MEDICAL TECHNOLOGY SA

The leukocyte apoptosis assay: A clinical predictor of radiosensitivity

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ABSTRACT
Approximately 6% of patients receiving radiotherapy show signs of late toxicity in normal tissue many months after treatment has been completed. The Leukocyte Apoptosis Assay (LAA) has been developed to predict intrinsic radiosensitivity of normal tissue based on the radiation-induced cytotoxic response of CD4 and CD8 lymphocytes. Apoptosis, or programmed cell death, can be observed after radiation exposure and can be evaluated using flow cytometry. The purpose of this study was to determine the reliability of the LAA assay and to standardise it in South Africa. Briefly, heparinised blood was collected and exposed *in vitro* to 0Gy (control), 2Gy and 8Gy gamma radiation. One sample was exposed to 0Gy, 2Gy, 4gy and 8gy gamma radiation and compared to 0Gy, 1Gy, 2Gy, 3Gy and 4Gy neutron radiation. After 48 hours, lymphocytes were collected and prepared for flow cytometric analysis using FITC-conjugated anti-CD4 and anti-CD8 monoclonal antibodies. Apoptosis was assessed by measuring internucleosomal DNA degradation using propidium iodide (PI) staining. Percentage radiation-induced apoptosis was determined for each sample. Results showed a clear dose response curve for both gamma and neutron exposure and apoptosis after neutron exposure was higher than at iso-equivalent gamma doses. Although inter donor variation was observed, intra donor variation was low where the mean SD was 1.61 for CD4 and 2.16 for CD8. Results were consistent across cell type and the study therefore yielded enough data to base radiosensitivity measurements on CD4 and CD8 lymphocytes. In the clinical setting, patients showing a low apoptotic response should be considered at risk for developing late effects whereas patients who do not present as radiosensitive may receive increased doses of radiation. The LAA would be able to identify radiosensitive patients, thereby enabling the oncologist to stratify each patient to plan an effective treatment regime.

INTRODUCTION

Radiotherapy uses various types of radiation to treat patients with malignant disease. The goal of radiotherapy is to destroy malignant tumours while minimising damage to normal tissue, using accurately directed modern linear accelerators (Symonds, 2001). Different treatment modalities, such as gamma and neutron irradiation, are selected depending on the type and location of the tumour. Linear energy transfer (LET) is the rate at which energy is deposited in the form of a charged particle as it travels through matter. This differs for gamma (low-LET) and neutron (high-LET) radiation and for a given charged particle, the lower the LET, the lower the consequent biological effect (Dainiak, 2002).

Radiation as a curative agent also poses the risk of serious damage to normal tissue. Radiation effects on normal tissue are usually divided into early and late reactions (Williams *et al*, 2003). Early reactions include any treatment-related morbidity and occur typically within 90 days after the start of radiotherapy. Characteristic symptoms include mucositis, dermatitis and depletion of the cellular compartments of the bone marrow. Late reactions occur several months or even years after radiotherapy (Williams *et al*, 2003). Typical examples are chronic myelopathy, lung or subcutaneous fibrosis, telangietasia, bone necrosis and radiation nephropathy, most of which are irreversible and may jeopardize the success of therapy. The Radiation Therapy Oncology Group /European Organization for Research and Treatment of Cancer (RTOG/EORTC) classification system was designed to document late toxicity side effects in more detail and classify symptoms and treatment using a six-point scale ranging from nil (0) to fatal (6) (Hoeller *et al*, 2003).

Cells confronted with increasing DNA damage by agents such as radiation, show one of two outcomes, *viz* to repair or to undergo apoptosis. Apoptosis or ‘programmed cell death’ is a physiological cellular response to agents such as radiation and cells undergoing apoptosis will shrink, show degradation of DNA, proteins and mitochondria and break apart into small fragments. Apoptosis involves a protein constellation associated with DNA in the cell and inhibition or mutations of these proteins can result in compromised apoptosis (Crompton *et al*, 2001). Although apoptosis occurs spontaneously in cultured human peripheral blood lymphocytes, it may be enhanced by exposure to ionising radiation and can be quantified using a number of markers.

Crompton and Ozsahin (1997) developed the leukocyte apoptosis assay (LAA) to predict intrinsic radiosensitivity of normal tissue based on the radiation-induced apoptotic response of CD4+ and CD8+ T-lymphocytes. Of significance is that radiosensitive individuals have an abnormality in their ability to recognise or repair DNA double-strand breaks induced by ionising radiation, leading to enhanced toxicity and a predisposition to cancer (Crompton *et al*, 2001). Approximately 6% of patients receiving radiotherapy show signs of late toxicity (RTOG/EORTC scale) many months after therapy has been completed, however as these patients have not been previously identifiable, radiotherapy doses have been conservative.

The purpose of this study was to standardise the LAA assay in South Africa. Donor lymphocytes were exposed to 0Gy (control), 2Gy and 8Gy of gamma radiation. A dose response curve was also plotted to confirm the sensitivity of the assay and exposure to both gamma and neutron radiation was done to compare the apoptotic response between low-and high LET modalities. Intra-nucleosomal DNA degradation was measured using flow cytometry and 10 000 cells or more were rapidly and objectively assessed for the parameter of interest. FITC-conjugated anti- CD4 and anti-CD8 monoclonal antibodies were used to gate the leukocytes of interest and propidium iodide (PI) was used to detect dead cells as it is prevented from entering intact cells (King, 2000).

Should the LAA be able to identify radiosensitive patients, the oncologist would be able to stratify each patient and plan an effective radiotherapy regime. Patients who do not present as radiosensitive could receive a higher dose whereas those presenting as sensitive would have the advantage of receiving alternative treatment options.

MATERIALS AND METHODS

Heparinised blood was collected from 9 volunteer subjects. The blood sample was diluted 1:10 in RPMI 1640 medium containing 20% foetal calf serum and exposed in tubes to 0Gy (control), 2Gy, and 8Gy gamma radiation using the iThemba LABS facilities. One sample was also irradiated at 0Gy (control), 2Gy, 4Gy, 6Gy and 8Gy to obtain a dose response curve. The same sample was also exposed to neutron radiation at 0Gy (control), 1Gy, 2Gy, 3Gy and 4Gy to compare the apoptotic response after exposure to low-LET (gamma) and high-LET (neutron) radiation.

Gamma irradiation was performed using a ^{60}CO g-source (Eldorado 76, Atomic Energy of Canada Ltd), delivering a dose rate of 0.30Gy/min at a source surface distance (SSD) of 70cm, a field size of 30 x 30 cm^2 and a gantry angle of 180°. All tubes were placed side-by-side in the centre of the radiation beam on a 0.5mm thick Perspex sheet with a 5cm thick Perspex block on spacers serving as backscatter material. The dose at the position of the tubes was verified by using a Farmer ionisation chamber.

Irradiated samples were incubated for 48hrs at 37°C, 5% CO2 and then treated for 20 minutes with 20μl of anti-CD4 and anti-CD8 Fluorescein Isothiocyanate (FITC)-conjugated monoclonal antibodies (Becton Dickinson). 4ml FACS Lysing solution (Becton Dickinson) was added to lyse erythrocytes as well as permeabilise leukocyte membranes. The samples were then washed and centrifuged and leukocytes were resuspended in 400μl of FACS Flow (Becton Dickinson). DNA within permeable leukocytes was stained using 5μl of propidium iodide (PI) for 5min before reading.

The assay was standardised at the Cape Peninsula University of Technology (CPUT) and flow cytometry was performed using a FACScan (Becton Dickinson). Briefly, cells flow through a laser beam, which excites the bound fluorochromes, giving green fluorescent emissions from FITC and red fluorescence from the PI and the intensity of colours for each cell is measured. Lymphocytes were identified and gated as region 1 (R1) on a 2D-scatter plot (Fig 1a). A second 2D-scatter plot of antibody fluorescence (FL1-height) vs. cellular DNA content (FL2-height) (Fig 1b) was used to identify either CD4 or CD8 positive lymphocytes in region 2 (R2). A third 2D-scatter plot of cellular DNA content (FL2-height) vs. cell size (FSC-height) was then used to determine the proportion of apoptotic cells in region 3 (R3) (Fig 1c and 1d) by identifying the population of cells with reduced DNA content and slightly reduced cell size. The percentage radiation-induced apoptosis was calculated by subtracting background apoptosis at 0Gy from apoptosis measured at 2Gy and 8Gy.

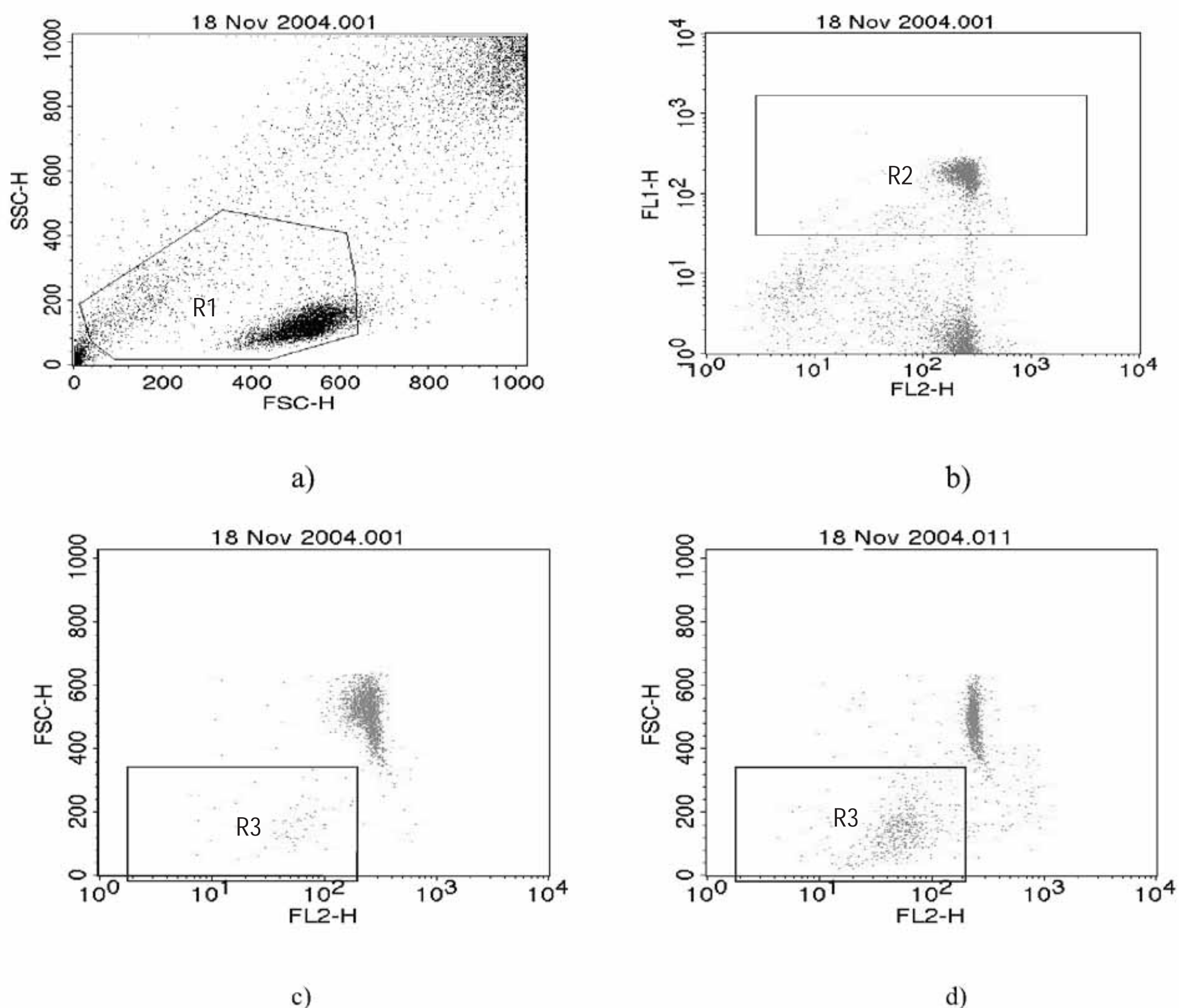

Figure 1. *Flow cytometry standardisation showing selection of all lymphocyte in region 1 (R1) using a forward scatter vs side scatter dot plot* ***(a)*** *; selection of CD 4 lymphocytes (FITC +) in region 2 (R2) using an FL2-H vs FL1-H dot plot* ***(b)****; apoptotic lymphocytes in region 3 (R3) using an FL2-H vs FSC dot plot at 2 Gy* ***(c)*** *and at 8 Gy* ***(d)***.

RESULTS

The age of the 9 volunteer colleagues ranged from 22-58 years with a mean age of 49.6 years. Although inter-donor variation was observed (Fig 2) apoptotic responses were consistent across cell type. As an example, donor 6 showed a low apoptotic response for both CD4 and CD8 lymphocytes at 2Gy and at 8Gy. Table I shows the results of radiation-induced apoptosis for CD4 and CD8 lymphocytes (after the background apoptotic level was subtracted from the total measured apoptosis) following gamma radiation.

Table I. *Percentage radiation-induced apoptosis of donor CD4 and CD8 lymphocytes after background level (0Gy) subtraction.*

Radiation-induced apoptosis for Donors 1-9						
	CD4			CD8		
	0Gy Control	2Gy	8Gy	0Gy	2Gy	8Gy
Donors						
1	7.49	9	16.72	7.98	12.62	30.66
2	8.21	10.6	20.83	13.27	14.76	24.38
3	3.76	9.02	12.96	8.63	16.45	20.45
4	5.6	5.72	15.6	5.82	10.39	34.57
5	7.31	9.42	13.16	12.39	16.8	21.2
6	1.7	2.56	9.28	2.21	2.68	14.47
7	3.69	7.01	19.85	4.81	11	38.77
8	0.73	10.7	13.85	0.59	12.11	24.25
9	3.45	9.04	21.24	4.34	11.46	23.97

The anti- CD4 and anti-CD8 antibodies gave meaningful discrimination as was shown in the standardisation (Fig 1b) and a clear increase could be seen in the apoptotic portion as the dose increased (Fig 1c and 1d). The results for each volunteer donor are presented graphically in Figure 2. CD4 0Gy (control) ranged from 0.73-8.21% (mean 4.61%) and for CD8 0Gy (control) it ranged from 0.59-13.27% (mean 6.82 %). Radiation –induced apoptosis at 2Gy for CD4 ranged from 2.56-10.7% (mean 8.03%) and for CD8 it ranged from 2.68-16.8% (mean 11.77%). Radiation-induced apoptosis at 8Gy for CD4 ranged from 9.28-20.83% (mean 15.83%) and for CD8 ranged from 14.47-38.77% (mean 25.51%). Intra-donor variation was low: mean variation for CD4 was 1.61%, range 0.30-5.37% and mean for CD8: 2.16%, range 0.07-7.21% (Fig 3).

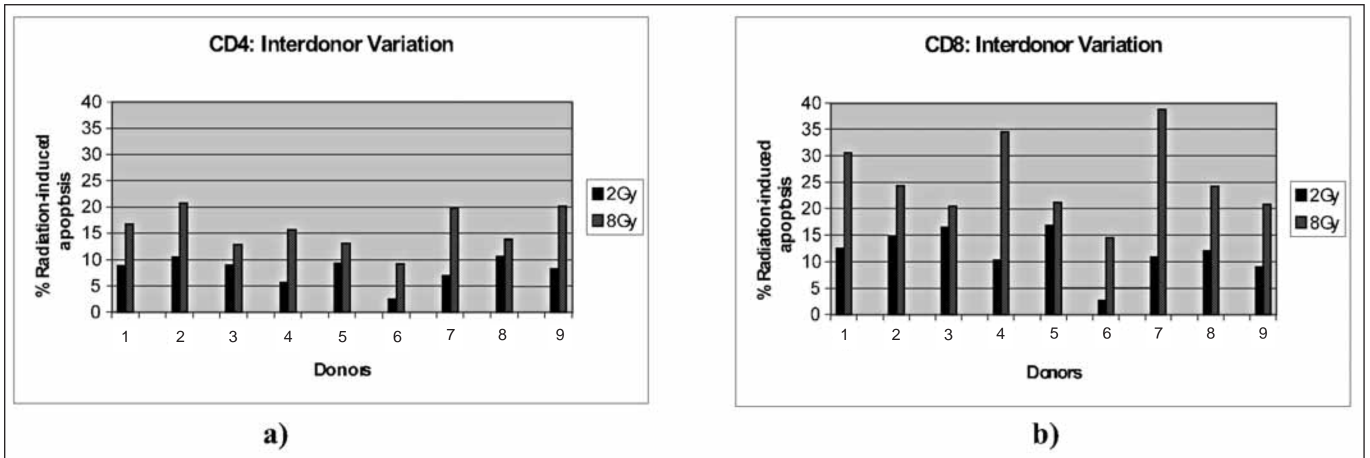

Fig 2. *Inter-donor variation of radiation-induced apoptosis at 2Gy (solid bars) and 8 Gy (striped bars)in CD4 lymphocytes* ***(a)*** *and CD8 lymphocytes* ***(b)****. An increased radiosensitivity was seen in CD8 lymphocytes compared to CD4 lymphocytes.*

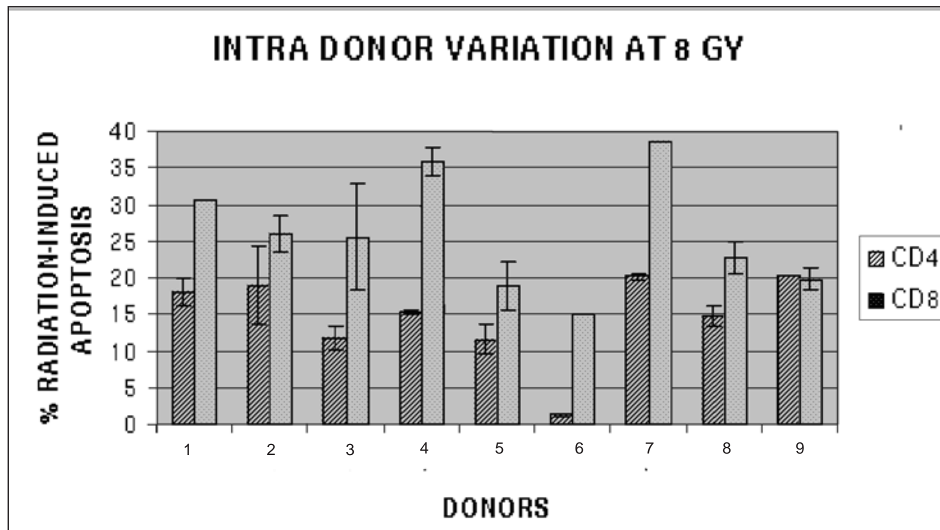

Fig 3. *Intra-donor varaition of 9 donors at 8Gy for CD4(striped bars) and CD8 (dotted bars). Error bars represent 1SD.*

The LAA also showed a clear dose response curve (Fig 4**)**. CD4 was 3.45% at 0Gy and increased to 24.69% at 8Gy and CD8 was 4.34% at 0Gy and increased to 28.31% at 8Gy. This indicates a clear increase in apoptotic response with increasing doses of gamma radiation. Similarly after neutron radiation, a clear dose response curve was observed (Fig 4) as CD4 showed 4.19% at 0Gy and increased to 28.68% at 8Gy, CD8 was 4.85% at 0Gy and

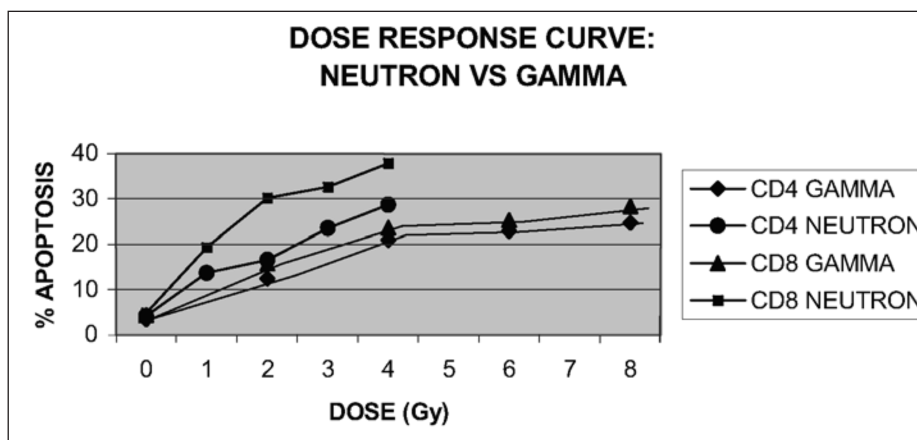

Fig 4. *Dose response curve of one donor after exposure to gamma(0-8Gy) and neutron (0-4Gy) radiation, showing increased damage after neutron exposure.*

increased to 37.89% at 8Gy. When comparing the apoptotic response of gamma and neutron radiation, there was increased damage by neutron radiation as at 2Gy gamma, CD4 was 12.49% but measured 16.64% at 2Gy neutrons. Similarly at 4Gy gamma, CD4 was 20.99% compared to 28.68% at 4Gy neutrons. The apoptotic response for CD8 also showed increased values for neutron radiation as at 2Gy, gamma showed 15.8% compared to 30.19% at 2Gy neutrons and 4Gy gamma showed 23.63% compared to 37.89% at 4Gy neutrons. The relative biological effectiveness (RBE), which is the ratio of the doses needed to create an equal level of biological damage, is increased in this comparison. This shows that equivalent doses of neutron and gamma radiation do not have the same biological effect.

Variance between individuals was found to be higher than variance within individuals as can be seen in Fig 2 and Fig 3 where inter-donor variation exceeded intra-donor variation. These results are supported by Schmitz and co-workers (2003) who reported similar findings. Results of the study showed CD8 lymphocytes to be more sensitive to radiation-induced apoptosis than CD4 lymphocytes (Table I), at 2Gy and 8Gy for all 9 donors. The data is supported by Wilkins and co-workers (2002) and Crompton and Ozsahin (1997) who also observed clear differences in radiosensitivities between CD4 and CD8 lymphocytes.

DISCUSSION

The purpose of this study was to determine the reliability of the assay and to standardise it in South Africa. Nine samples were exposed to 0Gy (control), 2Gy and 8Gy of gamma radiation and the apoptotic response of CD4 and CD8 lymphocytes was measured using the LAA. One sample was exposed to 0Gy, 2Gy, 4Gy and 8Gy gamma radiation and compared to 0Gy, 1Gy, 2Gy, 3Gy and 4Gy neutron radiation.

Results showed an increase in percentage apoptosis as the dose increased for both gamma and neutron radiation modalities. The study compared the apoptotic response to *in vitro* gamma (low–LET) and neutron (high–LET) radiation and increased damage by neutrons was observed which therefore needs to be investigated further. This study was unique in the sense that testing for radiosensitivity using the LAA with neutron radiation has not been reported before. This may result in better treatment regimes for patients suffering from advanced cancers not easily treated with gamma radiation such as salivary gland tumors and soft tissue sarcomas.

The study yielded enough data to base radiosensitivity measurements on CD4 and CD8 lymphocytes and from the above results, one can see that this pilot study can thus be applied to a standard curve and retrospective study in South Africa.

As lymphocytes display 6.5% less apoptosis with each ten years of life, age also plays a role in radiation-induced apoptosis (Radojec and Crompton, 2001 and Crompton *et al*, 1999) and this should be further investigated in the standard curve study conducted in South Africa. The data obtained from South African donors will also help to establish whether or not radiosensitivity is influenced by gender and especially the different ethnic groups in South Africa.

An assay for radiosensitivity will have numerous applications in the clinic and would help with management of acute responses, prediction of normal tissue toxicity and individualisation of patient radiosensitivity (Ozsahin *et al*, 1997). As cellular response to DNA double-strand breaks is a major factor influencing radiosensitivity in humans (Shiloh, 1997) the leukocyte apoptosis assay, which measures physiological response (in this case, apoptosis) at a cellular level, may be a good predictor of the response to radiation therapy. In addition, as this assay measures the cytotoxic response of cells, it could in future also be used to investigate patients' response to chemotherapy

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