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Human inter-individual variability in metabolism and genotoxic response to zidovudine

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Abstract

A mainstay of the antiretroviral drugs used for therapy of HIV-1, zidovudine (AZT) is genotoxic and becomes incorporated into DNA. Here we explored host inter-individual variability in AZT-DNA incorporation, by AZT radioimmunoassay (RIA), using 19 different strains of normal human mammary epithelial cells (NHMECs) exposed for 24 h to 200 μM AZT. Twelve of the 19 NHMEC strains showed detectable AZT-DNA incorporation levels (16 to 259 molecules of AZT/10^6 nucleotides), while 7 NHMEC strains did not show detectable AZT-DNA incorporation. In order to explore the basis for this variability, we compared the 2 NHMEC strains that showed the highest levels of AZT-DNA incorporation (H1 and H2) with 2 strains showing no detectable AZT-DNA incorporation (L1 and L2). All 4 strains had similar (≥80%) cell survival, low levels of accumulation of cells in S-phase, and no relevant differences in response to the direct-acting mutagen bleomycin (BLM). Finally, when levels of thymidine kinase 1 (TK1), the first enzyme in the pathway for incorporation of AZT into DNA, were determined by Western blot analysis in all 19 NHMEC strains at 24 h of AZT exposure, higher TK1 protein levels were found in the 12 strains showing AZT-DNA incorporation, compared to the 7 showing no incorporation ($p=0.0005$, Mann–Whitney test). Furthermore, strains L1 and L2, which did not show AZT-DNA incorporation at 24 h, did have measurable incorporation by 48 and 72 h. These data suggest that variability in AZT-DNA incorporation may be modulated by inter-individual differences in the rate of induction of TK1 in response to AZT exposure.
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Keywords: AZT; Nucleoside analogs; Thymidine kinase 1

Introduction

The nucleoside reverse transcriptase inhibitor (NRTI), 3′-azido-3′-deoxythymidine (AZT) comprises part of the first-line therapy for HIV-1 infection worldwide (DHHS, 2006), and is specifically recommended by the Centers for Disease Control and Prevention for inhibition of mother-to-child HIV-1 transmission (Centers for Disease Control and Prevention, 2003) and post-exposure prophylaxis in health care, laboratory and rescue workers (Cardo et al., 1997; Centers for Disease Control and Prevention, 1999). The genotoxicity of this drug has been studied extensively, and numerous reports indicate that incorporation of the drug into DNA, clastogenicity and mutagenicity are consequences of AZT exposure in cultured cells, animal models and humans (IARC, 2000; Poirier et al., 2004; Escobar et

Abbreviations: AZT, 3′-azido-3′-deoxythymidine, zidovudine; BLM, Bleomycin; BLU, Bright Luminescence Unit, an arbitrary unit of luminescence; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; ECL, Electrochemiluminescence; FITC, Fluorescein isothiocyanate; HRP, Horse Radish-peroxidase; H1 and H2, NHMEC strains M99005 and M98018, respectively, the strains that incorporated the highest levels of AZT into DNA when measured by AZT-RIA, after 24 h of exposure to 200 μM AZT; L1 and L2, NHMEC strains M98016 and M98040, respectively, two of the strains that incorporated undetectable levels of AZT into DNA when measured by AZT-RIA, after 24 h of exposure to 200 μM AZT; NHMEC, Normal Human Mammary Epithelial Cell; NRTI, Nucleoside reverse transcriptase inhibitor; PBS, phosphate buffered saline; RIA, Radioimmunoassay; RIPA buffer, Radio-immune precipitation assay buffer; TE, 10 mM Tris, 1 mM EDTA buffer; Tdt, Terminal deoxy-nucleotidyl transferase; TK1, Thymidine kinase 1.

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al., 2007; Olivero, 2007). Transplacental carcinogenesis in mouse offspring exposed to AZT during the last week of gestation was documented in multiple organs at 1–2 years of age (Olivero et al., 1997; Diwan et al., 1999; NTP, 1996; Walker et al., 2007). The studies revealed dose-related increases in incidences of liver, lung and reproductive organ tumors, and raised some concerns regarding potential cancer risk in human infants exposed to the NRTI drugs during development.

Mechanisms underlying human inter-individual response to AZT may be important for considerations of drug efficacy and toxicity. Here we explore the genotoxicity of AZT in 19 mammary epithelial cell strains derived from human breast tissue taken from 19 donors at reduction mammoplasty. These normal human mammary epithelial cells (NHMECs) have been characterized previously (Keshava et al., 2005), and constitute an ideal model to assess human inter-individual variability.

The antiretroviral nucleoside analog drug AZT becomes incorporated into nascent HIV-1 viral DNA via reverse transcriptase, and into the host DNA via classical polymerases. Prior to this incorporation the drug must be phosphorylated, by thymidine kinase 1 (TK1) followed by thymidylate kinase and nucleoside diphosphate kinase (Furman et al., 1986). Loss of TK1 activity has been observed in cultured cells exposed long-term to AZT (Avramis et al., 1993; Wu et al., 1995; Vazquez et al., 2004) and in patients exposed chronically to therapeutic AZT doses (Avramis et al., 1993). To understand inter-individual variability in genotoxic insult and metabolic capacity, with the intention of elucidating clinical efficacy and resistance, we investigated AZT-DNA incorporation in a panel of 19 different NHMEC strains and correlated AZT-DNA incorporation values with protein levels of the enzymatically-active (24 kDa) TK1.

Material and methods

Cell culture, AZT exposure and DNA preparation. Normal human mammary epithelial cells were cultured from organoids derived from tissues obtained at reduction mammoplasty from 19 different individuals by the Cooperative Human Tissue Network. Cells were grown at 37 °C in 5% CO_2 and serum free Mammary Epithelial Cell Medium (Cambrex, Rockland, ME) supplemented with growth factors, insulin and pituitary extracts (Cambrex) (Keshava et al., 2005). The cells were grown out to passage 6 to remove stromal components. AZT (Sigma-Aldrich Co, St Louis, MO) was dissolved in phosphate buffered saline (PBS) pH 7.2 (Biosource, Rockville, MD) and the final AZT concentration was calculated from absorbance at 266 nm. NHMECs from 19 different individuals were cultured to passage 6 and exposed to 200 μM AZT for 24 h, rinsed twice with PBS, removed from the flask by trypsin (Cambrex) and treated with trypsin-neutralizing agent (Cambrex) before centrifugation. Cell pellets were washed with PBS and then processed for DNA extraction using phenol-chloroform (Sambrook et al., 1989). This experiment was performed twice.

Measurement of AZT-DNA incorporation by AZT radioimmunoassay (RIA). DNA quantity was determined by absorbance at 260 nm in DNA aliquots from 19 NHMEC strains exposed on two separate occasions to either 0 or 200 μM AZT for 24 h. Samples were diluted to 30 μg DNA/ml using 10 mM Tris, 1 mM EDTA (TE) buffer, sonicated for 30 s, warmed at 42 °C for 15 min, heated at 99 °C in a thermomixer (Eppendorf, Hamburg, Germany) for 2 min, and placed immediately on ice.

The incorporation of AZT into NHMEC-DNA was determined by AZT-RIA (Olivero et al., 1994). Briefly, a rabbit polyclonal anti-AZT antibody (Sigma-Aldrich Co), which also recognizes AZT in DNA (Olivero et al., 1994), was reconstituted, diluted 1:7500, and incubated with NHMEC-DNA for 90 min at 37 °C. An aliquot (100 μl) containing approximately 20,000 cpm of [^{3}H]AZT tracer (16 Ci/mmol, Moravek Biochemicals Inc. Mountain View, CA) was added to each tube together with 100 μl of the secondary antibody, goat anti-rabbit immunoglobulin G (Sigma-Aldrich Co.). The mixture was incubated for 25 min at 4 °C, centrifuged at 3000 rpm for 15 min at 4 °C and the resulting supernatant was decanted. The pellets were dissolved in 0.1 M NaOH and counted in a liquid scintillation counter. The amount of standard AZT, added to 3 μg of NHMEC control DNA, required to inhibit antibody binding by 50% was 176.7±34.4 (average±SD, $n=5$) molecules of AZT/10^6 nucleotides. The lower limit of detection was 16 molecules of AZT/10^6 nucleotides.

NHMEC-DNA samples from the 19 strains, obtained from cells exposed to 200 μM AZT for 24 h on two separate occasions, were assayed in duplicate in 3 separate RIAs. In addition to the 24 h exposure, NHMEC L1 and L2 cells were exposed to 200 μM AZT for periods of 48 and 72 h, and incorporation of AZT into DNA was determined by RIA.

Cell viability, micronuclei and apoptosis in 4 NHMEC strains exposed to bleomycin. Selected end points were examined in a subset of 4 NHMEC strains. NHMEC strains M99005 and M98018, designated H1 and H2 respectively, incorporated the highest levels of AZT into DNA when measured by AZT-RIA, after 24 h of exposure to 200 μM AZT.

NHMEC strains M98016 and M98040, designated L1 and L2 respectively, were two of the strains that incorporated undetectable levels of AZT into DNA when measured by AZT-RIA, after 24 h of exposure to 200 μM AZT. For cell survival studies, these 4 NHMEC strains were grown to semi-confluency (~80% cell growth) in 10 mm Petri dishes, at which time 3 dishes of cells of each strain were exposed to either 0 or 200 μM AZT for 24 h. The experiment was repeated twice. Dishes were rinsed twice with PBS, trypsinized, neutralized, and an aliquot was taken and counted twice in a Coulter Counter (Coulter Electronics Ltd, Luton, England). Cell counts in drug-exposed cultures were compared with those in the unexposed dishes, and survival in exposed cells was expressed as percentage of survival in unexposed cells.

The four NHMEC strains were exposed for 4 h, on two separate occasions, to 36 μM bleomycin (BLM, Sigma-Aldrich Co). Cells were rinsed with PBS, cultured in fresh media for an additional 20 h, trypsinized, collected by centrifugation and resuspended in media containing serum to produce spreads on slides. Cells were fixed with Carnoy's fixative (3:1 methanol:glacial acetic acid), and stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and evaluated microscopically. The terminal deoxynucleotidyl transferase (Tdt) mediated dUTP nick end labeling (TUNEL) assay was performed to evaluate apoptosis in strains H1, H2, L1 and L2 using the Apoptag, Fluorescein *In Situ* Apoptosis Detection kit (Intergen, Purchase, NY). Apoptotic figures were scored in cells exposed to 0 or 36 μM BLM for 4 h, and then incubated for 20 h with fresh media. Apoptotic figures were visualized and photographed using a Nikon Eclipse E-400 (Nikon, Inc, Melville, NY) microscope with fluorescence capabilities. Micronuclei were observed with the use of a UV-2E/C DAPI filter and excitation and barrier filters of 330–380 nm, and 400 nm respectively. Scoring of apoptotic figures was performed with the use of a triple pass filter with excitation filters of 390–402 and 478–495 for DAPI and FITC, respectively, the barrier filters used were of 462 and 523 nm for DAPI and FITC respectively.

Cell cycle analysis by flow cytometry. In two independent experiments NHMEC strains H1, H2, L1 and L2 were plated in six-well plates, and, for each plate, three wells were unexposed and three wells were exposed to 200 μM AZT for 24 h. Cells (10^6) were harvested, pelleted and washed with culture medium without serum before they were fixed in ice-cold ethanol (1 ml; 70%) while vortexing. Following an overnight fixation at 4 °C, cells were pelleted by centrifugation and incubated with Ribonuclease A (Sigma-Aldrich Co) at room temperature for 20 min. Propidium iodide (20–50 μg/ml) (Molecular Probes, Eugene, OR) was added to each cell suspension and cells were kept in the dark at 4 °C overnight. Cells were passed through a fluorescence activated cell sorter (FACSCalibur, BD Biosciences, San Jose, CA) using the doublet discrimination module, and data were acquired using CellQuest (BD Biosciences) software. The cell cycle was modeled using ModFit software (Venty Software, Topsham, ME). Percentages of cells in G_0–G_1, S and G_2–M phases were calculated directly by the software.

Thymidine kinase 1 (TK1) protein quantitation by Western blot. Aliquots of cells from each of the 19 NHMEC strains were lysed in Radio-immune