Lipid Peroxidation Correlates with HIVmRNA in Serodiscordant Heterosexual HIVpartners of Nigerian Origin

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Abstract We recruited 59 individuals of known HIV serostatus after informed consent however, 44 were serodiscordant heterosexual partners [serodiscordant seronegative (SSN group) and serodiscordant seropositive (SSP group)] while 15 were seronegative healthy individuals (SNH). In the case–control study we choose to determine Malondialdehyde (MDA) concentration as a marker of lipid peroxidation index (oxidative stress) spectrophotometrically and quantify HIV mRNA by Real Time-nucleic acid sequence based amplification assay (RT-NASBA). Here our result show for the first time a high concentration of lipid peroxidation product (MDA, 116.6%) with a significant ($P < 0.05$) increase in HIV serodiscordant seropositive subjects over their seronegative partners. However, Spearman rank correlation statistics of SSP group showed a positive correlation value ($P < 0.01$, $r = 0.89$) between MDA and mRNA and a negative correlation between MDA and T-cell ratio ($P < 0.01$, $r = 0.96$). The study may strongly indicate a possible lipid peroxidation product threshold for predicting HIV infection and progression in serodiscordant heterosexual partners.

Keywords HIV/AIDS · Serodiscordant · MDA · mRNA · Nigeria · Oxidative stress

Introduction

Though the prevalence of human immunodeficiency virus (HIV) in serodiscordant infection has not been documented in the Nigerian population, about 0.5% (1 in 200) people are reported in America [1]. HIV serodiscordant infection exists where one sexual partner after being repeatedly exposed to the HIV seropositive partner remains seronegative [2]. These are individuals who are repeatedly exposed to HIV through different routes of exposure but persistently remain seronegative [3]. They are thus referred to as serodiscordant-seronegatives or elite controllers possessing natural resistance to HIV infection [4] though the HIV infection has a highly variable latent period of disease that averages 9–10 years in length [5, 6]. In about 80% of HIV infected individuals, AIDS develop within the median time of 10 years, thus are referred to as typical progressors (TP), in about 10% it develops within 2–3 years of infection “rapid progressors” (RP) [7]. It has also been observed that about 5–10% of infected people remain asymptomatic for about 7–20 years after infection despite being on no anti-retroviral therapy. Their immune function is well controlled with CD4+ lymphocyte counts above 600/mm³ and also a low plasma HIV-mRNA.

High viral load (HIV mRNA level) has been strongly associated with the heterosexual transmission as noted in HIV serodiscordant sexual partners of African origin. However, transmission was shown to be rare at viral load <1,500 copies/ml [8]. The higher the concentration of human immunodeficiency virus in the blood stream of an individual the greater the risk of transmitting the virus to a

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sexual partner during heterosexual intercourse. Consequently, it has been demonstrated that the no of copies (1,000 copies/ml as being potentially infectious threshold) of messenger ribonucleic acid (mRNA) in the blood, an indicator of viral load was a better predictor of transmission of HIV when compared to other factors, such as host, viral factors and the presence of sexually transmitted diseases [9]. Present data indicates that in a heterosexual encounter in which one of the sexual partners is HIV-positive, the probability of transmitting the virus to an uninfected partner is 0.11%, according to data analysis from rural Rakai, Uganda [10].

Some studies (11–14) from three distinct laboratories renewed the optimism in finding a predictive test for HIV infection. Separately they generated sets of data to support the fact that increased expression of HIV messenger RNA (mRNA) in peripheral blood mononuclear cells predicts HIV infection and progression to AIDS. Indeed, detection of HIV mRNA in peripheral blood mononuclear cells (multiply spliced) has been found to strongly predict HIV infection and progression to AIDS. Although, generally correlating with the immunologic status of the person being tested, the results of this test had a predictive power independent of CD4 cell counts. However, data associating lipid peroxidation to HIV mRNA and T-cell ratio as a predictive power of the infection process and progression rate is rare.

Oxidative stress and over production of inflammatory cytokines has also been shown to increase disease progression of HIV/AIDS patients. This may result from poor nutrition (mostly diet deficient in antioxidants) [15], psychological stress [16], chemical toxicity from dye and environment [17], and microbial activity (from multiple or concurrent infections). The lipid peroxidation index malondialdehyde (MDA) is used as a measure of oxidative stress in cells. Lipid peroxidation being a free radical reaction occurs when hydroxyl radicals possibly oxygen reacts with the unsaturated lipids of bio-membranes resulting in the generation of lipid peroxide radical ($\text{ROO}^\bullet$), lipid hydroperoxide (ROOH) and fragmentation products such as Malondialdehyde (MDA) [18, 19]. To infect cells, the membrane enveloped HIV transfers its genome both across the viral and cellular membranes, causing them to fuse and provide portal of entry [20]. Membrane lipids therefore are required for HIV-entry. Peroxidation of lipids can have numerous effects including; increased membrane rigidity, decreased activity of membrane bound enzymes, altered activity of membrane receptors (sodium pumps), altered permeability (increased permissibility), formation of hydrophobic centers which, may approach the external phase, alteration of protein structure, mutagenicity (resulting from DNA/RNA damage or bonding), and inhibition of growth and protein synthesis. This is due to the high nucleophilic properties, which enable them to react with

electrophilic sites such as amino and thiol groups. Reactive oxygen species (ROS, hydroxyl radicals ($\text{HO}^\bullet$), superoxide anions ($\text{O}_2^\bullet$), nitric oxide (NO) and hydrogen peroxide (H_2O_2), are constantly generated in aerobic organism in response to both external and internal stimuli [21].

In this study we hypothesized that lipid peroxidation may have a threshold in the effective HIV infection process and disease progression thus, may serve as a potent predictive tool for HIV infection amongst black populations of African origin.

Methods

Sample Collection and Identification

Serodiscordant heterosexual partners recruited for this study were from HIV centres in South-south and North-central Nigeria, both in government and religiously owned voluntary confidential counselling and testing centres. The partners were recruited from those undergoing HIV screening for the first time, either as a pre-requisite for marriage consummation or for medical diagnosis and also from individuals who on routine HIV-status check-up (in which case they were requested to bring along their sexual partners for HIV screening). In all the partners, sexual activity was confirmed through oral discussion and counselling. Venipuncture heparin anticoagulated peripheral blood (PB) samples from a total of 59 individuals were obtained after informed consent according to the Nigerian National Ethics and Operational Guidelines for Research on Human subjects (NNEOGRHS). All of the partners had had an unprotected sexual relationship for at least 6 months [6] and presented no other risk factor than sexual exposure to the HIV-infected partner. They are antiretroviral naive.

Study Design

The case-control study had fifty-nine (59) samples comprising 27 serodiscordant partners ($n = 44$) and 15 healthy normal individuals of average age 37 ± 6 . They were assigned into three groups; [serodiscordant seronegative (SSN) and serodiscordant seropositive (SSP)] and seronegative healthy individuals (SNH).

HIV Screening and Confirmation

The samples (plasma) collected were screened at various centres mention earlier to detect antibodies for HIV-1 and 2 using the determine test kit (Abbot determine HIV-1/2 kit, Abbot, Japan) and confirmation by enzyme immuno assay method (ImmunoComb 11, version 434/Eb, Organic, Israel).

Assays

HIV mRNA

Extraction

NucliSens Magnetic extraction method was used. This is based on Boom chemistry [22], which uses magnetic silica particles (silicon dioxide particles). The sample was lysed using guanidine thiocyanate lysis buffer and a magnetic extraction instrument (NucliSens miniMAG) was used for the extraction. These particles (silica) act as a solid phase and non-nucleic acid components removed by several washing steps performed in the NucliSens miniMAG. Nucleic acid components were then eluted from the solid phase.

Amplification and Detection

Nucleic acid based amplification and detection assay (NASBA) (NucliSens EasyQ HIV version 1.1, Netherlands) was used. For pre-amplification, 5 µl of the extracted sample was added into each tube. Ten microliter (10 µl) of the primer mix (primer acusphere: synthetic molecular beacon probes, synthetic primers, dithiothreitol, nucleotides, KCl and MgCl₂) was lyophilized and 90 µl of the primer diluent (DMSO) added onto the bottom of each tube containing the eluate, this was immediately vortexed until clear. A fresh tip was used for every sample. Incubation was done at 41°C. Five microliter (5 µl) enzyme solution [Avian Myeloblastosis virus reverse transcriptase (AMV-RT), RNaseH, T7-RNA polymerase, XCFF, BSA] diluted with γ-irradiated water was added and centrifuged for 2 s using Mini-Strip centrifuge. Amplification was achieved by using NucliSens Easy analyzer. This was monitored using programmed CD-ROM software.

Lipid Peroxidation

Lipid peroxidation product was assayed by the method of Wallin [23]. Thiobarbituric (TBA) reacting substances, in this case malondialdehyde (MDA) reacts with TBA to give a red/pink colour, which absorbs maximally at 532 nm. TBA-reacting substances were quantified as lipid peroxidation product by referring to a standard curve prepared using 1,2,3,3, tetraethoxypropane.

Calculation of T-cell Ratio

The ratio of T-lymphocytes was calculated as CD4/CD8. However, the CD4, CD3 and CD8 lymphocytes were determined by a FACScan flow cytometer (Becton Dickson Immunocytometry systems, San Jose, CA, USA). A three

tube two colour panel assay containing the reagents, fluorescein, isothiocyanate (FITC), FITC–mouse immunoglobulin, PE-mouse immunoglobulin and phycoerythrin was used. Fifty microlitre (50 µl) of whole blood and 20 µl of monoclonal antibodies were added to a tru-count tube, this was incubated for 20 min before adding 450 µl of lysing reagent (FACS lysing solution). All tubes were analysed using a FACScan flow cytometer. The two colour data were analysed using cell quest software. The result was expressed in cell U/l.

Statistical Analysis

Data were analyzed with a computer using SPSS version 17.0 (SPSS, Chicago, IL, USA) software package. Differences between means were assessed using analysis of variance (ANOVA). The acceptable level of significance was $P < 0.05$. Correlations were evaluated by Spearman's correlation significant at $P < 0.01$. Bonferroni correction factor was used to adjust values owing to the sample size used.

Results

HIV mRNA Concentration

Expression of HIV mRNA depicts the viral load of the subjects. The expression level of HIV mRNA in the three groups was shown in Table 1. In serodiscordant seronegative (SSN group) and seronegative healthy individuals (SNH group) HIV mRNA was not detected (lower detection limit). However, serodiscordant seropositive individuals (SSP group) showed a significant ($P < 0.05$) high expression level of HIV mRNA.

Concentration of MDA

MDA concentration (a lipid peroxidation product) detected by thiobarbituric acid reacting substances gives a measure of oxidative stress condition of the cell. The concentration of MDA (Table 2) was significantly ($P < 0.05$) elevated in SSP by 116.6% over the SSN group. However, comparison of SSN and SNH subjects revealed that the MDA concentration insignificantly ($P > 0.05$) increased in the SSN when compared with that of SNH by 12%.

Table 1 Plasma concentration of HIV mRNA

Group	mRNA (IU/ml)
SSN	<LDL (lower detection limit)
SSP	9800 ± 900
SNH	<LDL (lower detection limit)

Table 2 MDA concentration in SSN, SSP and SNH groups

MDA	SSN	SSP	SNH
Mean (μg/ml)	0.86 ± 0.3 ^a	1.85 ± 0.85 ^b	0.90 ± 0.1 ^a
Relative % mean	23.6 ± 2.3	49.9 ± 4.2	26.5 ± 1.92
% difference	–111.6*, –127**	87.6**	

Means with different superscript in the row are statistically significant ($P < 0.05$)

* % difference with respect to SSP group (seropositive control)

** % difference with respect to SNH group (seronegative control)

Table 3 Mean values of CD4, CD8, and CD3 positive T lymphocytes

T-lymphocyte	SSN	SSP	SNH
CD4	1348 ± 122 ^a	400 ± 108 ^b	1390 ± 121 ^a
CD8	1928 ± 120 ^a	795 ± 66 ^b	1140 ± 145 ^c
CD3	3442 ± 98 ^a	1001 ± 51 ^b	2800 ± 140 ^c

Means with different superscript in the row are statistically significant ($P < 0.05$)

Table 4 T-cell ratio

T-cell ratio	SSN	SSP	SNH
Mean	0.7 ^a	0.5 ^b	1.2 ^c
Relative % mean	29.2	20.8	50.2
% difference	+40*, –71.4**	–140**	–

Means with different superscript in the row are statistically significant ($P < 0.05$)

* % difference with respect to SSP (seropositive control)

** % difference with respect to SNH group (seronegative control)

Fig. 1 Shows the correlation of individual mRNAs and MDA

T- Lymphocytes (CD4, CD3 and CD8) and T-cell Ratio

The T-cell ratio was calculated as a ratio of CD4 and CD8 cells in the three groups. The results however (Tables 3 and 4) show a significant ($P < 0.05$) T-cell ratio increase in the SSN group by 40% when compared to that of SSP group. Comparison of SSP and SNH were significantly ($P < 0.05$) decreased by 140% in SSP group (Table 4).

Correlation

Correlation of MDA concentration and mRNA expression level (Fig. 1) in the serodiscordant-seropositive subjects showed a positive correlation ($P < 0.01$, $r = 0.89$). However, the correlation between MDA and T-cell ratio (Fig. 2) in the same subject was negative ($P < 0.01$, $r = -0.96$).

Discussion

We have correlated HIV mRNA and lipid peroxidation (measured as MDA) in 44 HIV serodiscordant heterosexual partners. This was done mainly with the view to determining the implication of oxidative stress (OS) in HIV Infection process and disease progression. Though, several other theories namely, viral and host factors have been proposed to explaining HIV natural resistance in some individuals. HIV replicative capacity (RC) has been studied as a component of viral fitness. This is a measure of the ability of the virus to replicate successfully in a given environment [24]. In long term non progressors (LTNPs) a

Fig. 2 Shows the correlation of individual MDA and T-cell ratio

shows negative correlation ($r=0.96$ at $P<0.01$)

number of genetic defects have been associated with the virus. Such documented defects or genetic lesions include the NF- κ B or SPI site and the nef mutant gene within the long terminal repeats of the virus. Some LTNP have been shown to possess this mutant gene (nef) however, this is rare in people of African origin [25].

A number of host factors may also influence HIV disease progression. Variations in HIV coreceptor molecules, notably cysteine cysteine receptor no. 5 (CCR5) (responsible for penetration and entry of HIV), influences both HIV susceptibility and disease progression. A mutant allele of CCR5 with a 32-base-pair deletion (CCR5-delta-32) has been found in the highly exposed group of seronegative individuals who are naturally resistant to HIV [26]. This is frequent in populations of European origin (15–20% of Caucasians are heterozygous, and 1% are homozygous), and encodes a non-functional truncated protein that is not transported to the cell surface. Those homozygote for the delta-32 allele exhibit a strong, complete, resistance to HIV infection, whereas heterozygotes delay progression to AIDS as seen in LTNP [27]. However, this mutation does not occur in populations of African and some Asian origins. From previous studies, vigorous virus-specific humoral and cell mediated immune responses have been detected in HIV challenged cells. High titers of potent neutralizing antibodies have been found in sera of LTNP's with strong CD8+ cytotoxic T-lymphocyte cells [28, 29]. Other host factors include members of the APOBEC family of cellular polynucleotide cytidine deaminases (e.g., APOBEC3G) which are potent inhibitors of HIV infection [30, 31]. However, these failed to give a holistic basis for such variation in HIV infection mostly, in black Africans that are naturally resistant to the virus. Other lipid peroxidation markers such as isoprostanes and 4-HNE have been

linked mostly in neurodegenerative diseases or neurologic conditions [32–34].

Increased lipid peroxidation has been severally reported in HIV infection [35–38]. However, the 116.6% increase of MDA found in the HIV seropositive subjects in this study may suggest an overwhelming lipid peroxidation process which consequently indicates high oxidative stress condition. The increased concentration of MDA values in HIV serodiscordant seropositive subjects thus observed is in consonance with the normally expected response that results in increased neutrophils and leucocytes that supposedly occur during acute HIV infection. This increase then indicates that excess reactive oxygen species (ROS) may have been generated in part by the respiratory bursts that occurs in neutrophils. Moreso, the production of oxidants may have resulted from the stimulatory effects of gp 120 (a HIV glycoprotein) and tat (the HIV viral-transactivator protein) during the course of infection, as Westendorp et al. [39] reported that HIV oxidant production is through the stimulatory effects of gp120 and tat, the viral transactivation protein. The increase in OS may have increased HIV replication as observed in the serodiscordant-seropositive partners. This could be through the tumor necrosis factor-alpha (TNF- α) induction possibly by H_2O_2 (an oxidant) which, activates Nuclear factor- κ binding (NF- κ B). NF- κ B promotes HIV replication indirectly by activation of gene products that further promote OS formation. Droge et al. [40] noted that OS increases the replication of HIV and the amount of certain cytokines, among them TNF- α through the activation of NF- κ B. We have to mention here the numerous reports on increased oxidative stress condition in HIV infection, both on anti-retroviral therapy (ART) patients which, was reported to increase protein oxidation and preexistent OS and also on

ART naïve subjects, these reports were mostly on the HIV disease progression [41, 42]. Suresh et al. [43] observed that the total antioxidant capacity (TAC) and lipid peroxidation evaluated in HIV-1 seropositive patients show severe oxidative stress conditions when compared with controls, and increased significantly with the progression of disease in HIV-1 symptomatic and asymptomatic subjects. However the distinguishing aspect of our work implies the use of the biochemical entities (MDA and mRNA) as a potent marker of HIV infection process as well as the progression of the disease using serodiscordant heterosexual partners of black origin as subjects.

Measurement of HIV mRNA is usually done using the polymerase chain reaction (RT-PCR) [44]. Detection and Measurement of cell-associated RNA species using reverse transcription and the polymerase chain reaction (RT-PCR) is not now routinely done in hospitals or commercial clinical laboratories. One significant question is whether this technology can be successfully transferred to the clinical laboratory. This however necessitated our use of nucleic acid based sequencing method for the study. Also, the need to handle cells and process labile RNA molecules complicates the task. It is of note that alternative tests capable of measuring viral RNA within circulating virions in plasma are becoming more widely available. According to Dewar et al. [45], NASBA has been introduced into medical diagnostics, where it has been shown to give quicker results than PCR, and it can also be more sensitive [46–48]. The NASBA technique has been used to develop rapid diagnostic tests for several pathogenic viruses with single-stranded RNA genomes, e.g. influenza A [49], foot-and-mouth [50]. However, the elevated HIV mRNA (9800 ± 900 IU/ml) and MDA concentration (1.82 ± 0.5 µg/ml) observed in the SSP subjects in this study probably support the assertion that OS increases HIV replication. The positive correlation of MDA and mRNA ($P < 0.01$, $r = 0.89$) in SSP subjects indicates a linear relationship. This tends to show that lipid peroxidation (OS) may facilitate HIV replication, thus the viral infection process.

Owing to the increased replication of HIV observed, the serodiscordant-seropositive subjects were unable to fight off the infection thus leading to establishment of acute HIV infection. Conversely, the serodiscordant-seronegative partners which, had an undetectable HIV mRNA and low MDA concentration, shows an effective immune system clearance of the virus and also an ineffective replication of HIV. This was further substantiated by the inverse relationship of helper/suppressor T-cell expressed as T-cell ratio. The T-cell ratio results (0.7, 0.5, 1.2) was consistent with some previously laboratory determined values. The result shows a decline in the T-cell ratio of the SSP subjects by 40% over their SSN partners and by 140% when compared to that of SNH subjects. This shows that there are

more CD8+ than CD4+ cells in the SSP and SSN groups when compared with the SNH group. As a measure of the immune status of an individual, this result suggests a stronger immune response to HIV infection in the SSN over their SSP partners. These results support the hypothesis that lipid peroxidation plays a role in the immunological abnormalities observed in experimental cells [51].

Therefore, the negative correlation ($r = -0.96$) of MDA and T-cell ratio in the SSP may further substantiate the strength of MDA as a possibly marker of immune system status in the HIV infection process.

In conclusion, we demonstrated here for the first time an increase of 116.6% of MDA in HIV serodiscordant-seropositive subjects over their seronegative partners thus indicating that increased OS (MDA) may play a significant role both in the infection process and progression of HIV/AIDS disease. However, the activities of tumour necrosis factor-alpha (TNF- α) induction, activation of NF- κ B and their role in activating gene products responsible for OS needs to be further investigated in serodiscordant HIV subjects.

Conflict of Interest There is no potential conflict of interest.

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