Effects of Zidovudine and Stavudine on Mitochondrial DNA of Differentiating 3T3-F442a Cells Are Not Associated with Imbalanced Deoxynucleotide Pools[▽]

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To test whether zidovudine (3'-azido-3'-deoxythymidine) (AZT) inhibition of thymidine phosphorylation causes depletion of the TTP pool resulting in mitochondrial DNA depletion, 3T3-F442a cells were differentiated in the presence of AZT and analyzed to determine mitochondrial DNA content and deoxynucleotide levels. These results suggest that AZT toxicity may not be related to deoxynucleotide pool alterations.

One of the more common and severe adverse effects of highly active antiretroviral therapy today is lipodystrophy (3, 13, 22, 24), characterized by loss of the peripheral adipose tissue with concurrent accumulation of central adipose. A variety of AIDS drugs have been associated with lipodystrophy, including the protease inhibitors and some nucleoside analog reverse transcriptase inhibitors (NRTIs), such as zidovudine (3'-azido-3'-deoxythymidine) (AZT) and stavudine (2',3'-dihydro-3'-deoxythymidine) (d4T).

The prevailing hypothesis for NRTI toxicity suggests that the NRTI triphosphate inhibits the mitochondrial DNA (mtDNA) polymerase γ (7, 11). While this hypothesis may hold true for the other NRTIs, it does not seem likely to be the mechanism behind AZT toxicity. Compared to the 50% inhibitory concentrations of other NRTIs, AZT-5'-triphosphate appears to be a poor inhibitor (11). This is compounded by the fact that AZT-5'-monophosphate is a poor substrate for thymidylate kinase, resulting in an insufficient amount of AZT-5'-triphosphate made within any given cell to be significantly inhibitory toward polymerase γ (6).

Previous work from this laboratory has led to an alternative hypothesis for AZT toxicity (9, 10, 12, 21). AZT inhibition of thymidine phosphorylation may deplete intracellular TTP. The imbalance of TTP compared to the other deoxynucleotides (dNTPs) could cause the observed mtDNA depletion in tissues affected by AZT toxicity, as imbalances in any of the dNTP pools can result in mtDNA deletions and depletion (1, 16, 18, 19).

Another NRTI thymidine analog, d4T, is much more potently toxic to polymerase γ (11) and has not demonstrated any inhibitory effects on thymidine phosphorylation in the isolated perfused heart (21) or in isolated mitochondria (E. E. McKee, unpublished data). As such, d4T provides a ready comparison to the effects of AZT, which may be utilizing a different mechanism of toxicity.

The 3T3-F442a cell line provides a good model system for this study, as both AZT and d4T have been associated with lipodystrophy (24). These cells are preadipocytes that can be induced to differentiate into adipocytes and have been shown to be sensitive to treatment with both of the proposed NRTIs used in this study (24). Thus, the goal of this work is to compare the effects of AZT and d4T on the dNTP pools and mtDNA of differentiating 3T3-F442a cells.

3T3-F442a cells were provided by Martine Caron (Université Pierre et Marie Curie, Paris, France). See Table 1 for growth and differentiation conditions. Beginning on day 0, the growth medium was supplemented with either 1 or 10 μ M AZT or d4T for the entire duration of each experiment, except in the controls where no NRTI was added. The medium was removed and replaced with fresh medium every 2 days.

To determine mtDNA copy number, a plate of cells was harvested by treatment with trypsin, and the total DNA was isolated using the Qiagen DNeasy kit. The samples were run on an ABI 7500 quantitative real-time PCR machine. The conditions of the run were an initial incubation at 95°C for 10 min, followed by 40 cycles in which 1 cycle consisted of 15 s at 95°C and 1 min at 60°C. The copy number of mtDNA and nuclear DNA (nDNA) was determined by comparisons to

TABLE 1. Growth and differentiation conditions used in this study

Exptl day(s)	Growth medium ^a	NRTI added to medium
−2	BGM + 10% newborn calf serum	None
0	BGM + 10% newborn calf serum	None or AZT (1 or 10 μ M) or d4T (1 or 10 μ M)
2	BGM + 5% newborn calf serum + 5% fetal bovine serum	None or AZT (1 or 10 μ M) or d4T (1 or 10 μ M)
4 to 10	BGM + 10% fetal bovine serum + insulin (1 μ g/ml medium)	None or AZT (1 or 10 μ M) or d4T (1 or 10 μ M)

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^a BGM is a basic growth medium that is composed of Dulbecco's modified Eagle medium (high glucose with L-glutamine, pyridoxine hydrochloride, and 110 mg/liter sodium pyruvate; Gibco) supplemented with 10 ml/liter antibiotic/antimycotic (100 $\times$; Invitrogen), 1.5 g/liter sodium bicarbonate, 176.4 μ M biotin, and 85 μ M pantothenic acid.

FIG. 1. Effects of AZT and d4T on mtDNA content in 3T3-F442a cells. The number of mtDNA copies per nDNA copy in the control cells (top) and cells treated with 1 and 10 μ M AZT (middle) or with 1 and 10 μ M d4T (bottom) was determined by real-time PCR as described in the text. Data represent the means plus standard errors of the means (error bars) from three independent experiments. The values for the control group (top) show the number of absolute mtDNA copies per nDNA copy on each day. The values for the AZT- and d4T-treated samples (middle and bottom) are calculated as the percentage of the control value for that day. The dotted lines in the middle and bottom panels represent the 100% control level. Statistical significance of the difference from the control (*) was calculated for each entire treated group versus the entire control group using a two-tailed Student's *t* test with $P < 1.4 \times 10^{-8}$ for 1 and 10 μ M AZT and $P < 8.4 \times 10^{-7}$ for 10 μ M d4T.

standard curves generated with purified PCR product from each of the amplified genes.

The primers and probes used for ND4 (positions 11133 to 11212) from the mouse mitochondrial chromosome were, respectively, 5'-CATCATCACTCCTATTCTGCCTAGC-3', 5'-AAGTCCTCGGGCCATGATTATA-3', and 5'-6FAM-CTCCAACCTACGAACGGATCCACAGC-BHQ1-3', where 6FAM is 6-carboxyfluorescein. The primers and probes used for polymerase γ (positions 1185 to 1332) from mouse chromosome 7 were, respectively, 5'-TTCTCGATACTATGAGCATGCACAT-3', 5'-GCTGGACCATTTGGCTTTCC-3', and 5'-6FAM-AAAGCGAGGGCAGAAAGTCCC-G-BHQ1-3'.

In order to measure the dNTP pools, the cells were lysed by

FIG. 2. Time course of total dNTP content in 3T3-F442a cells. Cells were grown and differentiated in the presence of AZT (1 or 10 μ M) or d4T (1 or 10 μ M) or not treated (control). Samples were taken every 2 days, and the amount of dNTPs present in each sample was determined using the assay described in the text. Data represent the total amount of dNTP (pmol) in each sample, determined by the sum of the amounts of dATP, dCTP, dGTP, and TTP in each sample. Data represent the means $\pm$ standard errors of the means (error bars) from three independent experiments.

treatment with 5% trichloroacetic acid for 60 min on ice. The acid-soluble fraction was removed and centrifuged. The supernatant was then neutralized with AG-11A8 resin. The dNTPs in the neutralized sample were measured using the protocol developed previously (17–19). The counts per minute (cpm) obtained were compared to a standard curve in order to calculate the moles of dNTP in each sample.

Depletion of the mtDNA was seen in the group of cells treated with 10 μ M d4T (Fig. 1); our experimental data confirm results previously obtained (24). No effect was observed when the cells were treated with 1 μ M d4T. However, both 1 and 10 μ M AZT resulted in a significant increase in mtDNA content (Fig. 1). This is contrary to what was found by Walker et al. (24), who observed mtDNA depletion at 1 μ M AZT. It is unclear why the AZT results differ. Our starting stock of 3T3-F442a cells was obtained from Walker et al., and our protocol is based on theirs.

AZT has been noted to cause an increase in mtDNA content in other cell lines as well (8). One possible explanation is that AZT is damaging the mtDNA, whether through AZT incorporation into the DNA, disruptions of the dNTP pools, or a different mechanism. The cell may try to upregulate mitochondrial function by increasing mtDNA replication. Normally, more mtDNA would allow more of the mitochondrial protein components of the electron transport chain to be made. However, much of the mtDNA present may be damaged and produce ineffective proteins, if any at all. While there is abundant mtDNA, the cell is still unable to fully function. Longer continual exposure to AZT could result eventually in depletion as the damaged and fragmented mtDNA is degraded.

Measurement of the dNTP pools revealed that the effects of

FIG. 3. Time course of dNTP in 3T3-F442a cells treated with AZT. Cells were grown and differentiated, as described in the text, in the presence of 0, 1, or 10 μ M AZT. Data represent the mean $\pm$ standard errors of the means (error bars) from three independent experiments and are expressed as the percentage of the total dNTP for that sample on that day. Data points marked with asterisks differ significantly from the value for the untreated control ($P < 0.05$), as determined by a two-tailed Student's t test.

AZT and d4T appear to be minimal. The size of the total dNTP pool in each sample increased significantly during the time course of the experiment (Fig. 2), related to an increase in cell number. However, it was not possible to normalize these data to cell number because the drugs, which decreased cell number, substantially increased cell size. This is in agreement with an AZT block in clonal expansion of differentiating 3T3-F442A cells reported by Stankov et al. (20). As a result, the individual dNTP pools are shown as a percentage of the total dNTP pool for that treatment on that day. The same general trends appear in the control group and all treatment groups (Fig. 3 and Fig. 4). While some points do show a statistically significant difference from the control group ($P < 0.05$), these differences do not show any general trends, with one exception. Treatment with 10 μ M d4T caused an increase in the percentage of TTP on days 4 to 8. The reason for this rise is unclear, and it might be related to a decrease in mtDNA replication resulting in the decreased mtDNA content observed in these cultures. Vela et al. (23) reported a similar increase in several dNTP pools with decreased mtDNA content in CEM-CCR5 cells treated with 10 μ M AZT.

The first time period sampled in our study was at 2 days, so very early changes in dNTP pools may have been missed. The lack of an effect of AZT on the TTP pool in 3T3-F442a cells indicates that these cells are able to synthesize TTP via a second pathway. This pathway may always be present in these cells, or it could be induced in response to an initial drop in TTP, as was noted by Fridland et al. (4). In the current protocol, the NRTI is added throughout the entire process of

FIG. 4. Time course of dNTP in 3T3-F442a cells treated with d4T. Cells were grown and differentiated, as described in the text, in the presence of 0, 1, or 10 μ M d4T. Data represent the mean $\pm$ standard errors of the means (error bars) from three independent experiments and are expressed as the percentage of the total dNTP for that sample on that day. Data points marked with asterisks differ significantly from the untreated control ($P < 0.05$), as determined by a two-tailed Student's t test.

differentiation, including when the cells are still actively replicating. During the replication phase, thymidine kinase 1 (TK1), ribonucleotide reductase, and thymidylate synthase are likely to be active, and the role of the thymidine kinases in supplying TTP might be modest. As a result, inhibiting this pathway with AZT may not be expected to alter the TTP pool during the replicative phase of differentiation. In this regard, TTP represented $54\% \pm 4\%$ of the dNTP pool in the control cells at day 2 but fell to $35\% \pm 3\%$ by day 10 ($P < 0.003$). This decline is likely related to the loss of expression of TK1, since TK1 is active only during S phase (14). As AZT also had no effect on TTP levels during the postreplicative phase of differentiation, this suggests that ribonucleotide reductase and thymidylate synthase may remain active in these cells. Ribonucleotide reductase has been found to have some activity in quiescent cultured fibroblasts (15). This activity is dependent on a p53-inducible R2 subunit (5) that has been associated with severe mtDNA depletion (2).

In summary, the toxic effects of AZT on the differentiation of 3T3-F442a cells into adipocytes do not appear to be related to alterations in the dNTP pools supplying mitochondrial replication.

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