Transplacental Infection of HIV-1 and the Associated Risk Factors In Utero

Shamala Moodley

Department of Biomedical Sciences, Mangosuthu University of Technology, South Africa

*Corresponding author: Shamala Moodley, PhD, Mangosuthu University of Technology, Umlazi, KwaZulu-Natal, South Africa, Tel: 27 31 9077641; Fax: 27 32 9433149; E-mail: shamala@mut.ac.za

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Abstract

Background: Identifying risk factors in "in utero" transmission of human immunodeficiency virus type 1 (HIV-1) is important when designing preventive interventions. Several lines of evidence suggest that Natural Killer (NK) cells have an important role in antiviral defence. However, the Human Leucocyte Antigen-G (HLA-G) molecules are inhibitory of these cell-mediated immune responses and could, therefore, promote the propagation of HIV-1 infection across the placental interface thus increasing the risk of vertical transmission.

Study design: A total of 55 women were enrolled into the study. Tests for viral loads, CD+4 counts, Natural Killer cells, p24 and HLA-G1 expression were performed. Using logistic regression analyses, a study of the risk factors were undertaken.

Results: Variables associated with in utero transmission were HIV-1 viral load and HLA-G1 expression. Mothers, with low haemoglobin values, were more at risk in transferring the virus to their babies. The tendency for the presence of high NK cells was indicative of improved immunity.

Conclusions: Patients, with low haemoglobin, were more likely to transfer the virus to their foetus. Viral RNA was a strong predictor of mother-to-child-transmission (MTCT). The expression of HLA-G1 was an additional risk factor in acquiring HIV-1 infection. Female babies were more at risk than male babies with MTCT.

Keywords: Maternal viraemia; Natural killer cells; HLA-G1; Placental tissue; Gender differences

Introduction

Mother-to-child transmission (MTCT) of human immunodeficiency virus type 1 (HIV-1) accounts for more than 95% of the cases of paediatric AIDS [1]. Accumulating evidence indicated that about one third of these infected infants will develop severe symptoms of the disease coupled with severe immune-depression by that about one third of these infected infants will develop severe symptoms of the disease. The disease is important when designing preventive interventions. Several lines of evidence suggest that Natural Killer (NK) cells have an important role in antiviral defence. However, the Human Leucocyte Antigen-G (HLA-G) molecules are inhibitory of these cell-mediated immune responses and could, therefore, promote the propagation of HIV-1 infection across the placental interface thus increasing the risk of vertical transmission.

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Materials and Methods

Patient sampling

Blood and placental tissue samples analysed in this study were collected from pregnant women attending the antenatal clinic (ANC) for a routine antenatal examination. HIV infection in the pregnant mothers was determined, with consent, for the presence of HIV antibodies (Determine, Abbott, USA). The HIV status was accomplished at 28 weeks of pregnancy by the attending gynaecologist. Inclusion criteria were antiretroviral naïve, HIV positive pregnant women.
women who were pre and post counseled before enrolling into the study. 

Enrolment evaluation, including the gestational age and demographic data were recorded. All mother-baby pairs were analysed for the study and results consolidated. 

Patients were divided into two groups post-delivery: 

HIV positive mothers, whose babies had a viral load of >400 RNA copies 

HIV positive mothers, whose babies had a viral load of <400 RNA copies 

Using the premise of Bryson et al., babies were considered infected “in utero” either, during pregnancy and before the onset of labour [19].

Blood and tissue sampling and transportation methods

Sample of blood specimens, from mothers (2x±2.5 ml EDTA vacutubes), and babies (±1 ml). Approximately 5 cm² placental tissue was obtained from two sides of the placenta to give an adequate representation of placental cells. These samples were placed into 10% formal saline and transported on ice, to the laboratories. Maintenance of adequate safety procedures, were observed.

Processing of blood specimens

CD4⁺ cell counts were done on all mother-baby pairs of samples using the automated FACS Count System instrument and reagent kit (Becton Dickson, USA) according to manufacturer’s instructions. The normal ranges for CD4⁺ counts were females: 600-1500 cells/mm³ and babies (<1 year) ≥1500 cell/mm³. 

Blood samples were processed for viral RNA copies a (Roche Amplicor Version 1.5, Germany with negative (normal uninfected human sera) and positive controls (heat inactivated human sera with antibodies to HIV-1 and HIV-2). Viral RNA thresholds of ≥400 copies/ml were used to determine the status of patients.

Immunohistochemistry staining for natural killer cells and p24 antigens

Duplicate sections of paraffin wax embedded placental tissue (4-5 µm thick) were examined by immunoperoxidase immunohistochemistry, as per manufacturer’s protocol. Monoclonal mouse Anti-Human Immunodeficiency Virus, p24 (DAKO, Denmark, Code No. M0857) and primary antibodies to CD56⁺ (Zymed, USA, Clone 123C3) for the presence of HIV antigens and NK cells (CD56⁺) were used. Commercially available CD56⁺ and p24 positive and negative controls (DAKO) were used to verify test specificity and sensitivity. Positive cases of NK cells (CD56⁺) and p24 antigens were regarded as negative in the absence of immunoreactive p24 antigen in distinct cells.

Experimental protocol for RT-PCR

An optimised experimental protocol RT-PCR assay was developed to detect the presence of HLA-G1. PCR mix for one 20 µl reaction was prepared in a 1.5 ml reaction tube held on ice. The following components were added to the capillaries in the order as mentioned: Water (PCR grade) 5.8 µl, MgCl₂ 1.2 µl, PCR primers 0.5 µl and 1.0 µl of Fast Start SYBR GREEN 1 (Roche Diagnostics, Germany). Real-time PCR was performed with the following cycle conditions: 10 minute at 95°C followed by 40 cycles of 10 seconds at 95°C, 50 seconds at 60°C, 16 seconds at 72°C and 5 sec at 85°C.

Statistics

Data with categorical variables are presented as percentages whilst values with continuous variables are presented as interquartile ranges and medians. To determine the significance of inter-related interactions and risk ratios an association of all variables was done using a logistic-regression analysis. Correlations and assessments of probabilities between variables and categories were performed using Pearson’s correlation test and the Chi Square test, respectively. Variables which altered the risk ratio were extensively evaluated. SPSS statistical software was used for all descriptive analyses in the study.

Results

Information on all mothers participating in the study was obtained by the attending gynaecologist from the antenatal and labour records. The median age of the mothers was 26 years with an interquartile range (IQR) of 23-28 years. There were 49 (89.1%) vaginal deliveries and 6 (10.9%) caesarian sections. Pregnancies ended in full term deliveries in 52 instances (94.5%). The median parity was 1 (IQR 1-2) and the median gravidity 2 (IQR 2-3).

All neonates were examined at birth by the attending paediatrician. The median Apgar score was 9.0 (interquartile range 1-9) and 10 (interquartile range 1-10) at 1 min and 5 min respectively. No statistical significance was observed in Apgar scores between babies considered infected and uninfected (p=0.540). There were 38 (69.1%) males and 17 (30.9%) females born to 55 mothers in the study. Seven (41.2%) females had >400 viral RNA copies/ml as compared to 8 (21.1%) males. The median weight of babies at birth was 3 kg (range 2.0-4.0 kg).

Mothers CD4⁺ cell values and viral load

Among the patients studied, association between CD4⁺ cell and viral load categories were performed to define the immune status of the patients and gauge the level of cellular response (Table 1). No significant association was observed between the mother’s CD4⁺ cell counts and viral loads (p=0.134). Despite the lack of statistical significance a trend was noted. Mothers (33.3%) with low CD4⁺ cell counts (<200 cells/mm³) had high viral loads (5 to 5.99 logs) compared with 7.7% with low CD4⁺ count and low viral loads (3 to 3.99 logs). Conversely, mothers with high CD4⁺ counts had low viral loads (53.8% in the 3 to 3.99 log category). Twenty three (41.8%) had high CD4⁺ counts (>600 cells/mm³) and viral loads (3 to 5 logs) which, may indicate recent infections. Mothers with CD4⁺<200 cells/mm³ had a mean log viral load of 2.2 whilst those mothers with >200 cell/mm³ had a mean log viral count of 1.86.
CD4+ cell counts and viral load of infected infants

Using the CDC classification (1994) the infected babies were categorized as: (i) no evidence of immune suppression (>1500 cells/mm³); ii) evidence of moderate immune suppression (750 to 1499 cells/mm³); iii) evidence of severe immune suppression (<750 cells/mm³). Of 15 babies considered infected in utero, 6 (40.0%) had log viral loads between 3 to 4 logs of which 1 (16.7%) had a CD4+ count <750 cells/mm³ whilst 5 (33.3%) had >1500 CD4+ counts. The remaining 9 (60.0%) infected babies had viral loads of >4 logs. Among the babies, with high log viral loads, low CD4+ counts were observed in 2 (22.2%). Two (22.2%) babies were found to have moderate immune suppression and 5 (55.6%) with high CD4+ counts showed no immune suppression. Overall, 66.7% of infected babies demonstrated a well preserved immune system (Table 2).

Assessment of mothers CD4+ counts, viral loads and babies viral load

An evaluation between HIV-1 infected mothers with <200 CD4+ cells/mm³ (AIDS) and those with >200 CD4+ cells/mm³ and the viral loads of their babies revealed a significant association between

<table>
<thead>
<tr>
<th>Mothers CD4+ T Cell Counts (mm³)</th>
<th>Number (%)</th>
</tr>
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<tbody>
<tr>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>&gt;600-1500</td>
<td></td>
</tr>
<tr>
<td>3-3.99</td>
<td>7 (53.8)</td>
</tr>
<tr>
<td>4-4.99</td>
<td>12 (36.4)</td>
</tr>
<tr>
<td>5-5.99</td>
<td>4 (44.4)</td>
</tr>
<tr>
<td>Total</td>
<td>23 (41.8)</td>
</tr>
</tbody>
</table>

Table 1: Maternal Viraemia and CD4+ Cell Values. N=55

<table>
<thead>
<tr>
<th>Mothers CD4+ cells/mm³</th>
<th>Babies CD4+ cells/mm³ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No suppression Category 1</td>
<td>21500</td>
</tr>
<tr>
<td>Moderate suppression Category 2</td>
<td>750-1499</td>
</tr>
<tr>
<td>Severe suppression Category 3 &lt;750</td>
<td>Total</td>
</tr>
<tr>
<td>&lt;200</td>
<td>3 (50.0)</td>
</tr>
<tr>
<td>≥200</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td>Total</td>
<td>5 (100)</td>
</tr>
</tbody>
</table>

Table 2: Comparison of Mothers and Infected Babies CD4+ Counts. N=15

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Variable not accepted into logistic regression: CD4\(^+\) count; EXP (B) denotes Odds Ratio values; Cat: Category

Haemoglobin values, viral load and CD4\(^+\) counts in HIV-1 infected pregnant women

Mothers (66.7%), with low haemoglobin (Hb) values (<10 g/l), reflected high viral loads (log 5 to 5.99). Conversely, 84.6% of mothers with Hb≥10 g/l had low viral loads (log 3 to 3.99). The association between Hb values and maternal viraemia was statistically significant (p=0.050). It was noted that mothers with low Hb values had high viral loads and vice versa (Figure 1). Using the CDC criteria (1993) 10 mothers with <200 CD4\(^+\) cells/mm\(^3\) were classified as having AIDS. Of these 6 (60%) had haemoglobin (Hb) values <10 g/l. Fifteen (33.3%) infected mothers with CD4\(^+\) counts ≥ 200 cells/mm\(^3\) had Hb values ≥ 10 g/l. In mothers with Hb values <10 g/l there was a difference of 26.7% between those with CD4\(^+\) counts <200 cells/mm\(^3\) and those with CD4\(^+\) counts ≥ 200 cells/mm\(^3\). Although not statistically significant (p=0.156) there was a trend for mothers with low CD4\(^+\) counts to have low Hb values (Figure 1).

Assessment of placental p24 antigens when compared with maternal viraemia and CD4\(^+\) cell counts

Plasma HIV-1 RNA levels of all infected mothers were analysed in conjunction with their CD4\(^+\) counts and p24 antigen, in the placenta. Parametric statistical analysis between maternal viral load (p=0.448), CD4\(^+\) cell counts (p=0.660) and the presence of placental p24 antigens was not significant. High p24 antigens with low or high viral RNA copies were evenly distributed. There was a small variance of 1.1% in p24 antigens (Figure 2A). Similarly, high p24 antigen values were observed with both high and low CD4\(^+\) cell values. The R Sq linear value 0.002 (0.2% variance) was negligible for any definitive conclusion (Figure 2B). The presence of p24 antigens, in placental tissue of HIV-1 infected mothers, is not influenced by their viral load or CD4\(^+\) cell count. It was also noted that viral load of the babies born to the 3 (5.4%) mothers whose placentas did not demonstrate the presence of p24 antigens, was <400 copies/ml.

A logistic regression equation was performed to evaluate NK immune response at the placental interface and the protection it confers on babies against HIV-1 infection (Table 3). The variables which were statistically accepted were the log viral load of mothers, gender of babies and NK cell values. The protective effect of NK cells was evaluated in category B (≤4 NK cells/30 hpf) and category C (10-19 NK cells/30 hpf) using category A (20-30 cells/30 hpf) as the baseline value. Category A was chosen, in the equation, as the baseline for evaluation, on the basis that having a low NK cell value is associated with the risk of infection when compared with a higher NK cell presence. Therefore, the highest category with the lowest risk was chosen as baseline.

Placental fold increases in HLA-G1 expression was observed in 37 (67.2%) women, whilst 18 (32.7%) demonstrated HLA-G1 placental values <1 fold. A positive correlation between maternal viral load and placental HLA-G1 was observed (p=0.038) (Figure 3).
A logistic regression analysis was carried out to determine the relationship between maternal viral load, HLA-G expression and vertical transmission of HIV-1 infection. There was a positive correlation between the maternal log viral load and transmission of infection to the baby (p=0.047; 95%CI 1.029-11.499). No significant correlation was noted with HLA-G1 and vertical transmission (p=0.066; 95%CI 0.983-1.712). However, the odds ratio (OR) indicated that the risk of infection was increased by 1.3 with every 1 unit increase in HLA-G1 expression (Table 3). Although no statistical significance was observed with gender of babies and vertical transmission (p=0.088; 95%CI 0.833-13.881) according to our regression analysis female babies were 3.7 times more likely to become infected than males.

**Effect of placental HLA-G1 on viral loads of the infants**

Altogether 27.3% of babies in the study were considered infected at birth. Correlation of placental HLA-G1 expression and plasma viral load of babies in both the infected and uninfected groups fall on the level of significance (p=0.05). The mean HLA-G1 fold increase in placentas of mothers with infected babies and uninfected babies was 2.17 (SD=3.04) and 0.55 (SD=2.57) with a median of 2.22 and 1.17 respectively. The absolute difference in median HLA-G1 values between the placentas of mothers with infected babies was approximately 1 which is considered statistically significant. The effect of HLA-G1 expression in vertical transmission between male and female babies was explored. The median HLAG1 fold change was higher in mothers with infected male babies (2.3) than in those with uninfected male babies (1.3) with a statistically significant association (p=0.013). Female babies showed no difference in values (p=0.963). Overall, HLA-G1 emerged as a risk factor for infection in males.

**Expression of up-regulated HLA-G1 in placentas of HIV-1 infected women**

Up-regulation of HLA-G1 expression was calculated as follows: mean value of HLA-G1 expression in mothers with infected babies (2.17) divided by the mean value of HLA-G1 in mothers’ with uninfected babies (0.55). HLA-G1 expression was up-regulated 3.95 times more in placental tissue of HIV-1 infected mothers with infected babies.

**Discussion**

Analysis of CD4+ cell counts and viral loads indicated no significant association between the viral load of mothers and their CD4+ cell counts. However, mothers with high viral RNA copies demonstrated a tendency to have low CD4+ cell counts whilst, mothers with high CD4+ counts had low viral loads. Some authors have found that there is an inverse, but variable correlation between plasma viral RNA levels and the level of CD4+ lymphocytes [20-23]. Similar to other studies data reveals that low CD4+ counts are indicators of a waning immune system [22,23]. Observation in the study found that HIV-1 infected individuals with CD4+ levels, below 200 cells/mm3 were classified as having AIDS [22]. Ninety percent of the women with AIDS had viral loads greater than 4 logs thereby, increasing the risk for transmission to the baby [23]. Other factors are also important for MTCT. The known risk factors are vaginal deliveries and low haemoglobin levels during pregnancy [24].

The implication of low haemoglobin concentrations in HIV-1 infected women and the risk of vertical transmission were evaluated. Sixty percent of the women classified as having AIDS, had low haemoglobin values with a corresponding low CD4+ count. Those with haemoglobin values greater than 10 g/l had CD4+ cell counts >200 cells/mm3. Although not statistically significant in this study, there was a trend for mothers with low CD4+ cell counts to have low haemoglobin values. A statistically significant association between maternal viraemia and maternal haemoglobin was observed. Mothers with haemoglobin values less than 10 g/l reflected high viral loads. Conversely, mothers with haemoglobin values greater than 10 g/l had lower viral loads. A study conducted in Zaire reported an association between risk of transmission and low haemoglobin concentrations in HIV-1 infected women [25]. Some studies found that HIV-1 infected women whose haemoglobin values were <10 g/l during pregnancy, were at an increased risk of transmitting the virus to their babies [26].

Overall, 20% of all infected babies were categorized as having evidence of severe immune suppression. In the same category 6.7% of babies were born to mothers with a diagnosis of AIDS. It has been reported that infants born to women with advanced disease and higher viral loads also tend to be rapid progressors [27-29]. Altogether, 66.7% of infected babies who were classified as no evidence of immune suppression demonstrated a better preserved immune system. The mothers CD4+ counts of 7 (46.7%) infected babies in this category were >200 cells/mm3. An observation that babies born to HIV-1 infected mothers, with immune deterioration had lower CD4+ counts than babies born to mothers with high CD4+ counts.

Quantitation of viral and cellular responses in placental tissue is a challenge because there are insufficient separation techniques to differentiate trophoblastic cells from non trophoblastic cells. Further difficulties are encountered in separating maternal cells from fetal cells at the placental interface. However, the presence of p24 and NK cells in placentas was analyzed in relation to its implication in vertical transmission using an immunohistochemical technique as measurement of viral invasion. Some studies, using HIV-1 infected placental tissue, concluded that because of the absence of p24 antigens, the placenta forms an efficient barrier to viral transmission [30].
However, cohorts in these studies were undergoing antiretroviral therapy during their pregnancy. Mothers in this study were antiretroviral naive during pregnancy which may account for the elevated detection of p24 antigens in 94.6% of placental samples. Other studies indicated that cells can be infected by HIV when coming into contact with infected leucocytes [31-34]. The substantial differences in detection levels may also be due to study populations from diverse geographical regions. Therefore, it is important to further interrogate contributing factors to HIV activity in placental tissue in different population groups.

Presence of p24 antigens and placental NK cells was evaluated against maternal viral load and CD4+ cell counts. The observation was that there was no influence between presence of p24 antigens in placental tissue, maternal viral load or CD4+ cell counts. These findings are in line with previous studies which reported that there was no relation between placental infections and, either CD4+ counts or plasma viral loads [35]. However, analysis of the functional activity of NK cells at the placental barrier induced by p24 antigen stimulation showed that there were lower median NK cell values in placentas of mothers with infected babies as compared with the uninfected cluster. Although not statistically significant, low placental NK cells were associated with the risk of infection when compared with higher NK cell presence. The risk of vertical transmission was increased 3.4 times more in placentas which had lower NK cell values. The association between placental NK cells and vertical transmission has not been conclusively established in other studies. In lieu of the limited information available for comparison with other studies, comments about the interaction and role of NK cells in vertical transmission can only be made from data in this study. Further studies are required to test the validity of our observations.

Now recognized as important cells with effector and regulatory functions, NK cells are likely to exert inhibition of viral peptide HLA-G complex manner by several cytokines. It has also been reported that HLAg expression has been up regulated with cytomegaly virus and HIV [39-41]. In agreement with these earlier comments this study found an up-regulation of HLA-G1 in HIV infected placental tissue. The odds ratio established that the risk of infection increased by 1.3 with every 1 fold increase in HLA-G1 expression. Although statistically significant, evidence of clinical significance between the two variables need to be established with larger studies. To the best of our knowledge this is the first attempt to explore the role of HLA-G1 in vertical transmission. The study found that median HLA-G1 values were higher in mothers with babies infected at birth when compared to the uninfected group of babies. It is reported here that an absolute difference of 1 in the median HLA-G1 values between the placentas of mothers with infected babies and uninfected babies, thus establishing statistical significance. According to logistic regression analysis females were 3.7 times more likely to acquire the infection. However, when gender susceptibility to HLA-G1 expression was explored graphically, a statistically significant association was observed between infected and uninfected male babies and HLA-G1 expression. No difference in HLA-G1 expression was observed between the infected and uninfected female babies. HLA-G1 emerged as a significant risk factor in males than in females. In view of these discrepancies a separate analysis needs to be conducted on male and female babies to establish the risk of infection. Other factors besides the presence of HLA-G1 could have increased the 3.7 odds ratio in favor of females. A recent study conducted by Biggar et al., reported that girls were at a higher risk of in utero HIV infection than boys. The author proposed that minor histocompatibility reactions between maternal lymphocytes and Y chromosome-n derived antigens reduce the risk of HIV transmission in boys [42]. The magnitude of these differences is still to be tested. Finally, expression of HLA-G1 was up-regulated 3.95 times more in placental tissue of mothers with infected babies than in mothers with uninfected babies. It raises the debate whether the expression of HLA-G1 at the placental barrier of HIV-1 infected women represents implication guilty by association or a symbiotic physiological interaction.

Conclusion

It was found that 27.3% of babies were considered infected in utero. Maternal immune competence in clearing or preventing p24 antigens from the placenta appears to be intact in 72.7% of mothers whose babies were born with undetectable levels of viral RNA copies at birth. This result is a tentative estimation, since the babies were not available for retesting. Therefore, it is difficult to speculate on how many of the babies will eventually succumb to the virus. The limitation of the study restricts comments on the final depth and range of immunological responses in vertical transmission. It creates a platform for more comprehensive future studies using larger sample size and stratification of study participants with different ethnic backgrounds.

References


