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Effector caspase activation, in the absence of a conspicuous apoptosis induction, in mononuclear cells treated with azidothymidine

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ABSTRACT

In the present study we focused our attention on the effect of AZT, at pharmacological and suprapharmacological concentrations, on some apoptosis-related key events and, particularly, on caspase activation in fresh human peripheral blood mononuclear cells (PBMCs). The main results can be summarized as follows: (i) AZT induced a strong, dose-dependent antiproliferative effect in mitogen-stimulated PBMCs, but low levels of cytotoxicity. in comparison with 5FU; (ii) low levels of cytotoxicity were coupled with a poor increase of apoptosis after AZT treatment in PBMCs; (iii) despite low levels of apoptosis, remarkable signs of both initiator and effector caspase enhanced expression with respect to control were detected by immunoblot analysis in AZT-treated PBMCs; (iv) enhanced caspase expression was associated with an increased expression of both anti-apoptotic Bcl-2 and pro-apoptotic Fas and p53 proteins, as detected by flow cytometry analysis; (v) combination treatment *in vitro* with AZT and anti-Fas significantly increased apoptosis in PBMCs with respect to single treatments. Overall, these results suggest that AZT treatment activates a complex, and apparently contrasting apoptosis-related signaling activity in PBMCs and that additional events are necessary to disrupt the balance induced by AZT towards apoptosis, on these cells.

1. Introduction

It is widely known that the dideoxynucleoside analogue 3′-azido-3′-deoxythymidine (AZT) exerts a potent role in the treatment and prevention of HIV infection, in combination with other reverse transcriptase (RT) or protease inhibitors. Beside its major role as antiretroviral, by directly inhibiting RT, AZT exerts a pleiotropic activity toward cells of different origin. One of the first reported observations was that AZT caused cell cycle arrest and accumulation in S phase in lymphoid cell lines [1,2]. Mechanisms involved in AZT-induced block in S phase were related to its capability to inhibit cell polymerases or to directly target proteins controlling the cell cycle [3]. Moreover, *in vitro* studies have shown that cell cycle arrest by AZT was associated to DNA damage. As a consequence, AZT has been shown to switch on repair mechanisms, which could potentially generate mutations [4,5].

Cells that undergo irreparable DNA damage are eliminated by various forms of cell death, but apoptosis plays a prominent role in this process [6]. Thus, it is plausible to hypothesize that cells exposed to excessive amount of AZT could be enrolled in this route of programmed cell death. In fact, some studies have highlighted the pro-apoptotic potential of AZT *in vitro*. In particular, it was reported that AZT induced both morphological and biochemical changes typical of apoptosis in mouse myeloma cell line [7]. Furthermore, *in vitro* data have shown that AZT initiated cell death by a caspase-3-dependent mechanism and altered metabolizing enzymes in human placenta cells [8]. In addition, it has been shown that in a few HIV-infected patients with discordant response to antiretroviral therapy (ART), both CD4+ and CD8+ lymphocytes were highly susceptible ex vivo to AZT-induced apoptosis [9]. In fact, only one report has shown that low concentrations of AZT both induced mitochondrial-mediated cell death of activated lymphocytes from healthy donors and sensitized target cells to receptor-mediated apoptotic signalling [10]. Thus, a supportive evidence for generalized apoptosis induction *in vitro* by AZT at pharmacological concentrations is lacking.

In contrast with results suggesting a pro-apoptotic activity of AZT, analysis of the immune response in individuals taking

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AZT and lamivudine as post-exposure prophylaxis showed that apoptosis susceptibility was actually inhibited by antiretroviral treatment [11]. Moreover, it has been reported that pharmacological and suprapharmacological concentrations of AZT, interferon and their combination failed to induce any significant apoptosis on two HTLV-I positive cell lines even in long-term culture [12]. Moreover, a recent study *in vitro*, focused on toxicity caused by antiretroviral combination therapy, showed and evident increase in mitochondria-derived ROS but no appreciable levels of apoptosis in HUVEC following treatment with AZT or indinavir, suggesting that the compromised mitochondrial function may be one important factor in endothelial dysfunction, without inducing an increase in apoptosis [13]. Thus, the effect of AZT on apoptosis is still controversial.

Aimed to understand the molecular mechanisms underlying discrepancies in induction of apoptosis by AZT, we then focused our attention on the effects of AZT after *in vitro* short term treatment, at pharmacological and suprapharmacological concentrations, on some apoptosis-related key events, and particularly on effector caspase activation, in human fresh mononuclear cells. Results revealed an evident activation of the effector caspase cascade, not associated with a corresponding apoptosis induction, in mitogen-stimulated mononuclear cells, following AZT treatment.

2. Materials and methods

2.1. Cell cultures

Human peripheral blood mononuclear cells (PBMCs) from healthy donors were separated by density gradient according to the standard technique by Lympholyte cell separation media (Cederlane, Ontario, Canada), cultured at a density of 10^6 cell/ml in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM glutamine (Hyclone, Cramlington, England, UK), 50 U/ml penicillin and 50 U/ml streptomycin (Hyclone), and stimulated by PHA at 5 μg/ml (Sigma, St. Louis, MO, USA), in presence or not of the dideoxynucleoside analogue 3′-azido-3′-deoxythymidine (AZT, Wellcome Research Laboratories, Beckenham, UK) at the various concentrations for a maximum of 72 h at 37 °C in a humidified 5% CO_2 atmosphere. Flow cytometry analysis showed that in this experimental conditions the majority of PHA-stimulated PBMCs independently of treatment, consisted of a quite homogeneous cell population, being mainly (>96%) CD3+ cells (data not shown). AZT was prepared as stock solutions in RPMI 1640 culture medium. The *in vitro* concentration of 8 μM AZT approximately corresponds to the pharmacological level utilized in ART *in vivo*, while upper concentrations of the drug (32 μM and 128 μM) were used as suprapharmacological concentrations. In some experiments, PBMCs were treated with 5-fluorouracil (5FU; Sigma) at the same concentration and at identical time of incubation than AZT. In the experiments on Fas-mediated apoptosis, 48 h after exposure to AZT cells were treated for 24 h with an agonist anti-human Fas mouse MAb (clone CH11) (Immunotech, Westbrook, ME, USA), at the concentration of 500 ng/ml. Following incubation in the above described culture conditions, cells were harvested and washed three times with cold phosphate-buffered saline (PBS) for 5 min, at 1200 RPM, +4 °C, and then processed for RNA and proteins extraction or for staining to evaluate protein expression and apoptosis by flow cytometry.

2.2. Cell death/viability and proliferation assays

Percentage or absolute number of dead/living cells was evaluated using the trypan blue dye exclusion test. A standard, thymidine incorporation assay was used to evaluate the antiproliferative effect of AZT. PBMCs were cultured at the concentration of $1 \times 10^5/100$ μl in a 96-well plate. AZT was added at the onset of the cultures at the concentrations of 8 μM, 32 μM, 128 μM. After 48 h in culture, ^{3}H-thymidine (Amersham Bioscience, United Kingdom) was added at 1 μCi/well. The cultures were harvested after a further 18 h incubation, for successive evaluation by radioactivity detection.

2.3. Evaluation of apoptosis

Apoptosis evaluation was performed by using two flow cytometry methods. The first method is based on flow cytometry analysis of isolated nuclei following detergent treatment and propidium iodide staining, as previously described by us [14]. Briefly, harvested cells were treated with a solution of propidium iodide at 25 μg/ml (PI, Sigma) plus 0.05% sodium citrate (Sigma) and with detergent at a high concentration (20% Triton X-100, Sigma), for 30 min. Isolated nuclei were then analyzed using a FACScan flow cytometry (BD Biosciences, Mountain View, CA, USA). Detectors and amplifier gains for forward and orthogonal scatter were adequately selected in order to simultaneously detect nuclei from viable, apoptotic and necrotic cells. Events were gated on forward versus orthogonal scatter in such a way that degraded DNA from cell debris or from doublets was excluded and nuclei from viable, apoptotic an necrotic cells were assayed. Data acquisition and analysis was performed using CellQuest™ software on a minimum of 5000 events for each sample (BD Biosciences, San Jose, CA, USA). The second method for evaluation of apoptosis consisted in double-staining of the cells with fluorescent annexin V and with PI solution. To this purpose, the "Annexin-V-FITC Apoptosis Detection Kit" (BD-Bioscence Pharmingen, San Diego, CA, USA) was used according to the manufacturer's instructions. Briefly, 5×10^5 cells were incubated for 15 min with annexin-V-fluorescein isothiocyanate (FITC) and then washed in annexin buffer. Cells were then stained with PI solution and analysed immediately after staining by flow cytometry analysis using a FACScan flow cytometer and Cell Quest™ software. This method is widely accepted to distinguish between early apoptosis and necrosis.

2.4. Immunostaining and flow cytometry analysis of cellular proteins

For the extracellular Fas receptor analysis, approximately 5×10^5 cells were resuspended in 50 μl of PBS with fluorescein (FITC) conjugated anti-human CD95 Fas/Apo-1 or irrelevant control antibody (BD Biosciences) and incubated at 4 °C for 30 min. After treatment, cells were washed twice in PBS and stored at 4 °C. For analysis of intracellular Bcl-2 and p53 proteins, 1.5 to 2×10^6 cells were fixed in 0.5 ml of 4% paraformaldehyde in PBS for 20 min at room temperature and permeability was obtained through incubation in 0.1% Triton X-100 in PBS with 0.1% FCS for 5 min. After washing in PBS with 3% FCS, permeable cells were incubated with FITC-conjugated anti-human Bcl-2 or PE-conjugated anti-human p53 or irrelevant control antibody (all from BD Biosciences) for 30 min on ice and washed twice in PBS. Flow cytometry analysis was performed by a FACScan flow cytometer and Cell Quest™ software.

2.5. Western blot analysis

For Western blot analysis, a total number of 5×10^6 PBMCs, treated with different concentrations of AZT for various times, were solubilized at 4 °C in lysis buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA pH 7.4, 1% Triton-X, 150 mM NaCl, 0.25% sodium deoxycholate, 1% NP-40 and, freshly added, 1 mM PMSF, 5 μM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 1 mM Na_3VO_4, 20 mM Na_3F, all from Sigma) and centrifuged at $10{,}000 \times g$ for 20 min. An amount of proteins obtained from $5x10^5$ cells was loaded onto a 10% SDS-polyacrylamide gel, subjected to