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DNA Double-Strand Break Induction by Gamma Radiation

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Liam M. Miller

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Student Name:	Liam M. Miller
Faculty Supervisor:	Dr. Linda Yasui
Faculty Approval Signature:	Linda Yasui
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ABSTRACT:

Radiation is a powerful tool in fighting breast cancer. However, the mechanism of cell killing is not fully understood. It is therefore important to characterize radiation so that it may be used more effectively. In the present study the relationship between gamma radiation and DNA double strand break induction is investigated. DNA double strand breaks are the focus of this study because the biological consequences of DNA double-strand breaks are significant, and can result in cell-killing/ apotosis (Heilmann 1995). DNA double strand break induction is surveyed using a constant-field gel electrophoresis approach previously described by J. Heilmann (Heilmann 1995). A linear increase in DNA double-strand breaks is reported here which is consistent with other models of DNA double-strand break induction. This study is part of ongoing research involving the delivery of a cytotoxic dose of radiation to cancer cells.

Abstract

Radiation is a powerful tool in fighting breast cancer. However, the mechanism of cell killing is not fully understood. It is therefore important to characterize radiation so that it may be used more effectively. In the present study the relationship between gamma radiation and DNA double strand break induction is investigated. DNA double strand breaks are the focus of this study because the biological consequences of DNA double-strand breaks are significant, and can result in cell-killing/ apotosis (Heilmann 1995). DNA double strand break induction is surveyed using a constant-field gel electrophoresis approach previously described by J. Heilmann (Heilmann 1995). A linear increase in DNA double-strand breaks is reported here which is consistent with other models of DNA double-strand break induction. This study is part of ongoing research involving the delivery of a cytotoxic dose of radiation to cancer cells.

Introduction

Ionizing radiation is harmful to cells. It can cause chromosome aberrations and DNA double-strand breaks which if left un-repaired could lead to cell death (Franks and Teich 1997). These effects, though detrimental in normal cells, provide a powerful tool in fighting cancer. If cancerous cells can be irradiated effectively they can be killed, or at least damaged enough so they cannot multiply. It is therefore important to investigate relationships between radiation and DNA double-strand break induction.

Ionizing radiation is any radiation with sufficient energy to ionize an atom by displacing one of its orbital electrons (Franks and Teich 1997). The type of ionizing radiation used in this study is gamma radiation. Gamma radiation is wave like in nature, and falls into the electromagnetic spectrum with X-rays. In fact, the only difference between gamma and X-rays is their origin. Gamma rays originate from atoms, whereas machines produce X-rays. Gamma emitters release a photon or "packet of energy" when an electron from a higher orbital falls to fill a void in a lower orbital, the energy of the wave or photon emitted is directly proportional to the difference in energy between the two orbitals (Franks and Teich 1997). Due to their wave-like nature gamma rays are more penetrating than many other types of radiation and in turn release their energy over a longer distance. This classifies gamma rays as low linear energy transfer radiation, or low

LET. Linear energy transfer is a measurement of energy deposition over a given distance. Particulate radiation such as alpha emitters are high LET radiation, due to the relatively massive nature of their emissions which slows the particle down, forcing it to deposit its energy over a shorter distance (Franks and Teich 1997). Gamma rays can cause damage in several ways, most notable however is the Compton effect. This effect occurs when a photon hits a cell and ionizes a molecule (most likely H_2O) and is deflected away with a lower energy, which in turn will ionize another molecule, and so on until the wave does not have enough energy to ionize any further. The molecules, which are now ionized, are highly unstable (reactive oxygen species) and can strip an electron from a neighboring molecule, this in turn, with the deflecting photon, produces multiple paths of secondary ionizations. Damage is most notable when DNA is ionized directly or indirectly from secondary ionizations, resulting in DNA double-strand breaks (Franks and Teich 1997). It has been shown by Rydberg that irradiation of mammalian cells results in multiple DNA double-strand breaks over a distance of perhaps a few kilobases (Rydberg 1996). Rydberg proposed that this is due to the organization of DNA in the cell. The ionizing radiation transverses the 30-nm chromatin fiber of DNA causing clustered DNA damage resulting in small DNA fragments that can be detected using agarose gel electrophoresis (Rydberg 1996). These small DNA fragments are the molecules of interest for the present study.

There are several different approaches to modeling DNA double-strand break induction, including the Distribution Shape model, Fraction of Activity Released model, and the Broken Stick model, all of which are addressed later. Pulsed-field gel electrophoresis and Constant-field gel electrophoresis are common techniques for these models; only the Distribution Shape model relies on a different technique (neutral sucrose gradient). The two types of gel electrophoresis rely on different principles to separate DNA in agarose gels, but share the same patterns of DNA migration, larger fragments of DNA move slower through the agarose than smaller fragments of DNA. Constant-field gel electrophoresis draws negatively charged DNA from the cathode to the anode by applying a constant voltage across the gel (Willis 1988). The electrophoretic mobility is known to be a sigmoidal function of the logarithm of the molecular weight of the DNA fragment (Willis 1988). Pulsed-field gel electrophoresis moves DNA molecules by changing the magnetic fields applied to the DNA. DNA is pulled toward the anode, forcing the molecules to re-orient themselves with respect to the magnetic field; larger DNA molecules re-

orient themselves more slowly than smaller ones (Chu 1990). Pulsed-field gel electrophoresis techniques are very sensitive to detection of DNA double-strand breaks at very low doses (less than 5 Gy) and work with a much larger size range of molecules, including whole chromosomes, where constant-field techniques only allow reliable separation to about 100kb (Wlodek 1991). Pulsed-field gel electrophoresis is more costly and time consuming than constant field techniques and require more skillful operation. However it has been shown by Wlodek et. al. that when constant-field techniques are applied to separate fragments of less than 100kb it is as reliable as pulsed field gel electrophoresis (Wlodek 1991). In the present study DNA fragments are restricted to between 100 and 1000 bp, so constant field gel electrophoresis serves as a more economical approach to surveying DNA double-strand breaks without sacrificing any sensitivity.

Materials and Methods

The assay used in this study has been previously described by Heilmann (Heilmann 1995) and is used here with minor modifications. The modifications include radiation source, cell line, and data collection methods. In the original Heilmann protocol accelerated carbon ions are used where gamma rays from a ^{137}Cs source are used here. Cells were irradiated on ice in PBS for 9 minutes 26 seconds per 25 Gy up to 100 Gy.MCF-7 cells, a human breast cancer, line was used for this study and were cultured and maintained using methods presented by Katzenellenbogen (Katzenellenbogen 1997).

Data is obtained in this study through liquid scintillation counting. Once the gel has been ran it is stained in ethidium bromide, then destained and photographed with molecular weight markers intact, using a UV box and a DS-34 Polaroid direct screen instant camera with a DS-H-7 0.7x hood with an orange filter. Each lane of the gel is then sliced into 1cm x 1cm cubes (minus molecular weight markers) and put into liquid scintillation vials with 5ml 1 N HCl and melted in a oven set at 70°C for 1 hour. After melting 5 mls scintillation fluid is added and then the vials are placed in the liquid scintillation counter. Count per minute data is obtained for each vial; background radiation is subtracted from each vial and then total counts for each lane is summed up. The counts per minute from the first cube of the lane, which corresponds to the well of the gel, is divided by the total counts per minute, resulting in the fraction of activity retained in the well or plug. Promega lambda/Hind III markers are used to produce a standard curve

(molecular weight vs. distance migrated), which is then used to estimate the average size of the DNA fragments at each dose.

Results

In the Heilmann study it is possible to determine actual numbers of DNA double strand breaks per dose unit using an equation presented in that paper (Heilmann 1995). The equation presents the average number of DNA double-strand breaks as a function of the fraction of activity retained in the well, where F_{ret} equals the fraction of activity retained in the well/plug; aD (alpha x Dose) equals the number of DNA double-strand breaks per unit dose; k is equal to the exclusion size of the gel (6Mbp); and N is equal to the mean size of the chromosomes (Heilmann 1995).

$$F_{ret} = e^{(-aD(k/N))}(1+aD(k/N)(1-(k/N))$$

However quantitative estimates of DNA double-strand break induction from this equation are not presented here.

Presented here is data from three separate experiments. Dose response curves are presented for each experiment as well as average fragment size data estimated from standard curves (standard curves not shown). It should be noted that base pair data is strictly an estimate and not statistically sound since the markers used did not flank the DNA bands on the gel. They are presented to confirm a general trend in the size of DNA fragments with regard to increasing dose. A better estimate could be achieved if proper molecular weight markers were utilized.

Table 1

		0 Gy	25 Gy	50 Gy	75 Gy	100 Gy
Exp. 1						
	FAR	**.993**	**.884**	**.830**	**.668**	**.634**
	Est. bp	**96783**	**45384**	**42491**	**39783**	**38495**
Exp. 2						
	FAR	**.980**	**.930**	**.855**	**.798**	**.735**
	Est. bp	**52420**	**36864**	**41233**	**38677**	**38063**
Exp. 3						
	FAR	**NA**	**.916**	**.764**	**.593**	**.605**
	Est. bp	**NA**	**37060**	**34040**	**32166**	**31268**

Presented below are graphical representations of the data in Table 1. Although the data is sometimes inconsistent, most likely these inconsistencies are the result of technical errors in the protocol. The FAR profiles show a linear increase of DNA double-strand breaks with increasing dose. DNA fragments also become smaller as dose is increased. These results are consistent with predictions made by several models of DNA double-strand break induction.

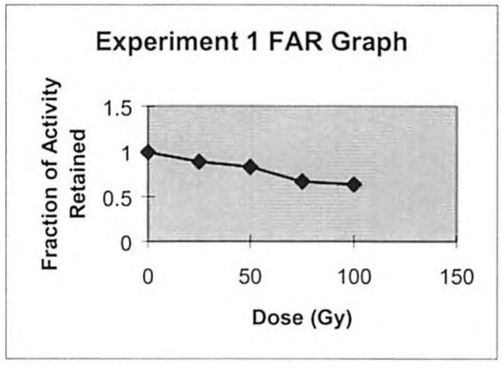

Fig1

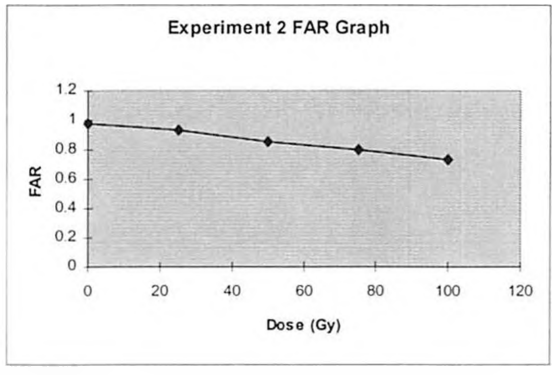

Fig2

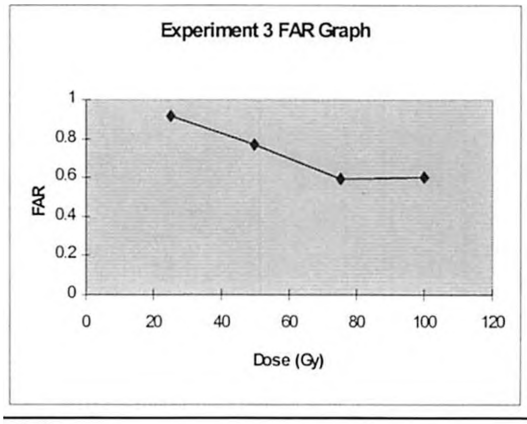

Fig3

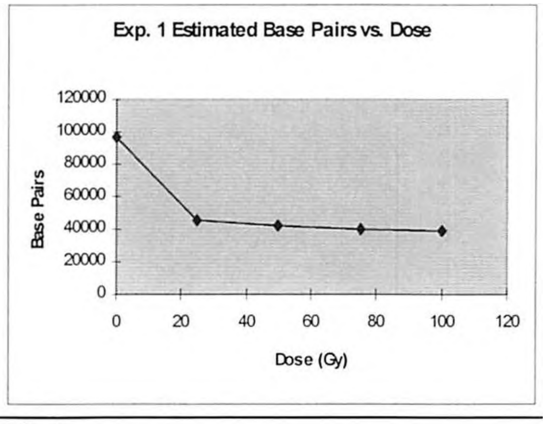

Fig 4

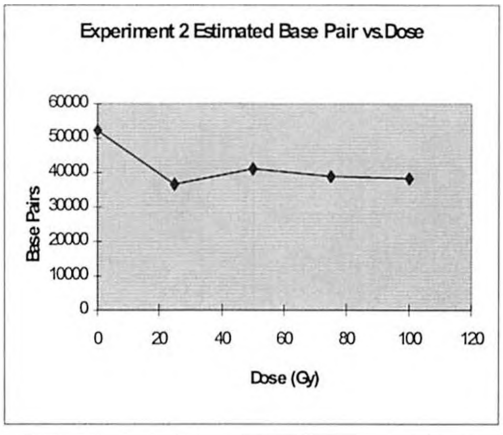

Fig 5

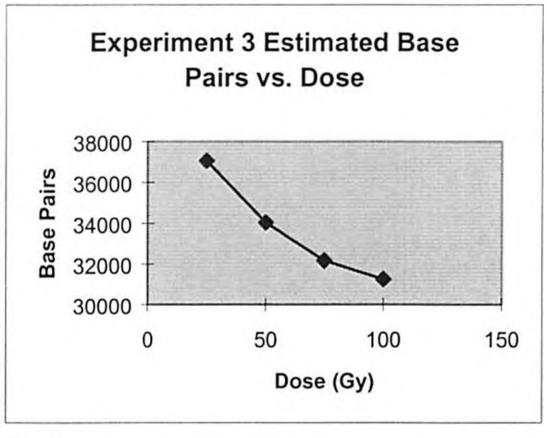

Fig 6

Discussion

From the experimental results various trends regarding DNA double-strand break induction are shown. First there is a linear induction of DNA double-strand breaks with increasing dose (Fig. 1-3). The FAR profiles indicate a good fit with a linear type response. Second it should be noted that with increasing doses, DNA fragments become smaller and smaller indicating cluster type DNA damage (Fig 4-6). These results are consistent with results reported previously by Rydberg (Rydberg 1996). Rydberg showed that small fragments of DNA are generated by ionizing particles (X-rays and accelerated ions) transversing the 30-nm chromatin fiber of DNA which generate multiple DNA double-strand breaks over a short stretch of DNA (within a few kilobases) and increase linearly with dose. Rydberg showed that small sizes of irradiated DNA would elute into the gel while the majority of the DNA at low doses will remain in the well. This suggests that the resulting DNA fragmentation is due to the organization of the DNA in the cell. The DNA double-strand breaks are localized and produce multiple DNA double-strand breaks over a short distance leaving the rest of the DNA relatively intact. This is evident when the cells are run on agarose gel, The DNA that has suffered few or no DNA double-strand breaks will be too large to elute into the gel and will remain in the well. This is what is reported here, up to 98% of the DNA is retained in the well at low doses in this study, suggesting large undamaged complexes are still present in the well. Upon increasing dose more and more DNA is fragmented by double strand breaks resulting in a larger proportion of the DNA that is able to move into the gel. Rydberg uses similar methods as described in this study to experimentally detect DNA fragments within the size range of interest (a few kb) but it should be noted that the amount of radiation used was in some cases as much as 10 fold more than used here (Rydberg 1996).

This along with the equation presented by Heilmann is essentially the Fraction of Activity Released model of quantifying DNA double-strand breaks, as it is applied today. We will look at the original FAR model by Blocher (Blocher 1990) a little later. The remainder of this discussion will focus on other models of DNA double-strand break induction.

The Distribution Shape Model of DNA double-strand break inductions, comes from a study of the effects of X-ray dosage on murine

cells. A.R. Lehmann and M.G. Ormerod published the study, titled "Double-Strand Breaks in the DNA of Mammalian Cells After X-ray irradiation", in 1970 (Lehmann 1970).

The authors use murine lymphoma cells (L5178Y) irradiated with X-rays and then the molecular weight of the DNA was assessed using neutral sucrose gradient techniques (Lehmann 1970). In this experiment irradiated cells are lysed on top of the gradient and centrifuged for several hours at varying speeds and times to produce the desired separation. The study makes a note about the effects of rotor speed upon sedimentation of the DNA; low rotor speeds need to be used for low doses of separation. This low rotor speed is used because at low doses the molecules that are run through the gradient are nearing the size of intact chromosomes, which will not freely sediment into the gradient (Lehmann 1970).

The molecular weight of the DNA that centrifuged through the gradient was determined by an equation from Lehmann and Omerod (Lehmann 1970).

$$(M/1.3x10^{8})^{.3456}=D/D_r$$

To use this equation, the authors run their sample of DNA with a sample of known molecular weight and solve for M using the ratio of the distance migrated between the known and unknown sample (Lehmann 1970).

The authors address three criteria that must be met for this method to produce meaningful results. The first is that sedimenting DNA is pure, not contaminated with any other cellular material. The second is that the DNA sediments according to theory and the last is that all the DNA must be recovered from the gradient. To ensure that these criteria are met some experiments were performed. One experiment included checking the sedimenting DNA bands for contamination. This is done by double-labeling techniques where DNA is labeled with one radiotracer, and lipids, RNA and other proteins are labeled differently. The study showed that there is no contamination of the DNA in these experiments (Lehmann 1970). Radioactivity profiles are also produced to assert the randomness of breaks. The assumption of the randomness of breaks is essential to the criteria that DNA sediments according to theory (Lehmann 1970). This assumption of random break induction is a central assumption in all of the DNA double strand break models presented here.

The results of Lehmann's study show a linear relationship between DNA double-stand break induction and dose (Lehmann 1970). These findings agree with the data presented in the present gamma studies,

however it should be noted that Lehmann used much more radiation than used in the gamma studies.

In the study " In CHEF electrophoresis a linear induction of dsb corresponds to a nonlinear fraction of extracted DNA with dose", D. Blocher investigates the use of pulsed-field electrophoresis as a tool to find a relationship between double-strand break induction and dose. This is in fact the original Fraction of Activity release model. Heilmann and Rydberg have applied this model for use with constant field gel electrophoresis. The author addresses the question " Are dsb induced predominantly linearly with dose or in a linear-quadratic manner?"(Blocher 1990). At this point in time, different methods had produced different results, Lehmann had reported a linear induction with dose using neutral gradient sedimentation (Lehmann 1970), where Randford and Hodgson reported a linear-quadratic double-strand break induction using neutral filter elution data (Blocher 1990). The author investigates this topic using the concept of size distribution of DNA fragments, and the results are compared with experimental data produced using CHEF electrophoresis (Blocher 1990).

The author derives a mathematical expression for the induction of double-strand break based on derivations from polymer equations described by Montroll and Simha (Blocher 1990). The equations begin by describing the total monomer mass of a polymer consisting of a finite number of monomers. Substitutions were then made to the equation in order to apply it to the mammalian genome. These equations are then summed up for all fragment sizes from 0 to k base pairs, which lead to an equation that describes the fraction of base pairs, which contribute to fragments with up to k base pairs (Blocher 1990). Next in the derivation, this fraction of base pairs is calculated for each chromosome, and multiplied by its weight contribution to the total weight of all the chromosomes (total nuclear content) then all of the terms are summed up for the entire genome (Blocher 1990). The result is this equation:

$$f_{<k} = (\Sigma_i n_i F_{<k,I}) / \Sigma_i n_i$$

This equation gives a fraction of DNA total mass below a certain number of base pairs as a function of absorbed dose, or essentially the fraction of DNA released into the gel. The authors then insert values for estimated random double- strand break induction, genome size and other variables. The theoretical results are graphed at various doses (0-100 Gy).

These results graph linearly, except they appear to have " shoulders at low doses" and "saturation at high doses" (Blocher 1990).

The author now produces experimental data using CHEF electrophoresis to compare to the theoretical data. The methods of CHEF electrophoresis are typical of methods used to quantify double-strand break induction: cells were labeled, mixed with agarose, irradiated in medium, put into lysis, DNA separated using pulsed-field gel electrophoresis, then labeled DNA is quantified in a liquid scintillation counter. The author quantified the amount of DNA left in the wells, and the amount that ran into the gel (Blocher calls this " the extracted DNA").

The results for the experimental data are in agreement with the theoretical data. Blocher uses the experimental results to confirm the theoretical results since the shoulder and saturation effects are seen in the graphed experimental results. Blocher suggest the saturation effects is due to the fact that at high absorbed doses of radiation all the DNA has been fragmented below k base pairs (Blocher 1990). Blocher then shows that if the theoretical expression contained even a small quadratic term, the results deviated from the experimental data (Blocher 1990). Blocher then concludes that the induction of double-strand break induction is linear as a function of absorbed dose, and irregularities in the graphical results are due to size distribution effects rather than a quadratic term in the mathematical expression (Blocher 1990).

Experimental data produced in this present study agrees with a linear induction of DNA double-strand breaks. However, since irradiation was limited to 100 Gy no saturation effect was observed for the studies involving gamma radiation.

In the study "A Quantitative Model of DNA Fragments Generated by Ionizing Radiation, and Possible Experimental Applications", Vincent E. Cook and Robert K. Mortimer derive a model of DNA double-strand induction. This model is known as the Broken Stick Model of DNA double strand break induction. In this model, they derive an equation for the observed frequency of DNA fragments as a function of size. In this experiment DNA fragments are generated at random using low-LET ionizing radiation and are then separated by pulse-field gel electrophoresis. This resulted in a smear of DNA fragments. These smears, measured as a function of their size, allow measurement of the parameters of the break induction. However, to look for specific fragments (i.e. genes or specific pieces of chromosomes) they used the Southern blot assay.

To model the break induction the authors made three assumptions. The first was that breaks at any given location in the genome is equiprobable, in other words the randomness of breaks. Under this assumption the authors predict that that the number of double-strand breaks is then proportional to the length of the molecule. Under this assumption the Broken-Stick distribution model describes the frequency of the fragment size for a given number of breaks (Cook 1991).

The second assumption was that the probe in the Southern blot hybridizes with only one fragment of the broken chromosome or molecule, and that fragment contains a point hybridization site. This assumption is only valid when the point hybridization site is small compared to the size of the molecule and small with regards to the resolution size of the gel system (Cook 1991).

The third assumption was that every probed fragment contributes equally to the measured smear intensity (Cook 1991). To validate this assumption the authors assert that the Southern blot assay must by very skillfully performed.

Under these assumptions, the model proposes that there are three cases that contribute to the overall frequency of the probed fragment F(x). There is the case where all breaks occur to the right of the hybridization site, and then conversely all breaks on the left of the site, and then when breaks are present on both sides. For this model the right side of the hybridization site is designated as the larger region, while the smaller region is designated as the left. For the first two cases, since the number of breaks can range from 1 to infinity and are located on only one side of the hybridization site the authors give us a summation (1to infinity) consisting of the product of the frequency for an unbroken chromosome (no double-strand breaks) and the probability of a fragment size for a given number of breaks (Cook 1991).

The contribution for the third case is more difficult. Since now there are breaks on both sides of the hybridization site, and either side can have between 1 and infinite breaks this now requires a double summation. This double summation consists of the products of the frequency of an unbroken chromosome and the convolution integral of the two independent Broken-Stick distributions for the left and right side. The contributions of all three cases added together yields an equation for the frequency of the probed fragments (Cook 1991).

The study takes an entirely theoretical approach yet they conclude the study by suggesting several experimental applications for this model. The

authors propose this model as an effective way to estimate DNA double-strand breaks per unit length of DNA (Cook 1991). They also state that with experimental determinations of DNA double-strand breaks vs. dose in this system, should be able to discriminate between linear and non-linear induction of DNA double-strand breaks. This would be the application of interest if it were to be applied to gamma studies.

The results reported for the present gamma radiation study, a linear induction of DNA double-strand breaks, is in close agreement with several published models of DNA double-strand break induction. This is an important step in the characterization of gamma radiation for its potential to deliver DNA double-strand breaks and ultimately cell death. Future research focus includes actual quantification of the absolute number of DNA double-strand breaks from the Heilmann equation as well as developing potentially more effective ways of delivering radiation to cancer cells.

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