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The Effect of HIV and Antiretroviral Therapy on Chromosomal Radiosensitivity

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Abstract

Introduction: Antiretroviral Treatment (ART) has led to an improvement in survival of HIV infected individuals. Some of them will develop cancer during the course of their infection and will require radiation therapy. HIV positive cancer patients have presented with adverse side effects of radiotherapy and elevated chromosomal radiosensitivity. This study investigated if ART has an influence on chromosomal radiosensitivity of HIV positive individuals.

Methods and Materials: Blood samples from 60 HIV positive individuals were *in vitro* exposed to doses of X-rays of 0, 2 and 4Gy and chromosomal radiosensitivity was assessed with the micronucleus assay. The micronucleus assay was also performed on lymphocytes of a group of non HIV-infected health care workers taking prophylactic post-exposure ART to measure the effect of these ART drugs on chromosomal radiosensitivity without HIV as a confounding factor.

Results: All HIV patients (those on ART and without ART) had significantly higher radiation induced Micronuclei (MN) than healthy controls. The MN yields increased in the HIV patients taking ART compared to HIV patients not on treatment. The evaluation of chromosomal radiosensitivity of health care workers on ART revealed no effects of ART.

Conclusions: HIV positive individuals show an increased chromosomal radiosensitivity. Antiretroviral treatment given to HIV positive individuals can lead to enhanced chromosomal radiosensitivity and therefore impose higher risks for radiotherapy side effects in these patients.

Keywords: Antiretroviral treatment; HIV; Chromosomal radiosensitivity

Introduction

In 2013, there were 5.6 million people living with Human Immunodeficiency Virus (HIV) in South Africa [1]. Antiretroviral Therapy (ART) has led to the improvement in health and survival of patients with HIV [2]. South Africa has one of the largest ART roll-outs in the world and in 2013 over 2 million South Africans were on ART [2]. The 2006 World Health Organisation's guidelines for treatment of HIV infection in adults recommended a first-line regimen of two Nucleoside Reverse Transcriptase Inhibitors (NRTIs) combined with one Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) [3]. Due to their cost effectiveness, Stavudine (d4T) and Zidovudine (AZT) were the most commonly prescribed NRTIs in low and middle income countries [4], however due its toxicity, the WHO updated guidelines recommended using Tenofovir (TDF) instead of d4T in first-line therapy [5]. In 2010 the South African Department of Health changed it guidelines to recommend TDF+lamivudine (3TC)/emtricitabine (FTC)+efavirenz (EFV) as first-line therapy for all new HIV patients and for those on d4T+3TC/FTC+EFV without toxicity to remain on that regimen [6]. According to the WHO 2013 guidelines [7] all patients should be switched to the TDF-based regimen and the transition of patients to a fixed-dose combination one-pill ART regimen containing TDF+FTC+EFV began in South Africa in 2013 [8].

ART is not only used in treatment of HIV but also in prevention. ART is administered prophylactically to non-infected persons after occupational exposure to HIV. A study in Health Care Workers (HCWs) has shown post-exposure prophylaxis to be protective after exposure to the HIV virus [9]. With the high incidence of HIV in South Africa, HCWs face constant risk of being exposed to HIV after being cut or pricked by sharps during routine patient care. A South African study showed that 69% of medical interns reported at least 1 percutaneous injury during their year-long internship [10]. The WHO guidelines recommend a combination of antiretroviral drugs for post-exposure prophylaxis based on two nucleoside reverse transcriptase inhibitors [11].

HIV infection is associated with a high risk of developing specific AIDS-related cancers such as non-Hodgkin lymphoma, Kaposi sarcoma and cervical cancer [12]. The prolonged survival of HIV infected individuals, largely owing to improved ART access, has also led to an increasing incidence of non-AIDS related cancers amongst these individuals [13,14]. Radiotherapy is a mainstay treatment for cancers in South Africa where cancer patients, many of which will be HIV positive, often present with advanced disease due to limited access to diagnostic centers in rural areas, lack of awareness and lower standards of healthcare facilities. As South Africa is also a country with

a prolific radiation industry and many radiation workers, the high number of HIV infected people and taking ART in the general South African population is also likely to be reflected in this workforce. The effect of HIV and ART on chromosomal radiosensitivity can have important implications for radiation protection in these workers.

A pilot study by Baeyens et al. (2010) has previously shown HIV positive patients to be more chromosomally radiosensitive than HIV negative controls [15]. Chromosomal radiosensitivity is increased vulnerability of cells to the DNA-damaging effects of ionizing radiation. It can be a result of impaired DNA damage repair. Associations between clinical response to radiotherapy and chromosomal radiosensitivity [16,17] and elevated radiotoxicity such as mucositis and neutropenia has been reported in some HIV patients receiving radiotherapy for several types of cancers (reviewed in [18]). As radiotherapy decreases CD4 cell counts [19], HIV positive people in clinical settings are advised to start ART before undergoing radiotherapy to minimise the risk of developing AIDS, particularly when the patient has low CD4 counts.

Since NRTIs and NNRTIs interact with host DNA, it is possible that they could have an impact on DNA damage repair and affect chromosomal radiosensitivity. AZT, 3TC and TDF have been shown to inhibit cell growth [20], to interfere with cell cycle arrest, induce cell death by apoptosis and inhibit telomerase activity [21-24]. Conflicting studies have shown AZT to act as a radioprotector or a radiosensitizer [25,26].

Chromosomal radiosensitivity can be tested through a variety of cytogenetic assays. A reliable and well-established method is the cytokinesis-block micronucleus assay (MN assay) that is commonly used in biological dosimetry and chromosomal radiosensitivity studies. Micronuclei (MN) are small nuclei that form in the cytoplasm when chromosomes or chromosome fragments are not incorporated into the daughter nuclei subsequent to cell division [27]. MN reflect the amount of DNA damage and DNA damage repair after exposure to radiation. The MN assay can be performed on lymphocytes, which are easily obtainable via venipuncture. The micronucleus assay is also considered a sensitive method to evaluate the cytotoxic potential of an active agent. The nucleoside reverse transcriptase inhibitors AZT, 3TC and d4T have been shown to produce an increase in MN in human lymphocyte cultures [28-30].

In this study the micronucleus assay on the lymphocytes of HIV patients not taking ART and of HIV patients on one of the 2 most commonly administered ART regimens in South Africa (d4T+3TC +EFV and TDF+3TC+EFV) was performed to assess the effect of HIV and these drugs on chromosomal radiosensitivity. The micronucleus assay on the lymphocytes of a group of non HIV-infected health care workers taking prophylactic post-exposure ART was also performed to measure the effect of these ART drugs on chromosomal radiosensitivity without HIV as a confounding factor.

Materials and Methods

Study population

Heparinised blood samples from 60 HIV positive individuals were obtained from the Themba Lethu HIV unit at the Helen Joseph Hospital in Johannesburg, South Africa. Twenty participants were HIV positive but not taking any ART medication. Twenty participants were on the d4T+3TC+EFV (30mg/150mg/600mg) regimen and twenty participants were taking the TDF+3TC+EFV (300mg/150mg/600mg) regimen. HIV patients were on ART for at least 6 months. Blood samples of 20 HIV negative healthy individuals, age-matched with HIV positive individuals, were included as controls. These were staff members and students from Charlotte Maxeke Johannesburg Academic hospital (CMJAH) where the study was undertaken. Population characteristics and treatment data are shown in Table 1. The CD4 counts, measured on the same day as the MN assay, were extracted from clinical records.

			HIV ART	HIV ART	
	Healthy individuals	**HIV without ART**	**D4T/3TC/EFV**	**TDF/3TC/EFV**	**Health care workers**
Gender					
female/male	14/6	15/5	13/7	18/2	8/2
Age					
mean ± SD	30 ± 7	36 ± 10	37 ± 7	41 ± 6	31 ± 11
range (min-max)	23 - 52	18 - 55	29 - 54	34 - 53	22 - 56
CD4 cell counts					
mean ± SD		480 ± 164 **	623 ± 240	649 ± 204	
range	(500 - 1200)*	268 - 858	202 - 1081	302 - 933	
Duration of ART (months)					
mean ± SD			51 ± 26	51 ± 28	1
range			13 - 100	6 - 99	
* normal range of CD4 counts in a healthy population					

** significantly lower than HIV ART groups

Table 1: Patient Characteristics.

For the second part of the study, heparinised blood samples of 10 health care workers on post-exposure ART prophylaxis were collected from CMJAH. All the HCWs were taking a combination of 2 NRTIs (300mg AZT + 150mg 3TC or 200mg FTC + 300mg TDF) for 28 days. Blood samples of these HCWs were *taken* at 3 time points: The first was the day of the needle-stick or sharps injury, just before starting prophylaxis treatment (T1); the second was 3-4 weeks after T1, after completion of the ART course of medication (T2); the third was between 3 and 4 months after T1 (T3). All blood donors signed informed consent and the study was approved by the Human Research Ethics Committee, University of Witwatersrand, Johannesburg, South Africa (M110361 and M120270).

Micronucleus assay

The micronucleus assay was performed as described by Baeyens et al. [15]. *In vitro* chromosomal radiosensitivity was measured by scoring MN in lymphocytes irradiated with doses of 2 Gray (Gy) and 4 Gy. A 2 Gy dose is frequently used in radiotherapy fractionation schedules. A 0 Gy dose was used as a sham-irradiated control (spontaneous MN) and also allowed us to examine the cytotoxic effect of ART on lymphocytes without irradiation. Lymphocyte cultures were set up by adding 0.5 ml blood to 4.5 ml of prewarmed RPMI (Roswell Park Memorial Institute) 1640 (BioWhittaker, Walkersville, USA) that was supplemented with 13% foetal bovine serum (Gibco-Invitrogen, New York, USA) and 0.01% antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin; Gibco-Invitrogen). The irradiations were done in the Radiation Oncology Unit at CMJAH. Culture flasks were placed in a Phantom-water tank at room temperature and irradiated with X-rays using a 6 MV photon beam from a medical linear accelerator (Siemens Healthcare, Erlangen, Germany). The distance from the culture flasks to the radiation source was 100 cm at an angle of 90 degrees. The field size at the depth of the sample was 10X10 cm and the dose rate was approximately 1.33 Gy/min. For each dose point, 2 co-cultures were set up. Subsequent to irradiation, lymphocytes were stimulated by adding 100 µl of phytohaemagglutinin (stock solution 1mg/ml, Sigma-Aldrich, St Louis, MO, USA). To block cytokinesis, 20 µl cytochalasin B (stock solution of 1.5mg/ml, Sigma-Aldrich) was added to the cultures at 23hrs. Cells were harvested 70hrs after stimulation using a cold (4°C) hypotonic shock with 7ml 0.075M KCl (Merck, Darmstadt, Germany). This was followed by fixation in methanol: acetic acid: Ringer (0.9% NaCl) solution (4:1:5) (Merck) at 4°C overnight. Thereafter cells were fixed another three times with methanol: acetic acid (4:1) (Merck). Cell suspensions were dropped on slides and stained for 1 minute with acridine orange stain (10µg/ml) (Sigma-Aldrich, St Louis, MO, USA) and immersed for 1 min in acridine orange buffer (Sigma-Aldrich, St Louis, MO, USA). Duplicate slides were made of each sample, coded and scored blindly by 2 experienced scorers using a Zeiss Axioskop fluorescent microscope (400X) (Carl Zeiss, Gottingen, Germany). At least 500 binucleated cells (BN) were scored per slide, according to the criteria of Fenech [31]. The results of both scorers were combined and normalised to 1000 BN. The number of mononucleated (N_1), binucleated (N_2), trinucleated (N_3) and polynucleated (N_4) cells per slide was scored and the nuclear division index (NDI) was calculated according to the formula: NDI= $(N_1+2N_2+3N_3+4N_4)$/Ntot where Ntot is the total number of cells per slide counted (Ntot = 500 cells).

Statistical analysis

Statistical analysis was performed with Graph Pad Prism 6. The non-parametric Mann-whitney test was used to compare means of MN counts and NDI values between the different population groups. For HCWs, differences between MN counts at different time points were tested for significance with the non-parametric Wilcoxon test. Significance was set at $p<0.05$.

Results

Effects of ART on chromosomal radiosensitivity in HIV positive individuals

The results of the MN assay for the four groups (HIV patients not on treatment, HIV individuals on ART regimens TDF+3TC+EFV and D4T+3TC+EFV and healthy controls) are shown in Table 2. In unirradiated cells (0Gy), there were no significant differences in spontaneous mean MN yields between the groups. Radiation-induced MN yields were calculated by subtracting spontaneous MN yields from MN yields in irradiated cells. The radiation-induced MN yields were significantly higher in patients (both regimens and without ART) compared to healthy controls at both 2Gy and 4Gy. Although both patient groups on ART had higher MN yields than HIV patients not on treatment, patients on TDF+3TC+EFV had the highest average MN yields, the increase being significant at 4Gy. The HIV patients without ART had significantly lower CD4 counts compared to those on ART. There was no correlation between CD4 counts, time on ART treatment and MN yields (data not shown). The mean NDI values for the unirradiated (0Gy) cultures were similar for all the groups, but the mean NDI for the irradiated cultures (2Gy and 4Gy) were significantly lower in the patients on ART compared to the HIV patients without ART and the healthy controls ($p<0.005$).

				HIV ART	HIV ART
		Healthy individuals	HIV without ART	D4T/3TC/EFV	TDF/3TC/EFV
0Gy	N	20	20	19	20
	Mean	14	14	12	10
	SD	6.4	6.0	7.4	5.8
2Gy induced	N	20	19	18	18
	Mean	300	355	368	386
	SD	58.3	71.4	58.9	58.2
	p values vs controls		0.0125	0.001	<0.0001
	p values vs HIV without ART			0.5414	0.1531

4Gy induced	N	20	18	16	19
	Mean	842	969	1018	1076
	SD	151.6	134.6	157.8	146.6
	p values vs controls		0.0098	0.0017	<0.0001
	p values vs HIV without ART			0.3407	0.0277

Table 2: MN yields (per 1000BN) for the HIV positive individuals not taking ART, HIV positive individuals taking 2 different ART regimes and healthy individuals.

Effect of ART on chromosomal radiosensitivity of health care workers

The MN yields for the 10 health care workers on prophylactic ART before starting ART (T1), at the completion of ART (T2), or 3-4 months after the completion of ART (T3) are shown in Table 3. There were no significant differences between the mean spontaneous and radiation-induced MN yields at the 3 measured time points.

		T1	**T2**	**T3**
0 Gy	Mean	10.3	13.4	10.9
	SD	3.5	5.2	4.4
	p values		0.234	0.836
2 Gy	Mean	307.7	297.1	296.1
	SD	90.9	78.8	82.7
	p values		0.625	0.547
4Gy	Mean	826.2	842.0	834.0
	SD	146.2	206.0	192.2
	p values		0.943	0.844

Table 3: Comparison of MN yields (per 1000BN) of health care workers taking prophylactic post-exposure ART before starting ART (T1), after 3-4 weeks on ART (T2) and 3 months after ART (T3).

Discussion

The present pilot study investigated if HIV and antiretroviral medication has an influence on chromosomal radiosensitivity. This is one of the first studies looking at the effects of ART on radiosensitivity in HIV infected individuals and is potentially very important for many cancer patients and radiation workers in South Africa exposed to radiation that will be HIV positive and on ART. Since NRTIs interact with host DNA, inhibit mammalian DNA polymerases and affect the cell cycle [21], it is possible that they have an impact on DNA damage repair and affect chromosomal radiosensitivity. Some studies have shown antiretroviral drugs to be radioprotective while others have suggested a radiosensitising effect [25,26]. For the first part of this study we investigated the chromosomal radiosensitivity of HIV patients not on ART, HIV patients on two common ART regimens (d4T+3TC+EFV and TDF+3TC+EFV) and compared it to healthy uninfected controls.

Micronuclei are considered a biomarker of cytotoxicity. In unirradiated cells no difference in MN yields between the four groups was detected, suggesting no cytotoxicity of the ART *in-vivo*. These findings are in agreement with those of Robbins et al. [32] who detected no biologically relevant changes in lymphocyte chromosomal aberrations in 26 HIV positive patients on combinations of ART (AZT, 3TC, d4T) across a number of time points. On the contrary, Shafik et al. (1991) [33] observed a high increase in chromosomal aberrations in AIDS patients treated with AZT and suggested that AZT induces genetic damage in lymphocytes of AIDS patients. None of the studied HIV positive patients were taking AZT, so this could explain the discrepancy between Shafik et al. (1991) [33] and the results presented here. Proliferation rates of lymphocytes from HIV infected individuals are known to be higher than HIV uninfected individuals [34], but this was not reflected in the NDI's calculated in this study. In lymphocytes irradiated with 2 and 4Gy, a difference in MN between the groups was noted that points to differences in chromosomal radiosensitivity. All HIV patients (those on ART and without ART) had significantly higher MN than healthy controls. This confirms earlier findings of Baeyens et al. [15]. The patients on the ART regimens had higher MN yields than HIV patients not on ART, with the TDF+3TC+EFV regimen presenting with significantly higher radiation-induced MN at 4Gy. The results suggest that substitution of d4T with TDF in the ART regimen of HIV patients leads to an enhanced chromosomal radiosensitivity. Cytotoxicity studies on TDF *in vitro* have been conflicting. Cihlar et al. (2002) [35] and Hecht et al. (2013) [36] have shown TDF to exhibit low cytotoxicity in various cell types, while a study by Bruning et al. [23] indicated that TDF induced DNA damage, reduced cell viability and induced cell cycle disturbances leading to apoptosis in cancer cells. This last statement could mean that TDF may be beneficial in radiosensitising tumour cells in HIV positive individuals, but its effect on normal tissue radiotoxicity, seen in the lymphocytes in this study, may be adverse. The significantly higher MN yields at 4Gy in the TDF group could reflect the un/misrepaired, severe DNA damage caused by the combination of HIV-infection, TDF and ionising radiation. Since studies have shown an association between clinical response to radiotherapy and chromosomal radiosensitivity [16,17], the effect of ART, especially TDF, on radiotoxicity requires further investigation.

For the second part of the study, we investigated the chromosomal radiosensitivity of 10 non-HIV-infected South African HCWs on the post-exposure prophylaxis ART to determine the effect of ART on chromosomal radiosensitivity without HIV as a confounding factor. All participants were on an ART regimen including two NRTIs. In unirradiated lymphocytes of these donors, no differences were detected in average MN yields before, during or after ART. The increase in chromosomal aberrations in AIDS patients taking AZT, seen in the study of Shafik et al. [33], was not noted in the HCWs taking AZT. The different results between both studies could suggest that not only AZT but the combination of HIV infection and AZT lead to increased chromosomal damage. The *in vitro* genotoxicity effects of AZT and 3TC seen in studies of Stern et al. [29], Lourenco et al. [28], and Gonzalez Cid et al. [30] were not confirmed in the current study. The fact that in these studies, ART was added in high concentrations for short terms *in vitro* to cell cultures, while in this study the ART was administered *in vivo* over a longer period, could explain the observed difference. No changes were noted in average radiation-induced MN yields of the HCWs at the different time points. Jagetia and Aruna [25], found that administration of AZT to Hela cells before exposure to different doses of γ-radiation resulted in a significant elevation in

the yield of MN. This was in contrast to Copaceanu et al. [26] who suggested radioprotection of AZT in Hela cell lines exposed to irradiation *in-vitro*. Several publications demonstrated that AZT and 3TC alter cell cycle kinetics and increase the proportion of cells in S phase [21,22]. Radiosensitivity of cells differs according to cell type and phase of the cell cycle the cells are in at the time of irradiation [37]. In both studies of Jagetia and Aruna [25] and Copaceanu et al. [26] irradiations were performed on rapidly-dividing Hela cell lines, while in the current study, lymphocytes were irradiated in the G0 phase of the cell cycle.

Following from the MN data on HCWs taking ART, we could not support that either AZT or 3TC have a radiosensitising or radioprotective effect on lymphocytes of healthy individuals. The fact that the increased chromosomal radiosensitivity, seen in HIV positive individuals on ART was not observed in the lymphocytes of HCWs on ART could be due to the following reasons: 1) The duration of the ART was different (more than 6 months for HIV positive patients versus 3-4 weeks in HCWs). 2) It is the ART and the HIV virus in combination with the radiation that led to increased MN yields in HIV-infected patients on ART. 3) It is different ART compounds or the combinations (d4T, TDF and EFV versus AZT) exerting different effects in the cells in response to ionizing radiation.

The results of this study suggest that HIV and certain recent ART (TDF+3TC+EFV) given to HIV positive individuals can lead to enhanced lymphocyte chromosomal radiosensitivity and therefore may impose higher risks for radiation-induced normal tissue complications in these patients. Further investigation of the different compounds of ART in combination with ionizing radiation is warranted, as HIV positive cancer patients undergoing radiotherapy and HIV positive radiation workers may potentially benefit from having their ART regimens altered according to the radiomodulatory effects of the various drugs.

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