lactic Acid Production by Molt-4F Cells—Several antimitochondrial bifunctional DNA intercalators have a similar effect on cell growth. The cytotoxicity of these compounds is characterized by an arrest of cell growth after seven to eight generations (10). Thus, the effects of ddC on mitochondria were examined. Mitochondrial deficiency is expected to induce an adaptive cellular response that results in the stimulation of glycolysis. The concentration of lactic acid, a product of anaerobic glycolysis, is expected to increase under this condition. Molt-4F cells treated with ddC for prolonged periods secreted more acidic metabolites into the culture medium than those not exposed to ddC. This was observed by the change in the color of the culture medium. There was a dose-dependent increase in the concentration of lactate in the culture medium after 3 days of exposure to ddC (Fig. 2). This result clearly indicates that glycolysis was stimulated in the ddC-treated cells and suggests that mitochondrial function was affected by ddC.

Mitochondrial DNA Content of ddC-treated Molt-4F Cells—To determine the fate of mitochondrial DNA after ddC treatment, total cellular DNA was extracted from Molt-4F cells. The DNA was treated with the restriction endonuclease BamHI, which recognizes a single site in the mitochondrial genome, a circular DNA with 16,569 base pairs (16). After BamHI treatment, the DNA was analyzed by agarose gel electrophoresis. The DNA in the gel was transferred to nitrocellulose paper and then monitored by hybridization either with mitochondrial or nuclear DNA probes. Fig. 3A shows that the mitochondrial DNA content dropped markedly after ddC treatment. After a 2-day exposure to 0.2 µM ddC, there was an approximately 5-fold decrease in the mitochondrial DNA content of Molt-4F cells. During the first 6 days of ddC treatment, the mitochondrial DNA content continuously diminished in spite of normal cell growth. The topoisomerase I gene was chosen as a control for the amount of genomic DNA.
The hypothesis that at low doses ddC can reduce mitochondrial DNA content (Fig. 3) was reprobed with topoisomerase I cDNA after removing the mitochondrial probe. BamHI cuts the topoisomerase I gene into two fragments. BamHI and analyzed on a 0.8% agarose gel. 5, 6.4, 9.5 kb, kilobase pair.

**FIG. 3. Mitochondrial DNA content of ddC-treated cells.** Molt-4F cells were treated with 0.2 μM ddC. The culture medium and ddC were changed every 2 days. Total cellular DNA was extracted from the cells after 0, 2, 4, 6, and 8 days of ddC treatment (lanes 1–5, respectively). The DNAs were digested with the restriction enzyme BamHI and analyzed on a 0.8% agarose gel. (A) the amount of mitochondrial DNA was determined as indicated. (B) the same nitrocellulose paper used for the mitochondrial DNA determination was reprobed with topoisomerase I cDNA after removing the mitochondrial probe. kb, kilobase pair.

BamHI cuts the topoisomerase I gene into two fragments. There was no significant alteration in the nuclear DNA content (Fig. 3B). These results are consistent with the hypothesis that at low doses ddC can reduce mitochondrial DNA content, which could lead to delayed cytotoxicity.

**Reversibility of ddC Effects—**After Molt-4F cells were treated with 0.2 μM ddC for 2 days, the cells grew normally following the removal of ddC from the culture medium. Mitochondrial DNA content was reduced after 2 days of ddC treatment and was able to recover to normal levels after 4 days after ddC was removed (Fig. 4B). Following 5 days of ddC treatment, complete recovery of mitochondrial DNA required 8 days (Fig. 4B). To test the reversibility of ddC effects after cell growth was affected, the drug was removed after 8 days of treatment. The cell growth returned to normal in 5 days (Fig. 4A). The cells were rechallenged with ddC to test the possibility of selection of resistant cells during ddC treatment. The results in Fig. 4A show that the cells were susceptible to ddC after recovering from the drug exposure. Therefore, in Molt-4F cells, the delayed cytotoxicity and mitochondrial effects of ddC are reversible.

**DISCUSSION**

The cytotoxicity of ddC has been evaluated by growth retardation assays that measure cell growth under the influence of drugs for 2 or 3 days. However, this assay does not detect the delayed cytotoxicity described in this paper. Clonogenic assays also have been used to assess toxic effects of drugs. Such assays may not be appropriate to evaluate delayed cytotoxicity since this type of assay depends on whether the drugs are constantly present and the number of generations of cell growth required to observe the delayed cytotoxicity. Since ddC is able to exert delayed cytotoxicity, whether other nucleoside analogs used in chemotherapy also cause this effect remains to be examined.

The cellular mitochondrial DNA content decreased approximately 5-fold after 2 days of ddC treatment, whereas cell number increased 4-fold. This suggests that the cells were growing without the replication of mitochondrial DNA. An increase in glycolysis, the compensatory mechanism for energy production, did not begin until day 3 after ddC treatment. However, the compensatory mechanism apparently was not sufficient to support cell survival with continued reductions in mitochondrial DNA content. Since the cellular content of mitochondria and the minimal number of mitochondria per cell required to support cell survival may differ from one cell type to the other, such delayed cytotoxicity of ddC could also vary. The thrombocytopenia observed during ddC clinical trials may be a result of this delayed cytotoxicity. Importantly,
Mitochondrial Toxicity of the Anti-HIV Compound 2',3'-Dideoxycytidine

this delayed cytotoxicity exerted by ddC is reversible in terms of cell growth and mitochondrial DNA content. Following an 8-day treatment with 0.2 μM ddC, Molt-4F cells survived, and cell growth returned to the control rate after switching the cell to medium without ddC. The mitochondrial DNA content also returned to the control level after ddC was removed.

One of the major side effects of ddC in clinical trials is a painful reversible peripheral neuropathy (7). Mitochondrial defects have been related to peripheral neuropathy (9). It is possible that the mitochondrial toxicity of ddC is also responsible for this major side effect observed clinically. In view of the results presented in this paper, the design of protocols for the clinical treatment of acquired immunodeficiency syndrome patients should take this effect into consideration. The challenge is to design a strategy that avoids the toxic effect of ddC without compromising its antiviral activity.

REFERENCES

1. Mitsuya, H. & Broder, S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1911–1915
2. Mitsuya, H., Weinhold, K. J., Furman, P. A., St. Clair, M. H., Lehrman, S. N., Gallo, R. C., Bolognesi, D., Barry, D. W. & Broder, S. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7096–7100
3. Mitsuya, H., Jarrett, R. F., Matsukura, M., Veronese, F. D. M., DeVico, A. L., Sarnaghadharan, M. G., Johns, D. G., Reitz, M. S. & Broder, S. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2033–2037
4. Starnes, M. C. & Cheng, Y. (1987) J. Biol. Chem. 262, 988–991
5. Balzarini, J., Kang, G-J., Dalal, M., Herdwijin, P., De Clercq, E., Broder, S. & Johns, D. G. (1987) Mol. Pharmacol. 32, 162–167
6. Zhang, H., Dalal, M., Cooney, D. A., Balzarini, H., Mitsuya, H., Broder, S. & Johns, D. G. (1987) Proc. Am. Assoc. Cancer Res. 28, 323
7. Yarchoan, R., Thomas, R. V., Allain, J-P., McAtee, N., Dubinsky, R., Mitsuya, H., Lawley, T. J., Safai, B., Myers, C. E., Perno, C. F., Kiecker, R. W., Wills, R. J., Fischl, M. A., McNeely, M. C., Pluda, J. M., Leuther, M., Collins, J. M. & Broder, S. (1988) Lancet 1, 76–81
8. Zimmermann, W., Chen, S. M., Bolden, A. & Weissbach, A. (1980) J. Biol. Chem. 255, 11847–11852
9. Pereshkhpour, G., Krapup, C., Buchthal, F., Dimaro, S., Bresolin, N. & McBurney, J. (1987) J. Neurol. Sci. 77, 285–304
10. Essnault, C., Roques, B. P., Jacquemin-Sablon, A. & Le Pecq, J.-B. (1984) Cancer Res. 44, 4355–4360
11. Balzarini, J., Cooney, D. A., Dalal, M., Kang, G-J., Curr, J. E., De Clercq, E., Broder, S. & Johns, D. G. (1987) Mol. Pharmacol. 32, 798–806
12. Balzarini, J., Pauwels, R., Baba, M., Herdwijin, P., de Clercq, E., Broder, S. & Johns, D. G. (1988) Biochem. Pharmacol. 37, 897–903
13. Ulman, B., Coons, T., Rockwell, S. & McCarvan, K. (1988) J. Biol. Chem. 263, 12391–12396
14. Cheng, Y., Grill, S. P., Dutschman, G. E., Nakayama, K. & Bastow, K. F. (1983) J. Biol. Chem. 258, 12460–12464
15. Medveczky, P., Chang, C. W., Oste, C. & Mulder, C. (1987) Biotechniques 5, 242–245
16. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R. & Young, I. G. (1981) Nature 290, 457–465