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PLACENTA
Placenta 27 (2006) 989–995

Down Modulation of TNF-α mRNA Placental Expression by AZT Used for the Prevention of HIV-1 Mother-to-Child Transmission

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Accepted 21 October 2005

Abstract

Mechanisms of HIV-1 in utero mother-to-child transmission (MTCT) protection provided by AZT are not completely understood. The placental cytokine network is involved in the control of HIV-1 in utero transmission but the effect of AZT on this network is unknown. To evaluate the effects of AZT on placental cytokine expression, the chorionic villi from HIV-1 uninfected women term placentae were cultured with 0, 100, and 2000 ng/ml AZT. Tissue fragments were harvested at days 1, 4, and 7 to determine the level of cytokine mRNA by real-time RT-PCR. The viability and morphology of the placental histocultures were monitored by the expression of beta-human chorionic gonadotropin (β-hCG) gene, lipopolysaccharide (LPS) activation, and microscopic examination. AZT at 2000 ng/ml significantly down-regulated TNF-α mRNA expression at day 1 and day 4, but had no effect on β-hCG, stromal cell-derived factor 1 (SDF-1), and IL-10 gene expression. AZT did not induce any deleterious impact on placental tissue structure. Furthermore, activation of chorionic villi by LPS for 24 h up-regulated IL-10 and TNF-α mRNA expression. Down-regulation of TNF-α mRNA could represent a mechanism through which AZT can decrease the risk of HIV-1 MTCT, in addition to its direct effect on HIV-1 replication.

Keywords: AZT; HIV-1; Histoculture; Placental cytokine expression; Mother-to-child transmission; Prevention

1. Introduction

The efficacy of AZT (3′-azido-3′-deoxythymidine) for the prevention of HIV-1 mother-to-child transmission (MTCT) was first established by the Pediatric AIDS Clinical Trials Group (PACTG) Protocol 076 and the ANRS 024 protocol [1]. Since then, it has been widely used for the prevention of HIV-1 MTCT in different settings [2–5].

However, the mechanisms of MTCT protection provided by AZT are not completely understood. One of the most important risk factors associated with increased perinatal transmission is a high maternal plasmatic RNA viral load [6]. Although the viral load decrease under AZT prophylaxis during pregnancy is not sufficient to fully explain its efficacy to prevent MTCT [7]. Furthermore in the PACTG 076 study, HIV-1 transmission was observed irrespective of the maternal plasmatic viral load, including women with undetectable levels [8]. It was thus proposed that, as AZT is metabolized and crosses the placenta [9,10], its presence in the fetus/infant prior to the exposure to HIV-1 may abort the first round of replication in the infant's cells and prevent infection from being established.

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doi:10.1016/j.placenta.2005.10.010

Several studies have reported that cytokines in placental environment are involved in the natural protection of the fetus against in utero infections [11–13]. HIV-1 and its antigens [14] as well as opportunistic microorganism [15–17] have been shown to up-regulate the placental pro-inflammatory cytokines, which in turn activate HIV-1 expression. This activation loop may result in increasing risk of HIV-1 MTCT.

Little is known about the activity of AZT on the expression of anti- and pro-inflammatory cytokines in the placental environment. We have previously described an in vitro placental histoculture system that maintains the complex interaction between the various cell subpopulations at the chorionic villi level and allows the analysis of the cytokine profiles expressed within the placenta [18]. We have thus investigated the effects of AZT on the placental environment using this system.

2. Materials and methods

2.1. Placentae collection

Term placentae were obtained, in accordance with French ethics guidelines, immediately after delivery from eight HIV-1 uninfected pregnant women, who delivered through elective caesarian section and were followed by the Department of Obstetrics and Gynecology at the Antoine Béclère Hospital, Clamart, France. Two placentae were reserved for studying the effect of LPS activation on β-hCG and cytokine mRNA levels in placental histocultures. Six placentae were reserved for studying the effect of AZT on β-hCG and cytokine mRNA levels. Morphological evaluations were performed on placental histocultures from one of the six placentae.

2.2. Placental histocultures and AZT effect

During the 2–3 h following collection, each of the six placentae was processed to isolate chorionic villi. First, the placentae were extensively washed at least three times with normal medium (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% L-Glutamine and 1% Penicillin/Streptomycin). Second, each placenta was cut to obtain 243 villi blocks of 2–3 mm. Each series of nine blocks was placed on top of one collagen sponge gel (1.5 cm^3, Gelfoam®, Pharmacia Upjohn, Kalamazoo, MI, USA). A total of 27 sponge gels were cultured in six-well plates (one sponge gel per well) at the interface between the media and the air. Each well contained 3 ml of histoculture medium (RPMI-1640 supplemented with 15% heat-inactivated FCS, 1% L-Glutamine, 1% Penicillin/Streptomycin, 1% Fungizone, 1% Non-essential amino acid, 0.1% Gentamycine) in the presence of AZT at a concentration of 0, 100, or 2000 ng/ml. Histoculture plates were maintained at 37 °C and 5% CO_2 for 1, 4, or 7 days, before villi block collection. Every experimental condition, dose of AZT and duration of histoculture, was studied in triplicate.

2.3. Placental histoculture and morphological evaluation

One representative placenta of the six placentae group was processed following the same procedures to obtain three additional wells per culture time point. Nine villi blocks were placed on top of each of the nine sponge gels on each well, cultured with 0, 100, or 2000 ng/ml AZT. After 1, 4, or 7 days of culture, villi blocks of each well were collected and fixed in 4% buffered formaldehyde and embedded into paraffin. Five millimeter sections were cut, stained with hematoxylin and eosin and observed under a light microscope.

2.4. Placental histoculture and 24 h LPS activation

The last two placentae were processed similarly. One hundred and sixty two villi blocks were cut per placenta. A series of nine blocks was placed on top of one sponge gel (one sponge per well) in six-well plates. A total of 18 sponge gels were cultured in the absence of AZT for 0 (no culture), 3, or 6 days in the conditions previously described (corresponding to six wells per experimental condition). Villi blocks were then pooled for each time point and cultured in three independent wells in presence of 0, 0.1, or 10 μg/ml of LPS (one concentration per well) from *Escherichia coli* (Serotype 055:B5, SIGMA, USA) in 1 ml histoculture medium. Plates were maintained at 37 °C and 5% CO_2 for 24 h before villi block collection.

2.5. β-hCG and cytokine mRNA expression

To assess the AZT effect, each series of nine villi blocks cultured in a given well was collected individually and mechanically homogenized (X620 CAT M Zipperer GmbH, Germany), leading to three replicates for a given experimental condition. Total RNA for each replicate was obtained by RNA extraction Kit (Rneasy® Mini Kit, Qiagen, France). cDNA was obtained using the Taqman® reverse transcription kit Reagent (Applied Biosystems, New Jersey, USA) and quantified by real-time PCR, using the ABI-PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA) with specific oligonucleotide primer sets and Taqman probes (Table 1). RNA levels were normalized by quantifying 18S RNA (20×, Applied Biosystems, Foster City, CA) in the same reaction. Calculation of gene expression in each sample was quantified by the comparative threshold cycle (CT) method. β-hCG and cytokine mRNA levels were expressed with respect to those in a reference term placental tissue (calibrator considered as the 1× sample), similar for each experiment and run in parallel for each analysis.

For the LPS activation study, the same procedure was used on the homogenate of each well obtained after collection of villi blocks cultured 24 h with each concentration of LPS. Triplicate evaluations of β-hCG and cytokine mRNA were performed from the same homogenate.

2.6. Statistical analysis

Experimental results were presented through mean ± standard deviation (SD) for each experimental condition.

To take into account the fact that all experimental conditions were evaluated on each placenta, the effect of each AZT dose after a given duration of histoculture on β-hCG or cytokine mRNA expression was expressed for each placenta as the ratio of the mean level of the three replicates, at this dose after this duration to the mean level of the three replicates with no AZT after the same duration of histoculture. Then, these ratios were analysed through analysis of variance of a factorial design including two factors [19], AZT dose (100 or 2000 ng/ml) and duration of histoculture (1, 4, or 7 days), and six placentae. First, it was tested if AZT dose effect varied with the duration of histoculture (interaction between AZT dose and duration of

Table 1
Primers and probes used to amplify β-hCG and cytokine mRNA from placental histocultures

Proteins	Primers and probes	bp
IL-10	(+) TTGCTGGAGGACTTTAAGGGTTAC	24
	(−) TCAGCTTGGGGCATCACC	18
	FAM-TTGCCAAGCCTTGTCTGAGATGATCCA-TAMRA	27
SDF-1 (α/β)	(+) CACTCCAAACTGTGCCCTTCA	21
	(−) CTTGTTTAAAGCTTTCTCCAGGTACTC	27
	FAM-CAACAACAGACAAGTGTGCATTGACCCG-TAMRA	28
β-hCG	(+) GCTACTGCCCCACCATGACC	20
	(−) ATGGACTCGAAGCGCACATC	20
	FAM-CCTGCCTCAGGTGGTGTGCAACTACC-TAMRA	26
TNF-α	Human TNF-α (20×) reagents Taqman® PE Biosystems, UK	

histoculture). Second, if no interaction was evidenced, the homogeneity of AZT effect across doses was tested independently of the duration of histoculture, whereas, in presence of interaction, the homogeneity of AZT effect across doses was tested for each duration of histoculture. Finally, the AZT dose effect was tested, either globally if homogeneity was evidenced or for each dose otherwise, by comparing the mean of the corresponding ratios to 1, using the residual error derived from the analysis of variance. No specific attention was given to the mean effect of the duration of histoculture, which was not studied per se and could not be easily interpreted due to the initial standardization of data, varying with the duration of histoculture.

To study the effect of LPS dose according to duration of histoculture, the same standardization as for AZT dose was used relatively to no LPS after the same duration, using the mean triplicate evaluations of β-hCG or cytokine mRNA expression on the same homogenate. Then, these ratios were analysed through a factorial design, similar to the one used for AZT dose, with two factors, LPS dose (0.1 or 10 μg/ml) and duration of histoculture (0, 3, or 6 days followed by 24 h of LPS activation), with two replicates.

The AZT or LPS dose effect was expressed as the mean of the corresponding ratios (mR) and its 95% confidence interval (95% CI). Therefore, an mR of 4.0 (or 0.25) for a given AZT or LPS dose means that the corresponding mRNA expression has been multiplied (or divided) on an average by 4 in the presence of this dose when compared to dose 0.

3. Results

3.1. AZT and/or histoculture duration did affect neither the morphology, LPS induced activation, nor the viability of placental chorionic villi

Histology analysis of histoculture villi showed that the morphology of placental chorionic villi was preserved regardless of the duration of histoculture (Fig. 1A–C, E). The large majority of nuclei were morphologically normal and no necrotic area was observed. The morphology of syncytiotrophoblasts and fetal macrophages (Hofbauer cells), as well as red cells

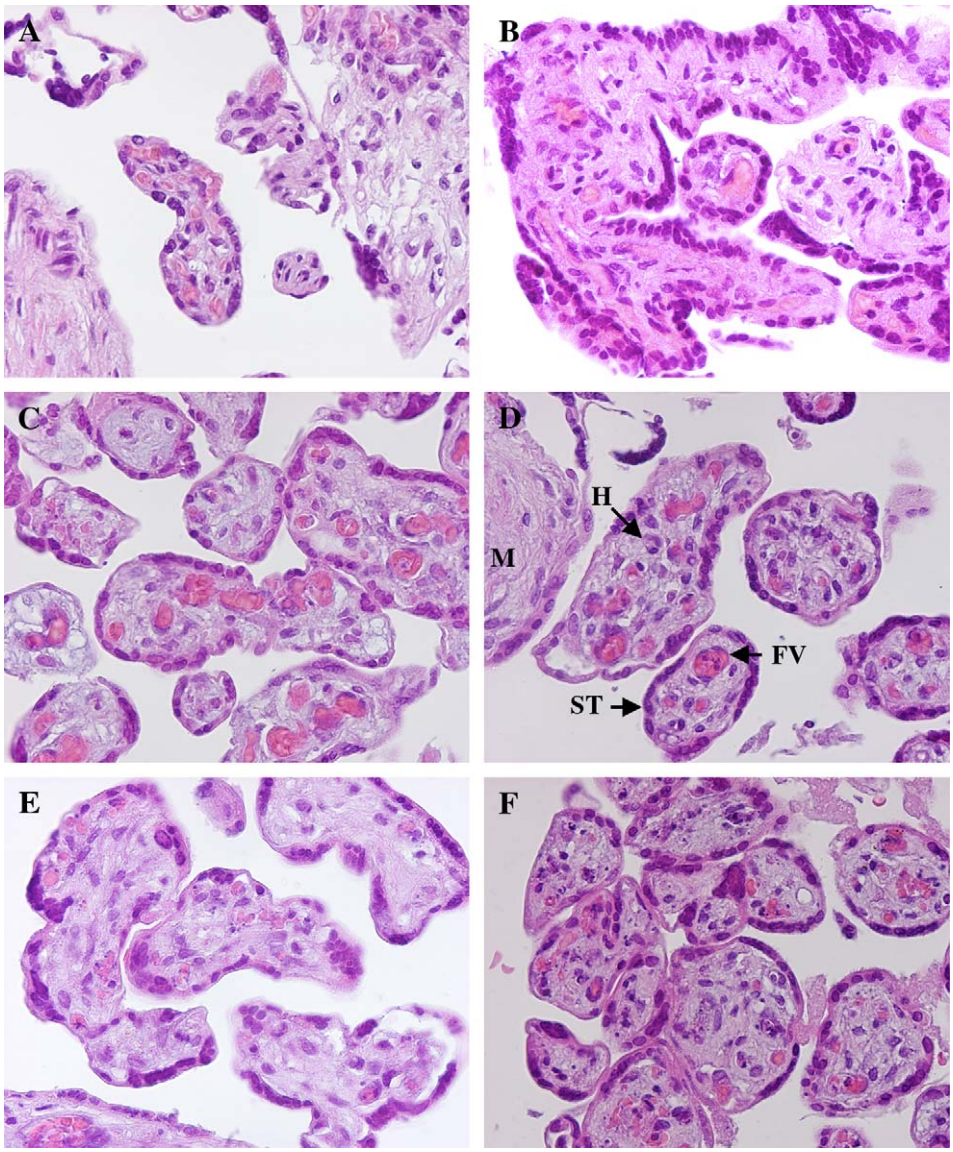

Fig. 1. 40× light-microscopic examination of chorionic villi stained with hematoxylin and eosin. Photographs A, B, C, and E are the chorionic villi at days 0, 1, 4, and 7 of histoculture without AZT, respectively, D and F the chorionic villi at days 4 and 7 of histoculture with 2000 ng/ml of AZT, respectively. H: Hofbauer cells, M: mesenchyma, FV: fetal vessel, ST: syncytiotrophoblast.

in fetal vessel was preserved throughout the experimental period, even at the highest AZT concentration (Fig. 1D, F).

After 24 h of LPS activation, placental chorionic villi showed a significant up-regulation of IL-10 ($p = 0.04$) and TNF-α mRNA ($p < 0.001$) levels, with a global mR of 3.4 (1.2–5.6) (Fig. 2A) and 4.2 (2.9–5.4) (Fig. 2B), respectively, but not of β-hCG ($p = 0.35$) and SDF-1 mRNA levels ($p = 0.28$), with a global mR of 1.9 (Fig. 2C) and 1.4 (Fig. 2D), respectively. The up-regulation or the absence of regulation for IL-10, TNF-α, β-hCG, and SDF-1 was observed regardless of the duration of histoculture tested ($p = 0.99$, 0.82, 0.54 and 0.85, respectively), and was homogeneous across LPS doses ($p = 0.69$, 0.59, 0.37 and 0.53, respectively).

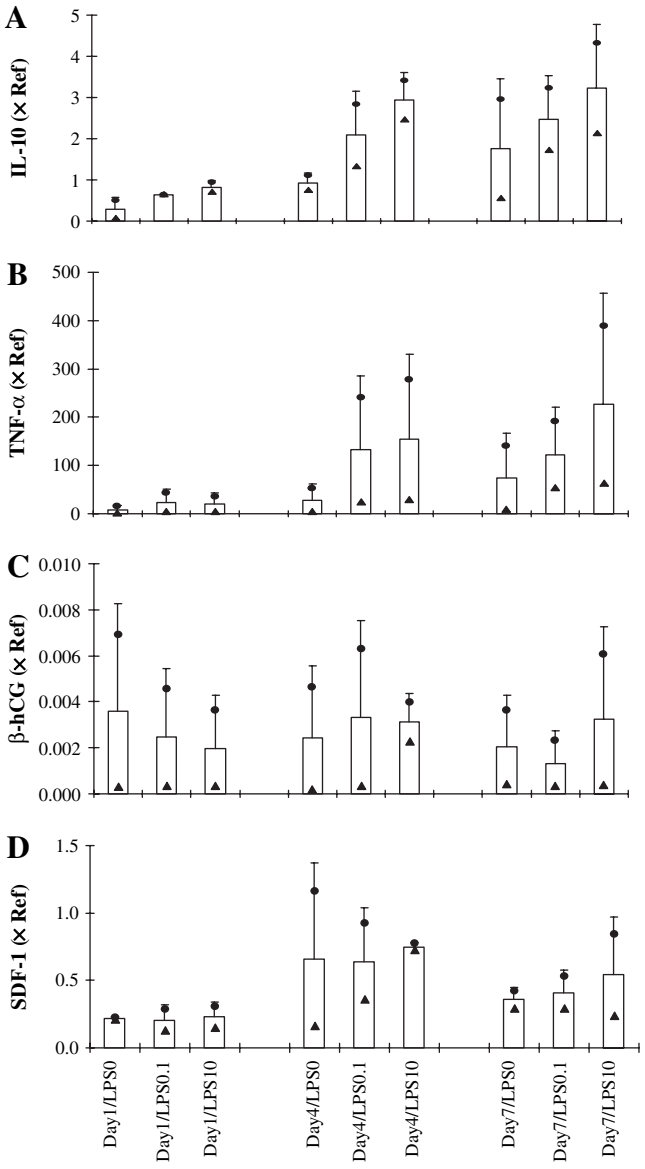

Fig. 2. β-hCG and cytokine mRNA expression from LPS activated placental histocultures. Chorionic villi from two term HIV-1 uninfected placentae were histocultured for 0, 3, or 6 days, then activated for 24 h with 0, 0.1, or 10 μg/ml LPS. The quantity of each cytokine mRNA was reported to the mRNA from a reference term HIV-1 uninfected placenta (× Ref). Results are presented as mean ± SD and symbols are individual placenta data.

When using the expression of β-hCG mRNA as a control of placental histoculture viability, no variation of the effect of AZT dose with the duration of histoculture could be found ($p = 0.37$), corresponding to the absence of interaction between AZT dose and duration of histoculture, as well as no heterogeneity of AZT effect across doses ($p = 0.46$) and no effect of the AZT dose ($p = 0.38$) with an mR of 1.1 (Fig. 3A).

3.2. AZT down-regulates the mRNA expression of TNF-α

The effect of AZT dose on SDF-1 and IL-10 mRNA expression did not differ with the duration of histoculture ($p = 0.44$ and 0.39, respectively), in contrast to the observation for TNF-α mRNA expression ($p = 0.04$).

The SDF-1 and IL-10 mRNA expression did not vary with the dose of AZT ($p = 0.87$ and 0.43, respectively), with an mR

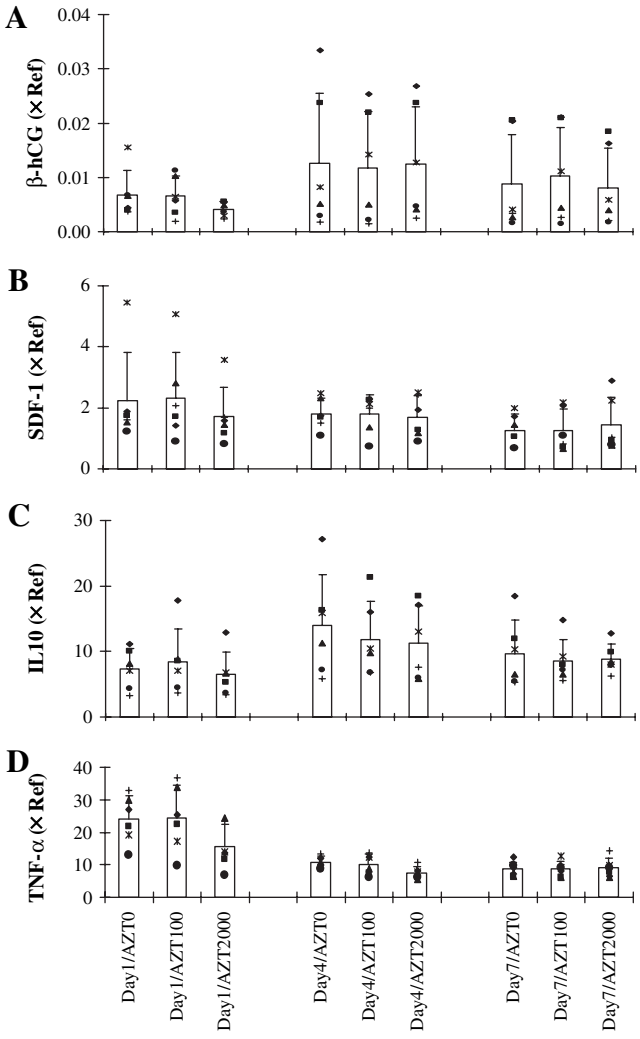

Fig. 3. Effect of AZT dose (0, 100, or 2000 ng/ml) and duration of histoculture (1, 4, or 7 days) on β-hCG, SDF-1, IL-10, and TNF-α mRNA expression in placental histocultures. Six term HIV-1 uninfected placentae were histocultured and three replicates were done per placenta for each experimental condition. The quantity of each cytokine mRNA was reported to the mRNA from a reference term HIV-1 uninfected placenta (× Ref). Results are presented as mean ± SD and symbols are individual placenta data.

of 1.0 (Fig. 3B) and 0.96 (Fig. 3C), homogeneous across doses ($p = 0.54$ and 0.47, respectively).

As expected from the interaction between the effects of AZT dose and of the duration of histoculture, only AZT dose of 2000 ng/ml significantly suppressed TNF-α mRNA expression at day 1 ($p < 0.001$) with an mR of 0.64 (0.46–0.82) and at day 4 ($p = 0.002$) with an mR of 0.69 (0.51–0.87), corresponding to about one-third reduction, but not at day 7 ($p = 0.44$) with an mR of 1.07 (Fig. 3D). In contrast, AZT dose of 100 ng/ml had no effect on TNF-α mRNA expression, regardless of the duration of histoculture ($p = 0.83$, 0.44, and 0.74 with an mR of 0.98, 0.93, and 1.03 at days 1, 4, and 7, respectively).

4. Discussion

Our results show for the first time that AZT at 2000 ng/ml (7.48 μM), concentration within the range observed in blood of women and fetuses receiving perinatal AZT as preventive therapy (0.1–10.0 μM) [20,21], down-regulates TNF-α mRNA expression in placental chorionic villi. In the same conditions, AZT did not affect neither SDF-1 and IL-10 mRNA expression, nor the structure and the viability of placental chorionic villi as indicated by the expression of β-hCG gene.

SDF-1 and β-hCG expression levels are essential for maintaining pregnancy and promoting normal fetal development [22–23]. Both have been shown to inhibit HIV-1 infection in human placental explants [24–25]. IL-10 is an anti-inflammatory cytokine which plays a role in graft tolerance and maintenance of normal pregnancy [26]. A reduction of circulating and placental IL-10 has been associated with neonatal growth retardation and pre-eclampsia [27,28]. Furthermore, IL-10 can block the production of TNF-α [29] resulting in suppression of HIV replication. AZT by maintaining the levels of SDF-1, β-hCG and IL-10 allows the survival and the normal growth of the placenta and the fetus and may decrease the risk of HIV-1 MTCT.

TNF-α is a pro-inflammatory cytokine which is important for pregnancy by controlling placental growth, cell death, immune privilege and hormone production [30]. TNF-α can stimulate HIV-1 expression in trophoblastic cells by activating HIV-LTR via the ubiquitous transcriptional factor NF-kB [31,32]. TNF-α also increases HIV infection and production after fusion of HIV-infected PBMCs with trophoblasts [33,34]. Thus, TNF-α may favor HIV-1 MTCT and its down-regulation by AZT could decrease the risk of HIV transmission. We observed a dose-dependent, down-regulation of TNF-α mRNA expression by AZT at day 1 and day 4 of histocultures, but not at day 7. This is most likely due to the lower level of TNF-α mRNA expression at this time point and not from a decrease of chorionic villi activation, since TNF-α and IL-10 mRNA could be up-regulated by LPS activation. Thus, our results support the hypothesis that AZT down-modulates TNF-α expression when TNF-α is highly expressed within the placenta. This high expression of placental TNF-α is frequently found in HIV-infected pregnant women co-infected with others microorganisms that could contribute in a higher risk of HIV-1 MTCT [35]. The histoculture system does not allow to detect low level of secreted cytokines by ELISA [18]. Since TNF-α is not secreted at high level within the placenta in non-pathogenic conditions, TNF-α secretion was as such not detectable in the supernatant of placental histocultures from our study even after 3 days of culture (data not shown).

In a multicentric study aiming to compare the cytokine profile in the placentae from HIV-1 uninfected and infected women, we have shown that significantly lower levels of TNF-α mRNA were detected in the term placentae from the HIV-1 infected women who received the AZT treatment earlier during pregnancy and who had a longer AZT treatment (S. Pornprasert et al., manuscript in preparation). These results support the ones described in this present study obtained in the histoculture system.

The mechanisms involved in the down modulation of TNF-α mRNA expression by AZT could be mediated through the inhibition of the activation of the transcriptional factor NF-kB. For AZT to be active, it needs to be phosphorylated intracellularly. AZT is metabolized within the placenta where it is phosphorylated to AZT mono-, di- and triphosphate. AZT monophosphate (AZTMT) is the major phosphorylated AZT metabolite of the intracellular AZT pool [36] and has been shown to inhibit phosphorylation and degradation of IkB by the IkB kinase complex [37]. AZT increases the reactive oxygen species (ROS; superoxide, hydrogen peroxide, and hydroxyl radicals) not only in placental cell lines but also in the first trimester and term explant placentae [38]. It has been shown that the ROS induce a decrease of the nuclear translocation of NF-kB via a decrease of IkB-α degradation resulting in down-regulation of TNF-α mRNA expression [39,40]. These may explain our findings. However, the exact molecular mechanisms involved need to be further investigated.

Our study may have potentially important physiological implications, since it demonstrates for the first time that AZT at concentration used to prevent HIV-1 MTCT down-regulates TNF-α mRNA expression in placental chorionic villi. If the effect of AZT on TNF-α mRNA is translated in vivo at the level of the protein, then the down-regulation of a cytokine known to increase HIV-1 replication at the placental barrier level may contribute to the efficiency of AZT to reduce HIV-1 MTCT, in addition to its direct antiviral activity on maternal viral load. Thus, our findings support the role of the placental cytokine network in the control of in utero MTCT of HIV-1.

Acknowledgements

We would like to thank the staff in the Department of Obstetrics and Gynaecology at the Antoine Béclère Hospital, Clamart, France for kindly providing placental samples. We particularly thank Michel Huerre and Patrick Ave from the histotechnology and pathology unit of the Institut Pasteur for the morphological evaluation of the placental histocultures. We also thank Intira Collins for correcting the English version of the manuscript.

This work was supported by Sidaction/Ensemble contre le SIDA. AF was supported by a Sidaction fellowship. SP holds a fellowship from the Thai Ministry of University Affairs and GD from the French Agency for AIDS Research (ANRS).

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