Reproductive Toxicology 50 (2014) 122–128

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Analysis of endoplasmic reticulum stress in placentas of HIV-infected women treated with protease inhibitors

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ARTICLE INFO

Article history:
Received 3 April 2014
Received in revised form
23 September 2014
Accepted 14 October 2014
Available online 25 October 2014

Keywords:
Protease inhibitor
Placenta
XBP1
Endoplasmic reticulum stress
Adverse effect
Mother-to-child transmission

ABSTRACT

Combined antiretroviral therapy has proven efficacy in decreasing vertical HIV transmission. However, endoplasmic reticulum stress is a known side effect of HIV protease inhibitors. We investigated endoplasmic reticulum stress in placentas of HIV-infected and uninfected mothers by PCR-based splicing analysis of the specific endoplasmic reticulum stress marker XBP1 in post-delivery placental samples of uninfected mothers and in HIV-infected mothers taking antiretroviral therapy. No elevated XBP1 splicing could be detected in placentas of uninfected mothers and most of the mothers receiving combined anti-retroviral therapy. However, markedly elevated XBP1 splicing was found in the placentas of three individuals on combined antiviral therapy, all receiving lopinavir or atazanavir. In vitro experiments confirmed induction of endoplasmic reticulum stress by lopinavir and atazanavir in trophoblast-derived cell lines. Since endoplasmic reticulum stress occurred in selective patients only, individual differences in susceptibility of HIV-infected mothers to protease inhibitor induced endoplasmic reticulum stress can be postulated.
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1. Introduction

Endoplasmic reticulum stress in various human tissues and cell types has been described as an adverse effect common to many HIV protease inhibitors [1–5]. The exact mechanism by which HIV protease inhibitors cause endoplasmic reticulum stress is not known. It is assumed that this is either mediated by their ability to interact with a yet unidentified endoplasmic reticulum-resident aspartic protease, with ensuing accumulation of incompletely processed proteins within the endoplasmic reticulum [5], or by their ability to induce oxidative stress [6], another well-known protein-damaging condition that leads to endoplasmic reticulum stress [7,8].

Accumulation of misfolded or incorrectly processed proteins within the endoplasmic reticulum leads to the recruitment of luminal endoplasmic reticulum chaperones and folding proteins such as BiP (binding protein) to these proteins in order to assure refolding of these proteins or to prevent formation of possible cytotoxic protein aggregates [7,8]. Recruitment of BiP to misfolded proteins is associated with its release from three endoplasmic reticulum membrane-bound signaling molecules, IRE1, PERK, and ATF6, which subsequently become activated and convey the endoplasmic reticulum stress signal. This includes signal transmission into the cytosol by inducing protein phosphorylation cascades and signal transmission into the nucleus by activation of transcription factors that subsequently transcribe cytoprotective molecular chaperones to cope with the cellular stress situation. However, long term impairment of the endoplasmic reticulum homeostasis can result in severe disturbances of cellular metabolism and can lead to cell death resulting in various pathological conditions found in humans [7,8].

Excessive endoplasmic reticulum stress has also been associated with the induction of autophagy or reticulophagy, a mechanism by which damaged regions of the endoplasmic reticulum might be removed [9,10]. The endoplasmic reticulum membrane-resident IRE1 molecule also causes an unconventional cytoplasmic splicing of the XBP-1 (X-box binding protein 1) mRNA, which results in the expression of the active form of the XBP-1 transcription factor. XBP1 is one of those transcription factors which lead to the expression of the characteristic endoplasmic reticulum stress response genes, and can also be used as a specific endoplasmic reticulum stress marker since its splicing by IRE1 can easily be detected by means of an PCR assay [5]. Using this assay, we analyzed and characterized

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http://dx.doi.org/10.1016/j.reprotox.2014.10.012
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the occurrence of the endoplasmic reticulum stress reaction in placenta tissue samples of HIV-infected women receiving combined antiviral therapy, of HIV-uninfected women, and in trophoblast-derived cell lines exposed to antiretroviral drugs.

2. Methods

2.1. Study participants

HIV1-infected pregnant women who took an antiretroviral combination therapy were prospectively enrolled in the Department of Obstetrics at the University Hospital Munich. The control group consisted of HIV-uninfected healthy pregnant women who delivered during the study period at the same institution. Patients' characteristics are summarized in Table 1. Delivery of HIV-infected and uninfected patients was all by Cesarean section. On the day of delivery, HIV drugs were taken by the patients in the same amount and frequency (twice per day) as during pregnancy. The study group consisted of women of various ethnicities, including Caucasian, African, and Asian. The protocol was approved by the local ethics committee. Written informed consent was obtained by all participants.

2.2. Cells and cell culture

The immortalized human 3A-subE placental cell line was purchased from ATCC (ATCC CRL-1584). JEG-3 human choriocarcinoma cells were kindly provided by Dr. Bernhard Ugele, University Hospital Munich. Both cell lines were cultured in fetal calf serum-containing RPMI1640 cell culture medium (Quantum 263, PAA, Pasching, Austria), supplemented with penicillin/streptomycin (PAA, Pasching, Austria) at 37 °C in a humidified atmosphere with 5% CO_2. The 3A-subE placental cell line (tPA30-1) was derived from "term placental cells" transformed with a tsA mutant form of the simian virus 40 (SV40) [11] (http://www.lgcstandards-atcc.org/Products/All/CRL-1584.aspx). JEG-3 cells were derived from the Woods strain of the Erwin-Turner tumor [12] (http://www.lgcstandards-atcc.org/Products/All/HTB-36.aspx).

2.3. Tissue preparation

After delivery, placentas were transported on ice to the laboratory (maximum transportation time: 5 min), and pieces of placental tissue were excised and frozen at −80 °C. For RNA extraction, small tissue pieces were cut with a scalpel from the frozen placenta tissues and transferred to a reaction vial placed in liquid nitrogen as previously described [5]. Frozen tissue samples were ground with a teflon pestle, thawed, and then dissolved in 1 ml Trizol reagent. Total RNA was extracted by the standard Trizol method as previously described [5]. mRNA was transcribed into cDNA with M-MLV reverse transcriptase (Promega, Mannheim, Germany) and oligo-dT as recommended by the supplier. RNA from placental cell lines was isolated with an RNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. From each placenta, 2 different samples were taken from different regions of the placenta and prepared and analyzed separately. Final data were presented as mean values generated from the 2 samples collected.

2.4. Quantitative and semi-quantitative PCR analysis

RNA extracted from placental tissues obtained after delivery was analyzed by an XBP1 splicing-specific real-time PCR assay to detect the occurrence of ER stress. Semi-quantitative PCR amplification was performed with PCR master mix (Promega, Mannheim, Germany) at 26 cycles (β-actin) or 32 cycles (XBP1) in a 25 μl PCR volume. Primer pairs used were 5′-GAAGAGGAGGCGGAAGCCAAG-3′ and 5′-GAATGCCCAACAGGATATCAG-3′ for XBP1, and 5′-GGAGAAGCTGTGCTACGTCG-3′ and 3′-CGCTCAGGAGGAGCAAT-GAT-5′ for β-actin amplification. Quantitative real-time PCR analysis was performed using the XBP1-specific primers described above in combination with the FAM-5′-TGCTGAGTCCCAGCAGGTGCA-3′-TAMRA probe and the β-actin-specific primers in combination with the probe FAM-5′-CCTTCCTGGGCATGGAGTC-3′-TAMRA. Real-time PCR was performed with a 7500FAST System real-time PCR cycler (Applied Biosystems, Darmstadt, Germany), using iTaq Fast Supermix with ROX (BioRad, München, Germany). Relative expression analysis was calculated by the $2^{-\Delta\Delta CT}$ method [13] using β-actin expression as a reference. Real-time PCR analysis was performed in duplicate on each placental tissue probe, resulting in 4 measured data for each patient. All primers were synthesized by biomers.net (Ulm, Germany).

2.5. Drugs and drug treatment

All antiretroviral medications for patients, including Kaletra (lopinavir + ritonavir), Reyataz (atazanavir), Norvir (ritonavir), Viramune (nevirapine), Truvada (tenofovir + emtricitabine), Viread (tenofovir), Combivir (lamivudine + azidothymidine), Epivir (lamivudine), and Zidovudine (azidothymidine) were provided by the University Hospital Pharmacy. For in vitro experiments, the tablets of fixed dose combination lopinavir plus ritonavir (Kaletra® 200 mg + 50 mg) were ground and extracted with dimethyl sulfoxide (DMSO) to reach a final concentration of 10 mg/ml, corresponding to 8 mg/ml (12.72 μM) lopinavir (MW 628.81) plus 2 mg/ml (2.77 μM) ritonavir (MW 721). Chemically pure atazanavir sulfate (MW 802.93), as used for in vitro experiments, was purchased from Selleckchem (Munich, Germany) and kept as a 10 mg/ml (12.45 mM) stock solution after direct solution in 100% DMSO. In cell culture experiments, except for the sample with the highest atazanavir concentration, additional DMSO was added to achieve a similar concentration of DMSO in samples and controls (0.2% DMSO in case that 20 μg/ml atazanavir was used as the highest concentration, and 0.15% in case that 15 μg/ml atazanavir was used as the highest experimental concentration). Similar supplementations were made for experiments with Kaletra, for which the highest concentration of DMSO used was 0.2%.

2.6. Autophagy staining

Autophagy was microscopically visualized with the fluorescent autophagy detection marker monodansylcadaverine (Sigma, Munich, Germany). For this, JEG3 cells were seeded on glass chamber slides 24 h prior to a further 24 h incubation of the cells with the corresponding protease inhibitors. All incubations were performed under standard cell culture conditions. After 24 h incubation with protease inhibitors, JEG3 cells incubated for further 30 min with 1 μg/ml of monodansylcadaverine (1 mg/ml stock solution in DMSO) in cell culture medium. After washing with PBS, slides were subjected to fluorescence microscopy using a DAPI-suited blue-cyan filter (365 nm excitation filter; 420–470 nm emission filter). Cells were then photographed as viable cells by means of a Zeiss Axiophot fluorescence microscope (Zeiss, Germany). For autophagy detection by FACScan analysis, JEG3 cells were seeded in 6-well cell culture plates (2×10^5 cells/well) and allowed to grow for 24 h under cell culture conditions before application of HIV protease inhibitors for further 24 h. Then, cells were incubated with a 1:1000 dilution of an autophagy detection dye (Cyto-ID Autophagy Detection Kit; Enzo Life Sciences, Lörrach, Germany) for 1 h, collected by trypsinization, and subjected to FACScan analysis (Beckman Coulter Epics XL), using a 488 nm excitation filter and a 525 nm emission filter.

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