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**Prediction of Radiosensitivity by Measurement of
Radiation-Induced Apoptosis in Human Blood using
the Comet Assay**

by

Narine Kizilian

A thesis submitted to the Faculty of Graduate Studies and
Research in partial fulfillment of the requirements for the
degree of

Master of Science

Department of Physics
Carleton University
Ottawa-Carleton Institute of Physics
Ottawa, Ontario
September, 1999
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in partial fulfillment of the requirements for the degree of
Master of Science

Chair, Department of Physics

Thesis Supervisor

Carleton University

Abstract

The purpose of this research was to determine whether levels of radiation-induced apoptosis in human blood would be predictive of individual radiosensitivity. Apoptosis was quantitated using the neutral comet assay, which was shown to be a valid method for detecting undamaged, apoptotic, and necrotic cells. Blood samples, exposed in vitro to x-rays (0-3 Gy), were monitored for apoptosis at different time points after exposure.

The degree of intra-individual variation in the levels of radiation-induced apoptosis was assessed using blood samples from four healthy individuals. The results indicated that the amount of variation differed from person to person. In order to assess the levels of inter-individual variation, blood samples were obtained from a group of radiotherapy patients who had demonstrated differential radiosensitivity. Apoptosis was measured in whole blood, granulocytes and mononuclear cells. When comparing the results from the normal and radiosensitive individuals, a consistent trend in the apoptotic response was seen for all x-ray doses between the two populations .

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To my Mom and Dad

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1.0 Introduction

Since the first use of ionizing radiation in the treatment of cancers it has become evident that each individual's response to radiotherapy is unique. Using the same treatment modality, the same treatment regimen on the same type of tumour does not guarantee an identical response from all individuals. Given this knowledge, current practices in radiotherapy try to establish *tolerance doses* for particular treatment regimens being used for a specific cancer. These tolerance doses are determined from population averages, obtained in clinical trials through follow-ups of patients, by establishing a dose that does not cause excess harm to normal tissue while maximizing the damage to the tumour. Based on this principal, groups of individuals are administered identical treatments, meaning that some individuals, who are more radiosensitive than others, are receiving beyond normal tissue tolerance doses, and others, who are more radioresistant, receive sub-tolerance doses. The ability to predict individual responses to radiotherapy would allow physicians to optimize the treatment plan for each patient.

Radiation response can be defined as an individual's capacity to recognize and respond to cellular damage caused by exposures to ionizing radiation. A small population of patients who receive radiotherapy have a lower tolerance to radiation damage and demonstrate a sensitive response. These radiosensitive individuals develop adverse tissue reactions to the radiation such as erythema, desquamation, atrophy, fibrosis, or necrosis, which can appear immediately or months to years after the initial treatment. In order to reduce the risk of developing complications when treating these patients, smaller doses should be

administered. However, the problem is in identifying these individuals before the treatment is given.

1.1 Predictive Assays

A major component of radiobiological research is in the area of predictive assays, that is, assays which are capable of predicting an individual's radiosensitivity. Currently the "gold standard" of predictive assays is the clonogenic assay, which measures radiosensitivity of a cell line by its ability to maintain its reproductive integrity. This assay has been performed on numerous cell lines established from both tumour and normal tissues. It has been found to be a useful tool when looking for differences in radiosensitivity of different types of tumours. However, it has not been found to be capable of explaining the observed differences in radiotherapy response in individuals with the same type of tumour[1]. Thus, research into the development of new predictive assays continues.

There are a number of qualities that an ideal predictive assay should possess. Of course, the most important of these qualities is the resolution of the assay. The assay should have a high capacity to distinguish between small changes in radiosensitivity between individuals. It should also be a fairly easy and quick assay so that patients would not have to wait long periods of time before their treatment can begin. Finally the cost of the assay is also an important factor. It should be inexpensive enough that it could be used on a daily basis in the clinic.

1.1.2 Normal vs. Tumour Tissue

A predictive assay can be performed on tumour cells or normal cells obtained from an individual. One might think that prediction of tumour response to radiation is more important since it is the target receiving the largest dose. However, since the limiting factor in radiotherapy is the normal tissue tolerance dose, it makes sense that prediction of normal tissue radiosensitivity would be much more useful. The radiosensitivity of tumour cells is less important information since, if it were possible, a very high dose would be delivered to destroy the tumour. The ability to use normal tissue would simplify a predictive assay significantly, since normal cells are more easily accessible than tumour cells. For example, human fibroblasts have been used widely in research for the development of predictive assays [2-4].

1.1.3 Use of Blood in Predictive Assays

The attractiveness of blood as a biological monitor has long been recognized. In medicine, it is the most commonly used tissue to determine the cause of ill health and disease. It can be easily collected and large numbers of cells can be obtained from small samples. Therefore it is understandable that blood would also be considered as a potential predictor of radiosensitivity. There are a number of studies that have investigated the potential of blood as a predictor of radiosensitivity. The majority of these studies look at the response of lymphocytes to ionizing radiation using the clonogenic assay, and rarely, apoptosis, or programmed cell death, is measured[5-8]. However, all of these publications call for further investigations before the applicability of blood as a predictor of radiosensitivity can be determined.

1.2 Consequences of Irradiation

In order to design new predictive assays it is important to understand the mechanisms through which ionizing radiation interacts with biological material and the consequences of these interactions. Only after we have a good understanding of the physics, chemistry and biology taking place as a result of radiation exposures will it be possible to identify measurable endpoints and investigate them for their ability to predict radiosensitivity.

1.2.1 Physics of Radiation

Today we have established a good understanding of the physics of radiotherapy, that is, the immediate events taking place when ionizing radiation interacts with biological material. As photon beams (x-rays and gamma rays) traverse biological tissues, they deposit some of their energy along their path, where it is used to produce fast moving electrons or positrons. There are three main processes which are responsible for the production of these high speed charged particles. These are the photoelectric effect, Compton incoherent scattering, and pair production. The energy of the photons entering tissues determines which of these mechanisms dominates. Figure (1) illustrates this concept.

Figure 1: The three mechanisms of photon interactions and their regions of dominance. (Taken from Krane, K.S., Introductory Nuclear Physics, 1988.)

The photoelectric effect is an interaction of a photon with a bound electron. The photon gives up all of its energy to overcome the binding energy of the electron and to provide it with kinetic energy. In biological tissues, which have a low atomic number, Z , the photoelectric effect is dominant at low photon energies. Compton scattering, also known as inelastic scattering, is the interaction of a photon with a free, resting electron. The energy of the initial photon is separated between the kinetic energy passed on to the free electron and to a new photon of lower energy. In biological tissues Compton scattering dominates at energies between 2keV and 2MeV. Pair production occurs when a photon interacts with the Coulomb field of a nucleus. The initial energy of the photon is used to produce two particles, an e^+ and e^- and to provide these particles with kinetic energy. In this type of interaction there is threshold energy of 1.022MeV required in order for the

interaction to take place. Therefore this interaction occurs only at high photon energies in biological tissues.

The charged particles produced through these processes, in turn, travel through the tissue and produce excitations and ionizations. In some cases these excitations and ionizations occur directly on the DNA molecule. However, most often, the charged particles interact with water molecules. The physical processes, beginning from the primary interaction of the photons with tissues to the final excitation and ionization, takes a very short time of approximately 10^{-16} seconds.

1.2.2 Chemistry of Radiation

Radiation exposures have been shown to produce many types of DNA damage. For example, chemical changes can be induced which may result in single strand breaks, double strand breaks, and intra- or inter-DNA strand crosslinks[9]. This damage may be produced when the radiation interacts directly with the DNA or indirectly, when DNA interacts with free radicals produced when nearby water molecules become ionized. Radiolysis of water causes the molecule to decompose into relatively unstable and highly reactive radicals. These free radicals are capable of diffusing into cells and damaging critical targets such as the DNA. The chemical processes resulting from radiation exposures take approximately 10^{-3} seconds.

1.2.3 Biology of Radiation

Depending upon the dose of radiation delivered and the repair capacity of the cells, there are three possible outcomes to the fate of an exposed cell: survival, mutation, or death.

Once a cell recognizes DNA damage, its repair mechanisms may become activated. If repair is initiated and carried out correctly, the cell will be returned to its original functioning state. However, if DNA repair mechanisms are not initiated or if the repair is not carried out accurately, the cell will either survive, carrying a mutation, or it will die.

A cell possessing a mutation introduces a risk of future cancer induction in the exposed tissue. However, if cell death occurs, the cell will be eliminated from the tissue taking with it any risk of cancer induction, implying that cell death may be a beneficial process.

These pathways are summarized in Figure 2. The biological events resulting from radiation exposures may take minutes or years to occur. Based on this information, we now have two candidates for potential use in predictive assays of radiosensitivity: measurement of repair capacity or measurement of cell death.

Figure 2: Consequences of radiation damage. (Adapted from Powell, S., McMillan, T.J.: *Radiother Oncol*, 19, 1990).

1.3 Repair Capacity

Biological research in the area of DNA repair has made many advances. We now have an understanding of different mechanisms of repairing different types of radiation-induced DNA damage. This knowledge has allowed for the development of many

techniques for the quantification of repair capacity. For example, gel electrophoresis techniques can be used to quantify changes in the amount of DNA fragmentation with time[10]. Repair capacity can also be quantified by measuring cell survival in split dose experiments[11; 12]. While all of these techniques can detect the presence of repair, they are not capable of determining if all DNA damage was repaired and if the repair was accurate. In fact, it is impossible to test for the complete accuracy of repair because this would require analysis of millions of genes in thousands of cells.

1.4 Cell Death

Cell death is a very simple endpoint to quantify since it requires only a “yes/no” answer, that is, yes the cell has died or no it has not. There are two forms of cell death, necrosis and apoptosis, which may result after exposures to ionizing radiation. These forms of cell death are morphologically and physiologically very different from one another. Due to these differences, many tools are available for their identification and quantification.

1.4.1 Necrosis

Necrosis is a form of cell death which results when cells are subjected to severe insult. A severe insult may be an exposure to high levels of ionizing radiation[13], a toxic substance, or some other damaging conditions such as severe hypoxia[14], hyperthermia[15], or direct cell trauma[16]. The main characteristic of a necrotic cell is its loss of membrane integrity. With impaired regulation of membrane permeability, necrotic cells experience an influx of extracellular material, which causes the cell to

display a swollen morphology. A build-up of Ca^{2+} develops in necrotic cells causing the activation of enzymes called phospholipases[17]. The function of phospholipases is to break down phospholipids, which are an integral component of cellular membranes. The activated phospholipases damage the membranes of all organelles in the cell, eventually causing all cellular components to rupture. This event is particularly important in the case of lysosomes, which burst and release hydrolases into the cell. [18]. As a consequence of this uncontrolled self-destruction, the DNA in necrotic cells is randomly cleaved into a range of sizes. [19; 20]. An inflammatory reaction is usually associated with necrosis which results in response to the release of cellular material, including hydrolytic enzymes, into the surrounding tissue and damaging neighbouring cells. Figure 3(a) summarizes these steps involved in necrotic cell death.

1.4.2 Apoptosis

In the late 1960's and early 1970's many investigators observed a form of cell death which was distinctly different from necrosis. Kerr, Wyllie and Curie named the process apoptosis in 1972[21]. Apoptosis occurs naturally in the body as the mechanism for maintaining homeostatic control of cell numbers. It is also the form of cell death which occurs in response to mild cellular stresses such as low levels of ionizing radiation[22; 23], or low levels of chemical and physical agents[24]. During the early stages of apoptosis, a cell will display cytoplasmic and nuclear condensation causing it to pull away from neighbouring cells. As a result of the condensation, the chromatin becomes very dense, collects into large masses and migrates to the edges of the nuclear membrane.

This is accompanied by the formation of protuberances on the cell membrane, commonly called blebs. In contrast to necrosis, membranes of apoptotic cells remain intact and all organelles retain their shape and membrane integrity.

DNA fragmentation during apoptosis is thought to occur in a controlled manner. The DNA is first cut into high molecular weight fragments (50-300kbases)[25] then into smaller fragments which are multiples of approximately 200 bases in length. The smaller fragments are produced by double strand breaks in regions of the DNA between nucleosomal cores[26; 27]. In the late stages of apoptosis, the cell breaks down into apoptotic bodies, which are small membrane-bound structures containing intact organelles and nuclear material. Surrounding cells quickly phagocytose these apoptotic bodies and thus avoid the development of inflammation in the tissue. Figure 3(b) summarizes the steps involved in apoptotic cell death.

Figure 3: Schematic summarizing morphological features of (a) necrotic and (b) apoptotic cell death. (adapted from Searle et al., 1982)

1.5 The Comet Assay

The comet assay, also called the single cell gel electrophoreses (SCGE) assay, was first introduced by Östling and Johanson in 1984[28]. It is a widely used tool for assessing DNA damage in the form of strand breaks. The main feature of the comet assay is that it allows for the visualization and quantification of DNA strand breaks at the level of individual cells. The technique is based upon a simple concept. Cells imbedded in agarose are lysed, to isolate DNA, and placed in an electric field. If strand breaks are

present then the negative charge of DNA will allow it to migrate to the anode in the presence of the electric field.

Presently there are two forms of the comet assay: the neutral and alkaline techniques.

The neutral comet assay allows for the measurement of double strand breaks, whereas the alkaline comet assay allows for the measurement of double and single strand breaks as well as alkaline labile sites. Recently it has become recognized that the comet assay, both neutral and alkaline techniques, can be used to distinguish between normal, apoptotic and necrotic cells[15; 22; 29; 30]. This method of detection is based on the different mechanisms of DNA cleavage between apoptotic and necrotic cells discussed above, and the lack of DNA cleavage in normal cells. Figure 4 shows all three types of cells observed in TK6 human B lymphoblast cells after performing the neutral comet assay and staining the samples with silver. These cells have been shown to undergo apoptosis in a time and dose dependent manner[22; 29].

Figure 4: Silver stained comets of (a) normal cell, (b) apoptotic cell, (c) necrotic cell obtained using the neutral comet assay.

1.6 Hypothesis

The aim of this project is to measure radiation-induced apoptosis in human peripheral blood obtained from a group of individuals who have previously undergone radiotherapy and demonstrated differential response to radiation. The radiosensitive individuals in the group developed late adverse tissue reactions. It is possible that these tissue reactions develop due to the inability of the individual's cells to initiate or undergo apoptosis and be eliminated in a controlled manner without causing tissue damage. Therefore, it is

hypothesized that radiosensitive individuals will have lower levels of apoptosis indicative of a reduced ability in recognizing radiation-induced DNA damage.

In order to test this hypothesis, three objectives were set for this project. First, it was necessary to verify the comet assay's ability of detecting apoptosis. This was done by comparing the comet assay with the standard cell morphology assay[24] for the detection of apoptosis. Second, an investigation of intra-individual variation was carried out to determine whether the levels of radiation-induced apoptosis within an individual changed with time. Finally, a comparison was made of the levels of apoptosis in blood from normal and radiosensitive individuals to determine whether a difference could be detected.

2.0 Materials and Methods

2.1 Neutral Comet Assay

The method described here has previously been reported by Kizilian et al.[31]

2.1.1 Chemicals and Solutions

Aqueous solutions, and all dilutions, were made with 18 M Ω water (Milli-Q plus PF unit, Millipore Corp., Bedford, MA). A 1% w/v agarose solution (Bio-Rad, high strength #162-0125, Hercules, CA) was prepared in Hanks balanced salt solution (HBSS). Lysis buffer (lithium chloride 1 M, urea 1 M, EDTA 10 mM, Tris base 50 mM, SDS 2%, Fisher Scientific, Fairlawn, NJ) was prepared at pH 7.3, filtered (0.2 μ m pore size, Nalge Company, Rochester, NY) and stored at ambient temperature. A proteinase-K stock solution (Boehringer-Mannheim Corp., Indianapolis, IN) was prepared at 20 mg mL⁻¹ in TE buffer (Promega Corp. Madison, WI), filtered (0.2 μ m pore size) and stored in 1 mL aliquots at -30°C. Electrophoresis was performed in TAE buffer (Tris-Acetate 0.04 M, EDTA 0.01 M, glacial acetic acid 0.02 M, Boehringer Mannheim). Trichloroacetic acid (TCA) was prepared as a 20 % w/v solution and filtered (0.2 μ m pore size). The methanol and glacial acetic acid were from Fisher Scientific, Fair Lawn NJ. TMSilver stain plus kit (Bio-Rad, Hercules, CA) was used, with a modified protocol, as a permanent stain for the "comets".

2.1.2 Embedding Cells in Agarose, Electrophoresis and Silver Staining

TMGelbond (FMC Bioproducts, Rockland, ME) was cut into 6.4 x 9.9 cm strips to fit onto an agarose C gel mold (part no. HE-4710, Hoefer Scientific Instruments, San Francisco, CA) and the TMGelbond-containing mold was placed into a casting tray (part no. HE33-B, Hoefer Scientific Instruments). To prevent curling of the TMGelbond rectangle, the long edges were secured in the mold by 2 thin Teflon strips (15 x 65 x 1 mm, Bio-Rad, Hercules, CA) so that the 1 mm edge forced the TMGelbond sheet to lie flat against the bottom of the mold. About $1.0\text{--}1.5 \times 10^5$ cells were added to 2.8 mL of 1% prewarmed agarose (50°C). The cell-agarose suspension was gently mixed with a 5 mL pipette and then spread onto the TMGelbond rectangle. At these dimensions, 3.0 mL of agarose-cell suspension produced a layer with a thickness of about 0.5 mm. The length of time the cells remained at 50°C was less than 10 sec. and did not cause any detectable damage to the control cells. When the agarose was fully solidified (~5 min), the gel/TMGelbond slab was carefully transferred from the casting tray to a tissue culture dish (150mm) and incubated at 50°C for 1 hr in 50 mL of lysing buffer containing $60 \mu\text{g ml}^{-1}$ proteinase-K. After lysis, the gel was washed twice for 20 min in TAE running buffer then transferred to a submarine agarose gel unit (model HE-33, Hoefer) for electrophoresis at 22 V for 5 min (Gibco-BRL Power Pack model 4001). Following electrophoresis, the gel was placed in a 150 mm culture dish and rinsed with 18 M Ω water, fixed in absolute ethanol for 1-2 hr, then dried overnight. The film was cut in two, one half was archived and the other half was silver stained according to the kit manufacturer (TMSilver Stain Plus, Bio-

Rad Laboratories Ltd., Hercules, CA), with the following modifications. The film was placed in a 100 mm culture dish and immersed for 1-2 hr in 9 parts TCA (20% v/v) and 1 part TMFixative Enhancer (a kit component) followed by two 15 min rinses in 18 MΩ water. The silver stain solution was prepared in subdued light from the kit components according to the manufacturer. All glassware and plastic coated magnetic stir bars used in the preparation of the silver stain were pre-washed with 50% nitric acid, followed by rinses in distilled water and then 18MΩ water. Briefly, 5 mL each of the TMSilver Complex solution, TMImage Development reagent and TMReduction Moderator solution were added, in order, to 35 mL of 18 MΩ water and gently mixed with a teflon-coated stir bar. This was immediately followed by addition of 50 mL of the TMDevelopment Accelerator solution. Approximately 25 mL of this final staining solution was added to each 100 mm culture dish containing a dried film. The dish was agitated 9-14 min. in a gyratory rocker set at about 60 cycles min.⁻¹. To stop the staining process, the gel was immersed for 20 min in an aqueous solution of methanol:glacial acetic acid:water (50:16:34) followed by 2 rinses in water. Overstained comets could be readily destained by following the procedure of Merril *et al.*[32]. The films were air dried and stored until analysis.

2.2 Measurement of apoptosis using the Cell Morphology assay.

Apoptotic and necrotic cells undergo a sequence of distinct morphological changes that allow the two modes of cell death to be distinguished under light microscopy. An independent assay for apoptosis, based on chromatin condensation with Giemsa staining

was used to confirm that the neutral comet assay's ability of apoptosis detection. For the cell morphology (CM) assay, 150 μL of a cell suspension ($2.5 - 8 \times 10^5$ cells mL^{-1}) was layered onto 125 μL of RPMI medium (30% FCS) in a cytocentrifuge cell holder and centrifuged at 1000 rpm for 6 minutes (Cytospin 3, Shandon Inc., Pittsburgh, PA). The slides were immediately fixed in ethanol:glacial acetic acid (3:1) for 1 min, allowed to air dry and then stained in 5% Giemsa for 5-10 min. Slides were dried and coverslips applied with TMPermout, (Fisher Scientific, Fairlawn, NJ). Cells were examined for chromatin condensation, membrane blebbing and apoptotic body formation under brightfield microscopy using a 100x oil immersion lens (Olympus, PlanApo, NA 1.40). At least 1000 cells were examined for each sample.

2.3 Cell viability

All samples were assessed for cell number and viability at each time point using a technique similar to that of Strauss *et al.* [33]. Briefly, a dye working solution was prepared by adding 30 μL of fluorescein diacetate (5 mg mL^{-1} in acetone) and 200 μL of ethidium bromide ($200 \mu\text{g mL}^{-1}$ in HBSS) to 4.8 mL of HBSS. Equal volumes (usually 30 μL) of the dye and cell suspension were pre-mixed, 10 μL was placed on the chamber of a hemocytometer and examined at low magnification by fluorescence microscopy (545nm ex.; 610nm em.). Cell number was assessed on the same grid under tungsten illumination and phase contrast optics.

2.4 Data collection and statistical analysis

The true rate of apoptosis was estimated by $p = A/N$, where A is the number of apoptotic cells and N the total number of cells counted. After N counts, the variance of the rate p is defined as

$$\sigma^2 = pq/N, \text{ where } q = (1-p) [34]. \quad [\text{Equation 1}]$$

The error bars on graphs representing single experiments show the standard deviation of each point calculated from this variance. When several experiments were averaged, the error bars represent the standard error of the mean between the experiments. Analysis of variance and t-tests were performed using the software package Instat.

2.5 Patients

There were 8 patients who participated in this study. All patients had previously received radiotherapy for different types of cancers. Among the patients, four experienced late radiotoxicity. Table 1 provides further patient information.

Table 1: Clinical information of patients.

Patient	Age and Sex	Clinical Condition	Radiotoxicity	Follow-up (months)
P1MN	78, male	Prostate cancer	None	34
P2MN	61, male	Prostate cancer	None	32
P3FN	55, female	Breast cancer	None	6
P4MN	52, male	Glottic larynx carcinoma	Early: erythema, mucositis Late: to be assessed	12
P5MS	52, male	Carcinoma of anal canal	Early: moist desquamation Late: severe fibrosis	120
P6FS	54, female	Papillary carcinoma of thyroid gland	Early: severe mucositis and esophagitis Late: esophageal stricture	20
P7FS	74, female	Bladder cancer	Early: ? Late: cystitis, proctitis	30
P8FS	70, female	Cancer of tonsil pillar	Early: confluent mucositis Late: tongue ulceration	12

2.6 Blood

2.6.1 Collection

Blood was drawn from the patients through venapuncture and collected into sodium heparin tubes.

2.6.2 Irradiation

Blood samples, in polypropylene tubes, were irradiated on ice. The samples were irradiated to give doses of 0, 1, 2, and 3 Gy using 250kV x-rays (Pentek, Bipolar Series 2, HF-320) at a dose rate of $168.3 \text{ cGy min}^{-1}$, at a constant current of 12.5mA.

2.6.3 Culture medium

Blood samples were cultured in RPMI 1640 (Gibco-BRL) culture medium supplemented with 10% FBS (Immunocorp Corp., Montreal, Que.), 2mM L-glutamine (Gibco-BRL), 25mM Hepes (Gibco-BRL), and 1% penicillin and streptomycin(Gibco-BRL).

2.6.4 Leukocyte Separation

Leukocytes were isolated from whole blood using a gradient density centrifugation technique. In a 15ml conical polypropylene centrifuge tube, 3ml of histopaque-1077 (Sigma diagnostics) were layered on top of 3ml of histopaque-1119 (Sigma diagnostics). Whole blood (6ml) was then carefully layered on top of the upper layer. The tube was centrifuged at $700 \times g$ for 30 minutes at room temperature. After centrifugation, two distinct opaque layers could be observed. The upper opaque layer contained lymphocytes, monocytes and platelets and the lower opaque layer contained granulocytes. The supernatant was removed to within 0.5 cm of the top layer and the

cells in this layer were aspirated and transferred to a clean 15 ml centrifuge tube. This procedure was repeated to obtain the cells of the second layer. The two populations of cells were washed twice by adding 10 ml of the culture medium and centrifuging at 200 x g for 10 minutes. The supernatant was removed and the cells were resuspended into 4 ml of culture medium.

2.7 Experimental Protocol

2.7.1 Validation of the Comet Assay

TK6 human B-lymphoblast cells were grown in RPMI-1640 (Gibco-BRL) medium supplemented with 10% fetal calf serum (Immunocorp Corp., Montreal, Que.) and 2 mM L-glutamine (Gibco-BRL). A fresh 20 mM H₂O₂ (J.T. Baker, Toronto, ON) stock solution was prepared in 18 MΩ water before each experiment. The concentration of the H₂O₂ was verified using a spectrophotometer (Spectronic 2000, Bausch & Lomb, Rochester, NY). A 10 ml aliquot of the TK6 cell suspension was placed into 5 tissue culture dishes (150ml) and treated with 0, 4, 6, 8, and 10 μM of H₂O₂ to induce apoptosis[35-37]. After 8, 10, 12 and 15 hours of incubation, two aliquots were removed from each sample and independently assessed for apoptosis using the neutral comet assay and the CM assay.

2.7.2 Intra-Individual Variation

Three blood samples were obtained from four healthy individuals (2 males, 2 females) over a period of 3 months. Detailed information of each of the donors is shown in Table 2. Whole blood samples (500ul) were immediately irradiated. After the irradiation, the

samples were diluted 10X using the blood culture medium and incubated in a 5% CO₂ incubator. The neutral comet assay was performed on each of the samples after 4, 24, 48, and 72 hours of incubation. To determine the fraction of apoptotic cells, the samples were silver stained and analyzed as described above.

Table 2: Sex and age of healthy blood donors.

Donor	Sex	Age
Donor 1	female	33
Donor 2	female	45
Donor 3	male	25
Donor 4	male	50

2.7.3 Inter-Patient Variation in Whole Blood

A single blood sample was obtained from each of the patients. The same procedure as described above in section 2.7.2 was used.

2.7.4 Inter-Patient Variation in Whole Blood and Leukocytes

A 500ul aliquot of whole blood was placed into four 2 ml polypropylene tubes. A 1ml aliquot of the mononuclear cell suspension was placed into four 2 ml tubes and similarly for the granulocyte suspension. All of the samples were irradiated at the same time. After the irradiation, the whole blood samples were diluted using the blood culture medium to a final volume of 5000 ul. The leukocytes samples were diluted to a final volume of 4000 ul. All samples were placed into a 5% CO₂ incubator. The neutral comet assay was performed on each of the samples after 4, 24, 48, and 72 hours of incubation. To determine the fraction of apoptotic cells, the samples were silver stained and analyzed as described above.

3.0 Results

3.1 Validation of the Comet Assay

Experiments were performed to validate the comet assay's ability of detecting apoptosis. This was done by comparing levels of apoptosis measured with the comet assay with the classical CM assay for detecting apoptosis. Apoptosis was induced using hydrogen peroxide. Figures 5 and 6 show the apoptotic fraction (AF) measured in TK6 cells using the comet and CM assay respectively, at varying concentrations of hydrogen peroxide and incubation times. The error bars represent the standard error of the mean of three experiments. Both assays showed an increase in the AF with increasing concentrations of hydrogen peroxide. It was also evident that the comet assay detected higher levels of apoptosis than the morphology assay. This is clearly shown in Figure 7, which presents the AF at 8 hours after treatment with increasing concentrations of hydrogen peroxide as measured by the CM assay and the neutral comet assay. The regression line has a slope of 0.83 ± 0.02 . The coefficient of correlation for the data is 0.997 indicating the data is highly correlated[31].

A one-way analysis of variance (ANOVA) was performed to determine if there was an increase in the AF with time. The results indicated that there was no significant change in the AF with exposure time, at any of the hydrogen peroxide concentrations in either of the assays.

Comet Assay

Figure 5: Fraction of apoptotic cells as a function of hydrogen peroxide concentration as measured with the comet assay at 8, 10, 12, 15 hours of incubation.

Morphology Assay

Figure 6: Fraction of apoptotic cells as a function of hydrogen peroxide concentration as measured with the CM assay at 8, 10, 12, 15 hours of incubation.

Figure 7: Fraction of apoptotic cells at 8 hours of incubation with hydrogen peroxide, measured using the CM assay and the neutral comet assay. The dashed line represents a slope of one.

3.2 Intra-Individual Variation

In order to investigate the intra-individual variation in the apoptotic response, blood samples from 4 individuals were analyzed for apoptosis 3 times over a period of 3 months. The individuals were considered healthy and their radiosensitivity was unknown since they had no previous history of radiation exposure. These results are shown in Figures 8 to 11. The error bars on each point represent the standard error of the mean of three experiments. The intra-individual variation was found to be different among the donors. The shape of Donor 2's apoptotic response curve indicating an increase in the AF with time. This is different from the responses of the other donors where the AF seems to plateau after 24 hours. Also in most cases, the variation in the unexposed samples was found to be smaller than the variation in the exposed samples.

Figure 8: Fraction of apoptotic cells as a function of time after exposure to 0, 1, and 3 Gy of x-rays, measured in whole blood from Donor 1.

Figure 9: Fraction of apoptotic cells as a function of time after exposure to 0, 1, and 3 Gy of x-rays, measured in whole blood from Donor 2.

Figure 10: Fraction of apoptotic cells as a function of time after exposure to 0, 1, 2, and 3 Gy of x-rays, measured in whole blood from Donor 3.

Figure 11: Fraction of apoptotic cells as a function of time after exposures to 0, 1, 2, and 3 Gy of x-rays measured in whole blood from Donor 4.

3.3 Inter-Patient Variations in Whole Blood

Figures 12 through 19 show the apoptotic response in whole blood obtained from the eight patients after *in vitro* exposures to 0, 1, 2, and 3 Gy of x-rays measured at 4, 24, 48, and 72 hours. The error bars represent the standard deviation calculated using Eq. 1.

These data were used to determine the inter-individual variation. The average response from the patients for each of the exposure levels is shown in Figure 20(a, b, c, d). The error bars represent the standard error of the mean of eight patient experiments. The largest inter-individual variation was observed at the 3 Gy exposure level after 24 hours.

In order to compare the data obtained from the normal patients with that of the radiosensitive patients the average apoptotic responses of the two groups of patients was calculated. The results are shown in Figure 21(a, b, c, d). The two populations were compared using an analysis of variance (ANOVA). The 3 Gy samples at the 24 hour time point showed a significant difference ($P=0.02$) between the two groups.

The inter-individual variation between the 8 patients is compared with that of the 4 healthy donors in Table 3, showing the mean fraction of apoptotic cells and the standard deviation. The standard deviations from the patient data are similar to that of the healthy donors. Table 4 allows a comparison to be made between the inter-individual variation of the patients with the intra-individual variation of Donor 4, showing the mean apoptotic fraction and standard deviations.

Figure 12: Apoptotic fraction vs. time after exposure to 0, 1, 2, 3 Gy of x-rays to whole blood of patient P1MN.

Figure 13: Apoptotic fraction vs. time after exposure to 0, 1, 2, 3 Gy of x-rays to whole blood of patient P2MN.

Figure 14: Apoptotic fraction vs. time after exposure to 0, 1, 2, 3 Gy of x-rays to whole blood of patient P3FN.

Figure 15: Apoptotic fraction vs. time after exposure to 0, 1, 2, 3 Gy of x-rays to whole blood of patient P4MN.

Figure 16: Apoptotic fraction vs. time after exposure to 0, 1, 2, 3 Gy of x-rays to whole blood of patient P5MS.

Figure 17: Apoptotic fraction vs. time after exposure to 0, 1, 2, 3 Gy of x-rays to whole blood of patient P6FS.

Figure 18: Apoptotic fraction vs. time after exposure to 0, 1, 2, 3 Gy of x-rays to whole blood of patient P7FS.

Figure 19: Apoptotic fraction vs. time after exposure to 0, 1, 2, 3 Gy of x-rays to whole blood of patient P8FS.

Figure 20: The mean apoptotic fraction measured in whole blood obtained from the eight patients after exposure to 0 Gy (a), 1 Gy (b), 2 Gy (c), and 3 Gy (d) of x-rays.

Figure 21: The mean apoptotic fraction measured in whole blood obtained from four normal and four radiosensitive patients after exposure to 0 Gy (a), 1 Gy (b), 2 Gy (c), and 3 Gy (d) of x-rays.

Table 3: Comparison of the mean apoptotic fraction and standard deviation of the inter-individual variation obtained from the eight patients and 4 healthy donors.

Time		4 hours		24 hours		48 hours		72 hours	
	Dose (Gy)	mean	SD	Mean	SD	mean	SD	mean	SD
8 Patients	0	0.011	0.006	0.281	0.062	0.284	0.050	0.260	0.093
	1	0.014	0.005	0.309	0.080	0.323	0.061	0.336	0.080
	2	0.016	0.005	0.323	0.074	0.369	0.060	0.390	0.084
	3	0.016	0.008	0.317	0.096	0.384	0.060	0.435	0.085
4 healthy donors	0	0.007	0.009	0.181	0.103	0.116	0.063	0.098	0.021
	1	0.009	0.008	0.209	0.092	0.189	0.073	0.227	0.029
	2	0.014	0.012	0.297	0.099	0.271	0.050	0.297	0.063
	3	0.008	0.008	0.256	0.101	0.270	0.082	0.348	0.071

Table 4: Comparison of inter-patient variation with the intra-individual variation measured in Donor 4.

Time		4 hours		24 hours		48 hours		72 hours	
	Dose (Gy)	mean	SD	Mean	SD	mean	SD	mean	SD
8 Patients	0	0.011	0.006	0.281	0.062	0.284	0.050	0.260	0.093
	1	0.014	0.005	0.309	0.080	0.323	0.061	0.336	0.080
	2	0.016	0.005	0.323	0.074	0.369	0.060	0.390	0.084
	3	0.016	0.008	0.317	0.096	0.384	0.060	0.435	0.085
Donor 4	0	0.004	0.001	0.210	0.033	0.165	0.041	0.117	0.034
	1	0.008	0.001	0.217	0.027	0.230	0.060	0.214	0.066
	2	0.006	0.001	0.227	0.027	0.236	0.106	0.252	0.094
	3	0.005	0.001	0.261	0.020	0.268	0.060	0.303	0.061

3.4 Inter-Patient Variation in Whole Blood and Leukocytes

A second set of blood samples was obtained from each patient in order to investigate the levels of apoptosis in whole blood and isolated leukocytes simultaneously. The levels of radiation induced apoptosis in whole blood, granulocytes and mononuclear cells are shown in Figures 22 to 29. The whole blood response was similar to the previously obtained results. Granulocytes were found to be very prone to apoptosis within the first 24 hours of incubation even with no radiation exposure. Mononuclear cells were found to show a dose-dependant response. The average apoptotic responses of the patients in the whole blood, granulocytes and mononuclear cells are shown in Figure 30, 31 and 32 respectively.

In order to compare the data obtained from the normal and radiosensitive patients, Figures 33, 34, and 35 show the average apoptotic response of the two groups from whole blood, granulocytes and mononuclear cells respectively. Again a reduced level of apoptosis in whole blood was obtained in the radiosensitive group at 24 hours for all x-ray exposure levels. However, the differences between the two groups was not found to be significant.

The data obtained from both whole blood samples from the patients was used to fit to the linear-quadratic model

$$SF = e^{-\alpha D - \beta D^2} \quad \text{[Equation 2]}$$

where SF is the surviving fraction which is equal to (1-AF), D is the dose, and α and β are constants. The x-ray doses given to samples were fairly low (1, 2, and 3Gy), therefore the second term of the equation can be ignored. Then Equation 2 becomes

$$(1-AF) = e^{-\alpha D} \quad [\text{Equation 3}]$$

Taking the natural logarithm of both sides gives

$$\ln(1-AF) = -\alpha D \quad [\text{Equation 4}]$$

The values of α were determined for both whole blood samples at 24 hours for each patient in order to investigate the possibility of identifying a better discriminator between the radiosensitive and normal individuals. The results are shown in Table 5. Again a significant difference in the mean values of α were found between the normal and radiosensitive patients using the data from the first blood sample. However this difference was not significant using the data from the second blood sample.

Figure 22: Apoptotic fraction vs. time after exposure to x-rays. (A) whole blood, (B) granulocytes, (C) lymphocytes, monocytes and platelets for patient P1MN.

Figure 23: Apoptotic fraction vs. time after exposure to x-rays. (A) whole blood, (B) granulocytes, (C) lymphocytes, monocytes and platelets for patient P2MN.

Figure 24: Apoptotic fraction vs. time after exposure to x-rays. (A) whole blood, (B) granulocytes, (C) lymphocytes, monocytes and platelets for patient P3FN.

Figure 25: Apoptotic fraction vs. time after exposure to x-rays. (A) whole blood, (B) granulocytes, (C) lymphocytes, monocytes and platelets for patient P4MN.

Figure 26: Apoptotic fraction vs. time after exposure to x-rays. (A) whole blood, (B) granulocytes, (C) lymphocytes, monocytes and platelets for patient P5MS.

Figure 27: Apoptotic fraction vs. time after exposure to x-rays. (A) whole blood, (B) granulocytes, (C) lymphocytes, monocytes and platelets for patient P6FS.

Figure 28: Apoptotic fraction vs. time after exposure to x-rays. (A) whole blood, (B) granulocytes, (C) lymphocytes, monocytes and platelets for patient P7FS.

Figure 29: Apoptotic fraction vs. time after exposure to x-rays. (A) whole blood, (B) granulocytes, (C) lymphocytes, monocytes and platelets for patient P8FS.

Figure 30: The mean apoptotic fraction measured in whole blood obtained from the eight patients (second set) after exposures to 0 Gy (a), 1 Gy (b), 2 Gy (c) and 3 Gy (d) of x-rays.

Figure 31: The mean apoptotic fraction measured in granulocytes obtained from the eight patients after exposures to 0 Gy (a), 1 Gy (b), 2 Gy (c) and 3 Gy (d) of x-rays.

Figure 32: The mean apoptotic fraction measured in mononuclear cells obtained from the eight patients after exposures to 0 Gy (a), 1 Gy (b), 2 Gy (c) and 3 Gy (d) of x-rays.

Figure 33: The mean apoptotic fraction measured in whole blood obtained from four normal and four radiosensitive patients (second set) after exposures to 0 Gy (a), 1 Gy (b), 2 Gy (c), and 3 Gy (d) of x-rays.

Figure 34: The mean apoptotic fraction measured in granulocytes obtained from four normal and four radiosensitive patients after exposures to 0 Gy (a), 1 Gy (b), 2 Gy (c), and 3 Gy (d) of x-rays.

Figure 35: The mean apoptotic fraction measured in mononuclear cells obtained from four normal and four radiosensitive patients after exposures to 0 Gy (a), 1 Gy (b), 2 Gy (c), and 3 Gy (d) of x-rays.

Table 5: Comparison of α values obtained from fitting patient data collected from two whole blood samples at 24 hours to Equation 4.

Patient	P1MN	P2MN	P3FN	P4MN	P5MS	P6FS	P7FS	P8FS
α (sample 1)	0.025	0.035	0.035	0.045	0.017	0.006	-0.035	0.023
	Mean = 0.035 SD = 0.008				Mean = 0.002 SD = 0.026			
	P = 0.0286 (considered significant)							
α (sample 2)	0.031	0.041	0.026	-0.021	0.026	0.012	0.040	-0.002
	Mean = 0.019 SD = 0.028				Mean = 0.019 SD = 0.018			
	P = 0.8857 (not significant)							

4.0 Discussion

4.1 Validation of the Comet Assay

Traditionally, the comet assay has been used to quantitate levels of DNA damage by measuring changes in comet tail length or tail moment[38]. Tail length is simply the distance DNA migrates during electrophoresis to form the tail and tail moment is a measure of the fraction of DNA which has migrated into the tail multiplied by the distance travelled. More recently, it has been reported that the comet assay can be used for the detection and quantification of apoptosis[22; 29; 30]. Although its ability to detect apoptosis has been compared to flow cytometry, it has never been validated with the classical CM assay. The CM assay is based on observing the distinct morphological changes occurring during apoptosis, such as chromatin condensation, membrane blebbing and formation of apoptotic bodies. However, this method is quite time consuming and requires a specially trained individual to perform the analysis of the cell preparations. The comet assay would be a much simpler alternative, and for this reason, the first aim of this study was to validate apoptosis detection by the comet assay using the CM assay.

For validating the comet assay, apoptosis was induced in TK6 cells using hydrogen peroxide. The use of TK6 transformed cells allows for better control of the experimental conditions, since both cell numbers and culture conditions could be maintained, and therefore, ensuring experiments were reproducible each time. Hydrogen peroxide was used in these experiments since it is a radiomimetic chemical and it has been used widely in apoptosis investigations in a number of different cell lines[35-37]. The results of

these experiments indicated that both assays measured a dose-dependent increase in apoptosis. However, the comet assay consistently detected higher levels of apoptosis than the CM assay. There may be two explanations for this discrepancy. It is possible that the identification of cells in the early stages of apoptosis, when the cells are condensing, is less efficient with the CM assay. This may be because they are more difficult to distinguish from normal cells. Another possibility is that the early DNA cleavage, into 50-300kb fragments, occurs before cell condensation is observed. The actual timing of DNA strand breaks is unclear, however this observation is in agreement with work by Patel *et al.* who detected DNA strand breaks using fluorophore end-labeling prior to the morphologic changes in the cell[39]. However, a very good correlation was obtained between the levels of apoptosis detected by the comet assay and the CM assay, demonstrating that the comet assay is an effective method of quantitating apoptosis[31].

4.2 Apoptotic Response Measured in Blood

4.2.1 Intra-Individual Variation

An effective predictive assay is one where the intra-individual variation is small, giving a more accurate representation of an individual's response and allowing for detection of smaller changes between individual responses. Intra-individual variation in the levels of radiation-induced apoptosis was investigated in peripheral whole blood samples obtained from four healthy individuals over a period of 3 months. Healthy individuals were used for these experiments in order to avoid the detection of variation in apoptosis levels which could be induced by changes in medications or treatments received by the

individuals during the course of the investigation. This is more likely to occur in patients who are under continued medical care than in healthy individuals.

It was found that the intra-individual variation was different from person to person. Some individuals produced very repeatable results, while others demonstrated large variations. The differing levels of variation were not found to be related to the sex or age of the individuals. There are two possible explanations for this result. First, the values for intra-individual variation are based on only 3 blood samples from each individual. Larger number of samples from each person would give a more accurate demonstration of this variation. Perhaps the variation is large, however, if more experiments were performed for each individual it may remove the difference in variation between the individuals. Second, the results may be indicative of the sensitivity of radiation-induced apoptosis to small changes in blood composition. It is possible that changes in blood composition due to lifestyle factors such as diet, exercise or alcohol consumption can actually affect the ability of radiation exposures to induce apoptosis in blood cells. Although it has previously been shown that different diet types can affect the baseline levels of DNA strand breaks in blood cells[40; 41], there are currently no studies available which have investigated the influence of lifestyle factors on the levels of apoptosis in blood and whether these factors influence the levels of radiation-induced apoptosis.

The results of these experiments also indicated that the intra-individual variation in the unexposed samples were usually smaller than that of the x-ray exposed samples. This

observation may be a consequence of the fact that apoptosis is being triggered by different mechanisms in the exposed and unexposed samples. The various pathways of triggering apoptosis have been summarized in a 1998 report by S. W. Hetts[42]. In unexposed samples, apoptosis is most likely induced due to the changes in the microenvironment of the cells after they have been drawn from patients, which are detected through a membrane signalling pathway. However, apoptosis of radiation exposed cells is due to the formation of direct or indirect DNA damage, as well as the changes in the cell environment. Therefore, if the sensitivity of the mechanism of inducing apoptosis through exposure to radiation is higher, this would be reflected in a higher intra-individual variation in the exposed samples than the unexposed samples.

4.2.2 Inter-Individual Variation in Whole Blood

The ideal method for testing the predictive power of an assay for radiosensitivity would be to track a group of patients for a long period of time, perhaps 2 to 3 years, and performing the assay early, upon diagnosis of the cancer, and repeat it following any type of treatment such as medication, chemotherapy, and radiotherapy. This would allow monitoring of the patient's response with time and it may also show if the response is altered by any of the treatments. The observed radiosensitivity of the patient after radiotherapy could then be compared to the results of the assay obtained before treatment commenced. However, this approach requires that a large population of patients be followed up in order to have a wide range of radiosensitivities to examine, since only 5% of radiotherapy patients are highly sensitive to radiotherapy. Due to the time constraints

on this study, it would not be possible to use this approach of testing. Therefore inter-individual variation was investigated in a group of 8 patients who had previously undergone radiotherapy and of which 4 had demonstrated late radiotoxicity as shown in Table 1.

For predictive assays a large variance in the inter-individual response is desirable, since this would allow for better resolution between small changes in radiosensitivity levels. From the patient data, the largest variation in the apoptotic response was found to be at 24 hours after exposure to 3 Gy. This point may allow for the best discrimination between individual radiosensitivities. However, when comparing the inter-individual variation with the intra-individual variation (Table 4), they were not found to be significantly different from one another. This result indicates that the fluctuations in the levels of radiation-induced apoptosis within one person are so large that they would cover a wide range of radiosensitivities.

When comparing the average response between the normal and radiosensitive groups a trend was observed at all the exposure levels. The radiosensitive group had lower levels of apoptosis than the normal group at 24 hours, equivalent response at 48 hours and higher apoptosis levels at 72 hours as shown in Figure 21. While this trend was observed at all exposure levels, only one sampling point showed a significant difference between the two responses when an analysis of variance was performed. At 24 hours after exposure to 3 Gy, the data indicated that the radiosensitive group had significantly lower levels of apoptosis ($P=0.02$) than the normal group. This finding suggests that

radiosensitive individuals may have a delayed or reduced ability to detect radiation damage. These results are in agreement with work done by Camplejohn *et al*, who reported a reduced apoptotic response in peripheral blood lymphocytes of individuals with a mutation in the p53 gene compared to normal individuals[6]. The p53 gene is known to play a major role in the recognition and response to DNA damage[43]. Studies on blood obtained from individuals with Ataxia Telecangiectia, who are extremely sensitive to ionizing radiation, also have found reduced levels of apoptosis[8; 44].

4.3 Leukocyte Response

Whole blood is composed of approximately 55% plasma, 45% erythrocytes or red blood cells, and less than 1% are the leukocytes and platelets[45]. Red blood cells contain no DNA because they are anucleate, or lack a nucleus. Therefore when apoptosis levels are measured in whole blood using the comet assay, it is only the response of the leukocytes which is monitored since they are the only blood cells which contain DNA and are capable of undergoing apoptosis. All components of blood which do not contain DNA are removed from the samples during the lysis step of the comet assay and do not contribute any information.

The leukocytes in whole blood consist of three subpopulations of cells. These are granulocytes, monocytes and lymphocytes. The different types of leukocytes and their development is shown in Figure 36. By investigating the apoptotic response of subpopulations of leukocytes it may be possible to better understand the shape of the response curves obtained from whole blood and perhaps provide an explanation for the

Figure 36: Summary of whole blood composition.

difference in the apoptotic response between the normal and radiosensitive individuals identified above.

4.3.1 Inter-Patient Variation in Whole Blood and Leukocytes

The individual rates of apoptosis in granulocytes and mononuclear cells, the latter consisting of monocytes and lymphocytes and platelets, were investigated to obtain a better understanding of their contribution to the overall response of whole blood. Blood samples were drawn a second time from the 8 patients and the levels of apoptosis in leukocyte subpopulations were monitored simultaneously with the whole blood. When performing these experiments, the granulocytes and mononuclear cells samples were irradiated after isolation from the whole blood rather than being removed after irradiation of the whole blood. Due to the existence of several chemicals in whole blood which are capable of scavenging radiation induced radicals[46], the amount of damage the leukocytes receive when exposed outside of whole blood is not equal to, but greater than, the levels of damage that would be obtained when exposed in whole blood[47]. However the separation process for the leukocytes requires approximately 1 hour of blood handling, which may be more stressful on exposed cells rather than unexposed cells and may potentially interfere with the radiation response of the cells. Therefore by performing the separation prior to the irradiation ensures all samples have been treated identically except for the differences in the x-ray doses received.

The apoptotic response of whole blood was found to be similar to the results obtained from the first set of samples. When comparing the levels of apoptosis between the normal and radiosensitive groups a reduced level of apoptosis was again observed at 24

hours at all x-ray doses. However, this time, the differences were not found to be statistically significant. Another attempt was made at comparing the whole blood data from the normal and radiosensitive patients by fitting it to the linear quadratic model. Since the x-ray doses were quite low, the β term was ignored. Solving for α , a significant difference was found between the normal and radiosensitive groups from the first blood samples and no significant difference was found from the second blood samples. This is most likely due to the fact that inter-individual variation was not found to be significantly higher than the intra-individual variation and also by the fact that each group only contained four individuals.

A large fraction of granulocytes were found to undergo apoptosis by the 24 hour sampling point, regardless of the radiation dose. This observation may be explained by the fact that granulocytes have a very short life of 4 to 8 hours in the circulatory system[48]. Therefore even undamaged granulocytes would die simply due to their age. When comparing the average response between the radiosensitive and normal patients, we obtain the same trend as was observed with the first whole blood samples, that is, reduced apoptosis levels at 24 hours, equivalent levels at 48 hours and slightly higher levels at 72 hours. Since granulocytes make up approximately 65% of leukocytes, it is understandable that they would make a big contribution to the overall shape of the whole blood apoptotic response curves. However, the differences between the normal and radiosensitive populations were not significant.

Mononuclear cells make up approximately 35% of the leukocytes. The main feature observed in the apoptotic response of mononuclear cells was the separation between the control and x-ray exposed samples. When all patient data was combined, a progressive increase was observed in the rate of apoptosis with increasing radiation dose as shown in Figure 31. This result suggests that the levels of apoptosis in mononuclear cells are sensitive to the radiation dose and may have potential for applications in biological dosimetry. Since mononuclear cells contain both lymphocytes and monocytes, only one of these cell types may be responsible for the demonstrated dose-dependent response. The response of lymphocytes to ionizing radiation has been studied extensively. They have been shown to be highly sensitive to ionizing radiation exposures[49-51]. Lymphocytes have also been found to undergo both radiation-induced apoptosis and necrosis depending upon the dose delivered to them[52]. Although monocytes have been reported to be able to undergo apoptosis[53], there is little data available on their response to radiation exposures. Further separation of the mononuclear cells into lymphocytes and monocytes should be performed to determine which of these cell populations, if not both, was responsible for the dose-dependent increase in the levels of apoptosis. When comparing the average levels of apoptosis of the normal and radiosensitive groups, Figure 35, the radiosensitive group consistently had higher levels than the normal group at all exposure levels. However, the differences were not significant.

5.0 Conclusions/Future Work

The purpose of this study was to investigate the possibility of predicting radiosensitivity by measuring differences in the levels of apoptosis of human peripheral blood after *in-vitro* exposures to ionizing radiation using the comet assay. The comet assay was found to be a sensitive method of detecting apoptotic cells based on their distinct DNA cleavage patterns. When compared with the CM assay, the levels of apoptosis detected with both assays were highly correlated.

Although the comet assay is a valid method of detecting and quantitating apoptosis, in the future it may be possible to improve the efficiency of the assay. For example, the possibility of processing multiple samples concurrently should be investigated. This may be done by decreasing the size of the molds in which the cells are embedded on and thus increase the number of samples that can be held on each strip of TMGelbond. It may also be possible to use fluorescent stains to visualize the comets and analyze the cells with a fluorescent microscope. Although the samples would not be stained permanently, this would decrease sample processing time significantly. Finally it may be possible to analyze samples for apoptosis using an automated counting system. By implementing a computer driven microscope stage and an image analysis program, samples could be analyzed without the need of an individual to operate the microscope. Researchers at Health Canada's Radiation Protection Bureau are currently investigating all of these possible improvements to the comet assay.

The intra-individual variation in the levels of radiation-induced apoptosis was found to differ among individuals. The variation was small for some individuals and large for others. Differences in variation among the individuals may be due to differences in blood content immediately before blood samples were drawn. This result stresses the need for data on the effects of lifestyle factors on both baseline and radiation-induced levels of apoptosis in blood. Investigation of intra-individual variation should be done on large groups of individuals, both males and females, of different ages and sampled numerous times over a long period of time. It may be necessary to examine subpopulations of blood cells. The difficulty with these investigations will be in identify a population of cells which do not show a variation in their apoptotic response with time within an individual but show a large variation among different individuals.

The inter-individual variation in the levels of apoptosis in whole blood was not found to be significantly larger than the intra-individual variation. This result indicates that radiation-induced apoptosis in whole blood does not have sufficient sensitivity for consideration as a predictive assay. However, when comparing the apoptosis levels in whole blood between normal and radiosensitive individuals, the radiosensitive population consistently showed lower apoptosis levels 24 hours after exposure. This finding suggests that radiosensitive individuals may have a delayed or reduced ability of recognizing radiation damage.

The shape of the apoptosis response curve in whole blood was found to be governed mainly by the granulocyte population since these cells make up such a large fraction of

the leukocytes. Mononuclear cells consisting of monocytes and lymphocytes were found to be sensitive to the radiation dose and may be responsible for the separation in the whole blood response curves between the different exposure levels. The mononuclear cells obtained from the radiosensitive population had slightly elevated levels of apoptosis at 24 hours at all exposure levels.

The data obtained from this study identifies the problem of large intra-individual variation, which must be dealt with when using blood to measure radiation-induced apoptosis levels. Further investigation into potential causes of the variation may provide new insight into ways of reducing the variation. The differing levels of radiation-induced apoptosis between normal and radiosensitive individuals have shown some potential for use in predictive assays of radiosensitivity, however, more studies are required which investigate larger groups of patients over longer periods of time before a final decision can be made on its applicability.

6.0 References

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