1

**PM-1 3 1/2" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT**

PRECISIONSM RESOLUTION TARGETS

**The prediction of patient radiosensitivity using the clonogenic assay and
asymmetric field inversion gel electrophoresis**

by

Ruth Caro Brown, MSc

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

Doctor of Philosophy

**Department of Physics
Carleton University
Ottawa-Carleton Institute of Physics
Ottawa, Ontario**

July, 1996

© copyright

1996, Ruth C. Brown

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa (Ontario)
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395 rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file / Votre référence

Our file / Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-19363-2

Canada

Name RUTH CARO BROWN

Dissertation Abstracts International is arranged by broad general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

RADIATION PHYSICS

SUBJECT TERM

0756

U·M·I

SUBJECT CODE

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS

Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION

General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0777
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523

PSYCHOLOGY

Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS

Language 0679
General 0289
Ancient 0290
Linguistics 0291
Modern 0401
Literature 0294
General 0295
Classical 0297
Comparative 0298
Medieval 0316
Modern 0591
African 0305
American 0352
Asian 0355
Canadian (English) 0593
Canadian (French) 0311
English 0312
Germanic 0315
Latin American 0313
Middle Eastern 0314
Romance 0370
Slavic and East European 0372

PHILOSOPHY, RELIGION AND THEOLOGY

Philosophy 0422
Religion 0318
General 0321
Biblical Studies 0319
History of 0320
Philosophy of 0322
Theology 0469

SOCIAL SCIENCES

American Studies 0323
Anthropology 0424
Archaeology 0326
Cultural 0427
Physical 0310
Business Administration 0310
General 0272
Accounting 0270
Banking 0454
Management 0318
Marketing 0385
Canadian Studies 0501
Economics 0503
General 0504
Agricultural 0505
Commerce Business 0508
Finance 0509
History 0510
Labor 0511
Theory 0358
Folklore 0366
Geography 0357
Gerontology 0357
History 0478
General 0478

Ancient 0579
Medieval 0581
Modern 0582
Black 0328
African 0331
Asia 0332
Australia and Oceania 0334
Canadian 0335
European 0336
Latin American 0337
Middle Eastern 0338
United States 0339
History of Science 0398
Law 0398
Political Science 0615
General 0616
International Law and 0616
Relations 0617
Public Administration 0617
Recreation 0814
Social Work 0452
Sociology 0626
General 0627
Criminology and Penology 0938
Demography 0631
Ethnic and Racial Studies 0628
Individual and Family 0629
Studies 0630
Industrial and Labor 0629
Relations 0630
Public and Social Welfare 0700
Social Structure and 0700
Development 0709
Theory and Methods 0999
Urban and Regional Planning 0453
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES

Agriculture 0473
General 0285
Agronomy 0475
Animal Culture and 0476
Nutrition 0359
Animal Pathology 0478
Food Science and 0479
Technology 0480
Forestry and Wildlife 0817
Plant Culture 0777
Plant Pathology 0746
Plant Physiology 0306
Range Management 0287
Wood Technology 0308
Biology 0309
General 0379
Anatomy 0329
Biostatistics 0353
Botany 0369
Cell 0793
Ecology 0410
Entomology 0307
Genetics 0317
Limnology 0416
Microbiology 0433
Molecular 0821
Neuroscience 0778
Oceanography 0472
Physiology 0786
Radiation 0760
Veterinary Science 0786
Zoology 0760
Biophysics 0786
General 0760
Medical 0760

EARTH SCIENCES

Biogeochemistry 0425
Geochemistry 0996

Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES

Environmental Sciences 0768
Health Sciences 0566
General 0300
Audiology 0992
Chemotherapy 0567
Dentistry 0350
Education 0769
Hospital Management 0758
Human Development 0982
Immunology 0564
Medicine and Surgery 0347
Mental Health 0569
Nursing 0570
Nutrition 0380
Obstetrics and Gynecology 0354
Occupational Health and 0381
Therapy 0571
Ophthalmology 0419
Physiology 0572
Pharmacology 0382
Pharmacy 0573
Physical Therapy 0574
Public Health 0575
Radiology 0575
Recreation 0575

Speech Pathology 0460
Toxicology 0384
Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences 0485
Chemistry 0486
General 0487
Agricultural 0488
Analytical 0488
Biochemistry 0488
Inorganic 0488
Nuclear 0488
Organic 0488
Pharmaceutical 0488
Physical 0488
Polymer 0488
Radiation 0488
Mathematics 0605
Physics 0606
General 0607
Acoustics 0608
Astronomy and 0608
Astrophysics 0608
Atmospheric Science 0608
Atomic 0608
Electronics and Electricity 0608
Elementary Particles and 0608
High Energy 0608
Fluid and Plasma 0608
Molecular 0608
Nuclear 0608
Optics 0608
Radiation 0608
Solid State 0608
Statistics 0608

Applied Sciences

Applied Mechanics 0346
Computer Science 0984

Engineering 0537
General 0538
Aerospace 0539
Agricultural 0540
Automotive 0541
Biomedical 0542
Chemical 0543
Civil 0544
Electronic and Electrical 0545
Heat and Thermodynamics 0546
Hydraulic 0547
Industrial 0548
Marine 0549
Materials Science 0550
Mechanical 0551
Metallurgy 0552
Mining 0553
Nuclear 0554
Packaging 0555
Petroleum 0556
Sanitary and Municipal 0557
Systems Science 0558
Ceramics 0559
Computations Research 0560
Plastics Technology 0561
Textile Technology 0562

PSYCHOLOGY

General 0621
Behavioral 0622
Clinical 0623
Developmental 0624
Experimental 0625
Industrial 0626
Personality 0627
Physiological 0628
Psychobiology 0629
Psychometrics 0630
Social 0631

The undersigned hereby recommend to
the Faculty of Graduate Studies and Research
acceptance of the thesis,

**The prediction of patient radiosensitivity using the clonogenic assay and asymmetric field
inversion gel electrophoresis**

submitted by

Ruth Caro Brown, MSc

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Chair, Department of Physics

Thesis Supervisor

External Examiner

Carleton University

ABSTRACT

Radiation therapy is usually administered with the assumption that all patients respond equally to treatment. This is not the case and researchers have been trying to find ways to predict how patients will respond to radiotherapy by predicting both tumour response and normal tissue response. In radiotherapy, however, the dose of radiation that can be delivered to the tumour is limited by the dose that can be tolerated by the normal tissue in the treatment field. Therefore, it is more important to predict normal tissue response in order to deliver the maximum dose possible to the tumour to maximize the probability of tumour control. Previous studies have used surviving fractions after low doses (2 Gy) of radiation, but it is doubtful whether differences in survivals at this low level of cell kill are measurable.

In this thesis, several radiation protocols were examined for predictive value including survival after high doses (6 Gy) at both high (112 cGy/min) and low (882 cGy/min) dose rates, fractionated doses (12 Gy in 6 fractions) and when 6 h of repair time is allowed after a single high dose rate irradiation (HDRI). Two pairs of cell lines were studied: a pair of melanoma cell lines, SKMEL3 and HT144, which are radioresistant and radiosensitive respectively and a pair of primary fibroblast cell lines grown from skin samples from humans. The OMB1 cell line was from an apparently normal volunteer and the S11358 cell line was from a patient who had an extremely sensitive reaction to radiation therapy. It was found that, with the clonogenic assay, the radiation protocol

which best distinguished between the sensitivities of the two cell lines was fractionation

The clonogenic assay, however, is too slow to be useful in the clinic and a faster assay is required. The asymmetric field inversion gel electrophoresis (AFIGE) assay which measures DNA double strand breaks was examined as a possible assay. This assay, which can be completed in a few days, was only able to resolve differences in radiation response between the melanoma cell lines after HDRI. It did, however, point out that cell lines have different sensitivities for a variety of reasons and that more than one assay will be needed to predict the radiosensitivity of a patient prior to therapy.

ACKNOWLEDGMENTS

In the completion of this thesis, I have had the assistance and encouragement of many people and would like to take this opportunity to thank them all. In particular, my thesis supervisor Peter Raaphorst who supported me, not only financially, but scientifically and provided me with the benefit of his scientific knowledge and experience. I am also indebted to Cheng Ng who gave me emotional support, encouragement and never ending good advice.

I would like to thank the Radiation Oncologists at the Ottawa Regional Cancer Centre who supported my research by providing me with skin samples from radiosensitive patients. These doctors include Dr. M. Reid, Dr. P. Cross, Dr. C. Lochrin, and Dr. L. Grimard. Also Dr. P. Drouin from the Notre Dame Hospital in Montreal and Dr. M. Brundage from the Kingston Regional Cancer Centre who also supplied me with tissue samples. I would also like to thank the patients who donated their tissue.

I am also grateful my fellow researchers at the Ottawa Regional Cancer Centre, from technicians to graduate students. In particular, I thank Alexandra Vincent for her biochemical expertise and help setting up the AFGE assay, patience as I ventured into a new field of research but mostly for her friendship. I would also like to thank Dong Ping Yang for her assistance in several experiments allowing me to make more efficient use of my time. I am grateful to Sheryl Cybulski who ran my flow cytometry samples, kept the lab running smoothly, made it a pleasant atmosphere in which to work and distracted me with coffee breaks.

Most importantly, I would like to thank Dave Wilkins. Although, at the beginning of my thesis, he was just a fellow graduate student, he has become my best friend and husband. He has been an essential editor of this thesis but more importantly has given me love, support and encouragement throughout the development of this research.

TABLE OF CONTENTS

Acceptance sheet	ii
Abstract	iii
Acknowledgements	v
Table of contents	vi
List of abbreviations	viii
List of figures	ix
List of tables	xi
 1 0 INTRODUCTION	 1
1 1 Predictive assays	1
1 2 Mechanisms of cell kill	3
1 2 1 Molecular effects	5
1 2 2 Cellular effects	7
1 3 The Clonogenic assay	7
1 3 1 Modelling cell survival	7
1 3 1 Clonogenic assay as a predictor of radiosensitivity	8
1 3 Asymmetric Field Inversion Gel Electrophoresis	10
1 3 1 Theory of gel electrophoresis	10
1 3 2 Measurement of DNA dsbs as a predictive assay	12
1 4 Hypothesis and specific aims	14
 2 0 METHODS AND MATERIALS	 16
2 1 Fibroblast growth	16
2 2 Melanoma Cell Lines	18
2 3 Clonogenic Methods	18
2 3 1 General tissue culture methods	18
2 3 2 Colony forming assay	21
2 3 3 Curve Fitting	22
2 4 AFIGE Methods	23
2 5 Irradiations	29

2 5 1 High Dose Rate	29
2 5 2 Low Dose Rate	30
2 6 Flow Cytometry	31
3 0 RESULTS	32
3 1 Clonogenic Results	32
3 1 1 Melanoma cell lines	32
3 1 2 Fibroblasts cell lines	46
3 2 AFIGE results	58
3 2 1 Melanoma results	58
3 2 3 Fibroblasts results	67
3 3 Clonogenic vs AFIGE	75
3 4 Other patients	80
3 5 Inter-patient variability	98
3 6 Time scale of the AFIGE assay	104
4 0 DISCUSSION	107
4 1 Clonogenic Assay	107
4 1 1 Melanoma cell lines	107
4 1 2 Fibroblast cell lines	111
4 2 AFIGE assay	113
4 2 1 Melanoma cell lines	114
4 2 2 Fibroblasts	118
4 3 Clonogenic vs AFIGE	120
4 4 Other patients	122
4 5 Inter-patient variability	123
4 6 Time scale of AFIGE assay	124
5 0 CONCLUSIONS	125
5 1 Future Directions	128
REFERENCES	130
APPENDIX I	142

LIST OF ABBREVIATIONS

AFIGE	asymmetric field inversion gel electrophoresis
AT	ataxia telangiectasia
ATCC	American Type Culture Collection
CHEF	clamped homogeneous field gel electrophoresis
DNA	deoxyribonucleic acid
dsb	double strand break
DRSE	dose rate sparing effect
FAR	fraction of activity released
FIGE	field inversion gel electrophoresis
FRD	fraction of rejoined damage
HDR	high dose rate
HDRI	high dose rate irradiation
LDR	low dose rate
LDRI	low dose rate irradiation
LQM	linear quadratic model
Mbp	megabase pairs
PE	plating efficiency
PFGE	pulsed field gel electrophoresis
PLD	potentially lethal damage
PLDR	potentially lethal damage repair
RNA	ribonucleic acid
RR	repair ratio
SEM	standard error of the mean
SLD	sublethal damage
SF	surviving fraction
SF ₂	surviving fraction after 2 Gy
SF _{3.5}	surviving fraction after 3.5 Gy
SF ₅₀	dose required to reduce survival to 50%
SF ₁₀	dose required to reduce survival to 10%
ssb	single strand break
T _{1/2}	half-time of repair

LIST OF FIGURES

	page
Figure 1 2 1 Schematic of DNA damage following ionizing radiation	4
Figure 1 2 2 Schematic of the major types of DNA lesions caused by radiation	4
 Figure 2 3 1 Growth curves of OMB1 and S11358	 20
Figure 2 4 1 Comparison of FAR between cells irradiated in plugs of flasks	24
Figure 2 4 2 Schematic of AFIGE apparatus	27
Figure 2 4 3 Diode box circuit diagram	27
Figure 2 4 4 Example of typical gel of SKMEL3 cells after HDRI	28
 Figure 3 1 1 Survival curves of SKMEL3 and HT144 after HDRI	 41
Figure 3 1 2 Survival curves of SKMEL3 and HT144 after LDRI	42
Figure 3 1 3 Survival curves of SKMEL3 and HT144 after fractionated irradiation	43
Figure 3 1 4 Cell cycle analysis SKMEL3 and HT144 during fractionated irradiation	44
Figure 3 1 5 Survival curves of SKMEL3 and HT144 after HDRI and 6 h of repair	45
Figure 3 1 6 Survival curves of OMB1 and S11358 after HDRI	52
Figure 3 1 7 Survival curves of OMB1 and S11358 after LDRI	53
Figure 3 1 8 Survival curves of OMB1 and S11358 after fractionated irradiation	54
Figure 3 1 9 Cell cycle analysis of OMB1 and S11358 during fractionated irradiation	55
Figure 3 1.10 Survival curves of OMB1 and S11358 after HDRI and 6 h of repair	56
Figure 3 1 11 Recovery ratios after a 6 Gy acute irradiation for fibroblasts	57
 Figure 3 2.1 FAR vs. dose of SKMEL3 and HT144 after HDRI	 63
Figure 3 2.2 FAR vs. dose of SKMEL3 and HT144 after LDRI	64
Figure 3 2 3 FAR vs. dose of SKMEL3 and HT144 after fractionated irradiation	65
Figure 3 2 4 FAR vs. dose of SKMEL3 and HT144 after HDRI and 6 h of repair	66
Figure 3 2 5 FAR vs. dose of OMB1 and S11358 after HDRI	71
Figure 3 2 6 FAR vs. dose of OMB1 and S11358 after LDRI	72
Figure 3 2 7 FAR vs. dose of OMB1 and S11358 after fractionated irradiation	73

Figure 3 2 8	FAR vs dose of OMB1 and S11358 after HDRI and 6 h of repair	74
Figure 3 3 1	Surviving fraction vs FAR for SKMEL3 and HT144 after HDRI	77
Figure 3 3 2	Surviving fraction vs FAR for OMB1 and S11358 after HDRI	78
Figure 3 3 3	SF vs FAR for SKMEL3 and HT144 after HDRI and 6 h of repair time	79
Figure 3 4 1	Survival curves of OMB1, OMB7 and S11358 after fractionation	86
Figure 3 4 2	Survival curves of OMB1, OMB8 and S11358 after HDRI	87
Figure 3 4 3	Survival curves of OMB1, OMB8 and S11358 after fractionation	88
Figure 3 4 4	Survival curves of OMB1, OMB9, OMB10 and S11358 after HDRI	89
Figure 3 4 5	Survival curves of OMB1, OMB9, OMB10 and S11358 after LDRI	90
Figure 3 4 6	Survival curves of OMB1, OMB9, OMB10 and S11358, fractionation	91
Figure 3 4 7	Survival curves of OMB1, OMB13 and S11358 after HDRI	92
Figure 3 4 8	Survival curves of OMB1, OMB13 and S11358 after LDRI	93
Figure 3 4 9	Survival curves of OMB1, OMB13 and S11358 after fractionation	94
Figure 3 4 10	Growth curves of OMB1, OMB14 and S11358	95
Figure 3 4 11	Survival curves of OMB1, OMB14 and S11358 after HDRI	96
Figure 3 4 12	Survival curves of OMB1, OMB14 and S11358 after fractionation	97
Figure 3 5 1	Growth curves of OMB12A and OMB12G	100
Figure 3 5 2	Survival curves of OMB12 after HDRI	101
Figure 3 5 3	Survival curves of OMB12 after fractionated irradiation	102
Figure 3 5 4	FAR vs dose of OMB12 after HDRI	103
Figure 3 6 1	FAR vs dose for cells assayed directly from tissue	106
Figure A1 1	Cesium sources before Theraplan	145
Figure A1 2	Cesium sources after Theraplan with shielding	145
Figure A1 3	Vial holder from above	149
Figure A1 4	Vial holder from the side	149
Figure A1 5	Final design of incubator and irradiator	150

LIST OF TABLES

Table 3.1.1 Comparison between the surviving fractions of SKMEL and HT144	34
Table 3.1.2 Doses which reduce cell survival to 50% and 10% for melanoma cells	35
Table 3.1.3 Ratios of SF after various radiation protocols for melanoma cells	37
Table 3.1.4 Ratio of doses which reduce cell survival to 50% and 10%	37
Table 3.1.5 Comparison between the survival of fibroblasts after various doses	47
Table 3.1.6 Doses which reduce cell survival to 50% and 10% for fibroblasts	47
Table 3.1.7 Ratios of SF after various radiation protocol for fibroblasts	49
Table 3.1.8 Ratio of doses which reduce cell survival to 50% and 10%	49
Table 3.2.1 Slopes of lines from AFIGE assay for SKMEL3 and HT144 in FAR/Gy	60
Table 3.2.2 FRD after various radiation protocols for SKMEL3 and HT144	61
Table 3.2.3 Slopes of lines from AFIGE assay for OMB1 and S11358 in FAR/Gy	68
Table 3.2.4 FRD after various radiation protocols for OMB1 and S11358	69
Table 3.3.1 FAR required to reduce the SF to 10% for SKMEL3 and HT144	76
Table 3.3.2 FAR required to reduce the SF to 10% for OMB1 and S11358	76
Table 3.4.1 Details of patients showing sensitive response to radiation therapy	84
Table 3.4.2 Summary of responses of radiosensitive patients	85
Table A-1. Cesium source identification and activity measured on June 28, 1992	142
Table A-2 Dose rate for varying distances from source plane	144
Table A-3. Dose-rates before and after installation of Cesium irradiator	150

1.0 INTRODUCTION

1.1 Predictive assays

Radiation therapy is usually administered with the assumption that all patients respond equally to treatment. In fact, this is not the case as has been shown in several case studies [1,2]. For many years, researchers have been trying to find ways to predict how patients will respond to radiotherapy by predicting both tumour response [3-9] and normal tissue response [10-16]. In radiotherapy, however, the dose of radiation that can be delivered to the tumour can be limited by the dose that can be tolerated by the normal tissue in the treatment field. It is, therefore, more important to predict normal tissue response since it is desirable to deliver the maximum dose possible to the tumour in order to maximize the probability of tumour control.

In the clinic, radiotherapy treatments are planned to treat to the maximum tolerance of the sensitive patients who make up only a small percentage of the population. The radiation dose delivered is given so that up to 5% of the patients could have a complication within 5 years of treatment (TD 5/5) [17]. If this 5% of sensitive patients could be identified prior to treatment, the dose given to the remaining patients could be increased which could potentially increase local control and cure by up to 20% [18]. The prediction of a patient's response to radiation could also allow treatment to be tailored to each individual patient. By reducing the dose given to radiosensitive patients, normal tissue complications could be minimized, while in radioresistant patients, better tumour control could be achieved.

It is thought that radiosensitivity has a genetic component expressed in all cells of a patient which, in certain inherited syndromes, dominates the overall response of the patient to radiotherapy [19]. This has been shown in patients with ataxia telangiectasia (AT), which is characterized by hypersensitivity to radiation [20,21]. Skin fibroblasts, along with other cell types, from these patients have shown this sensitive response in cell culture [22].

There is also clinical evidence of interpatient variability in the radiosensitivity of normal tissues in radiotherapy patients with no known genetic syndromes [23-25]. If the radiosensitivity of patients without genetic syndromes also has a genetic component, normal human fibroblasts should provide a system on which to measure differences in patients' responses to radiation. The method of measuring these interpatient differences in radiosensitivity is the focus of this work. An assay is required that is sensitive enough to detect interpatient variation but also fast enough to be clinically useful. This means that the assay must not delay the start of radiation treatment, however, the results might not be needed for the first few fractions since a patient found to be sensitive could have their dose reduced by shortening the treatment. The time limit would, therefore, be on the order of a few weeks. If found, this assay (or combination of assays) could be an important tool in providing radiotherapy which is optimized for each patient. Development of this assay requires an understanding of the mechanisms by which cells are damaged and killed by radiation.

1.2 Mechanisms of cell kill

1.2.1 Molecular effects

Radiation damage to an intracellular molecule occurs in several temporally distinct stages after the radiation event as summarized in Figure 1.2.1 [26,27]. Within 10^{-18} s, the energy of the radiation is physically deposited in the molecule. Energy can be transferred to the molecule either directly from the incident radiation or indirectly through another molecule. These two processes are called the direct effect and the indirect effect respectively and occur within 10^{-12} s.

The direct effect is the dominant process when radiations with high linear energy transfer (LET) such as neutrons or α -particles are considered [28]. With low LET radiation such as photons, the indirect effect accounts for most of the damage incurred. Since water accounts for approximately 70% of the mass of tissue, interaction of radiation with water is the predominant cause of damage by the indirect effect.

In water, the ionizing energy absorbed gives rise to primarily three free radicals ($\text{OH}^\bullet$, $\text{H}^\bullet$, e_{aq}^-) which are very reactive and can attack the DNA molecule either by oxidation by the $\text{OH}^\bullet$ radical or by reduction by $\text{H}^\bullet$ or e_{aq}^- . Single strand breaks (ssb's) are most often formed by the production of a radical (R) on the deoxyribose by loss of a hydrogen atom either by direct ionization and proton loss, or abstracted by a $\text{OH}^\bullet$ radical. The radical then reacts with oxygen forming a peroxy radical ($\text{RO}^\bullet$) which is highly reactive and will react with hydrogen to fix the damage and subsequently create an irreversible ssb [29].

Within 10^{-3} s of the initial event, unstable target radicals react in several ways: by a

Figure 1.2.1 DNA damage following ionizing radiation
(modified from Powell, S. McMillan, T.J.: Radiother Oncol, 19, 1990).

Figure 1.2.2 Major types of DNA lesions caused by radiation
(from Whitaker, S.J.: Eur.J.Cancer, 28, 1, 1992).

unimolecular reaction or a reaction with oxygen resulting in permanent damage, or a reaction with a sulphydryl compound (eg. glutathione) which will lead to chemical repair [30]

There are many types of damage that can occur in the DNA from radiation including base damage, sugar damage, ssb's, double strand breaks (dsbs's), and crosslinking either to other DNA strands or to proteins [31,32] (Figure 1.2.2). Although the mechanism for the production of ssb's in the presence of oxygen is well established, the complete mechanism of dsbs is not known. It is thought, however, that dsbs result from two ssb's caused either by one event breaking both strands or two separate events in close proximity on the DNA strand [33].

This damage may lead to cell death, cell mutation or be repaired. Although the exact mechanism of cell death following irradiation is still unclear, DNA dsbs are believed to be the most important lesion due to ionizing radiation that leads to cell lethality [34,35]. The evidence for this is based on a process of elimination: other lesions such as ssb's and base damage have been shown not to correlate with cell killing. Also, agents which produce dsbs specifically, e.g. restriction endonucleases, cause chromosome aberrations and cell killing in a similar way to ionizing radiation [36,37]. However, the specific relationship between DNA damage and cell killing has yet to be clearly established.

1.2.2 Cellular effects

When a population of cells is exposed to radiation, individual cells may a) not receive any damage in a critical target and be unaffected; b) accumulate enough damage in

critical sites to be lethal and will die in their next attempt of mitosis; or c) accumulate damage that, in itself, is not lethal. These cells can repair in time or, if more damage is accumulated before the initial damage is repaired, the cell could die. Most cells die either by necrosis or apoptosis (programmed cell death). Death by necrosis is characterized by the loss of membrane integrity of the cells before the DNA degrades. Conversely, during apoptosis, the DNA degrades before the loss of membrane integrity. Cells can also be irreversibly arrested in the cell cycle without ever having reached mitosis and, therefore, be reproductively dead but not metabolically [38].

These types of damage to mammalian cells can be classified into three types: a) lethal damage which is irreparable and leads to cell death; b) sublethal damage (SLD), which under normal circumstances will be repaired in a few hours unless more damage is added to the cell which can accumulate to result in lethal damage; and c) potentially lethal damage (PLD), which, under normal post-irradiation environmental conditions causes cell death but under ideal growing conditions can be repaired. It is believed that PLD is also repaired in sub-optimal conditions in which the cells cannot cycle, so that they do not have to attempt mitosis while their chromosomes are damaged. This allows the DNA to be repaired before mitosis [28].

Cells can also misrepair their damage [39,40], in which case the cells would not be able to progress through mitosis and would die in the process of dividing. This can occur by two damaged chromosomes recombining in such a way that the cell is no longer viable.

1.3 The Clonogenic assay

1.3.1 Modelling cell survival

Cell survival can be measured using the clonogenic assay in which each surviving cell after treatment grows and divides to form a colony (see methods). A survival curve is a plot of the fraction of surviving cells versus the dose. This method was originally described by Puck in 1957 [41,42]. There are many theories devised on how to model radiation survival curves and there is no consensus on which is best. It is, however, accepted that a two-component model is necessary to adequately describe cell survival *in vitro*. The linear quadratic model [43-45] assumes that there are two components of cell kill, one proportional to dose and the other to the square of the dose:

$$S = e^{(-\alpha D - \beta D^2)} \quad (1-1)$$

where S is the surviving fraction of the cell after a dose (D) of radiation, and α and β are constants which depend on the cell type and the irradiation conditions. In the past, the α component has been related to a dsb produced by a single charged particle track producing lethal damage and the β component to a dsb arising from a combination of two independently induced ssb's produced by uncorrelated particle tracks [46]. More recently, these components have been described as being related to chromosome aberrations [28,47]. Cells that experience exchange-type chromosome aberrations (dsb in two separate prereplication chromosomes which exchange their broken pieces to form a dicentric and an acentric fragment) are unable to survive. At low doses (α component),

these two breaks are a result of a single event set in motion by the absorption of an X-ray or γ -ray. The probability of the interaction between the breaks is proportional to dose. At high doses (β component), these two breaks may result from two separate electrons and the probability of this interaction is proportional to the square of the dose [28]. Either explanation predicts that α is independent of dose-rate while β is dependent on dose-rate and also on a repair time factor [48,49].

1.3.1 Clonogenic assay as a predictor of radiosensitivity

Several studies have shown that differences in radiosensitivity can be measured *in vitro* using the clonogenic assay for fibroblasts [14-16,18,22,24,50,51], lymphocytes [22] and tumour cells [3-5,8,9,52]. It has also been shown that the range of radiosensitivities in several fibroblast and lymphocyte cultures is larger than can be explained by sampling or methodology errors [10]. The radiosensitivity that is measured *in vitro* represents an intrinsic quality of the cell and may be representative of radiosensitivity *in vivo* and, therefore, the patient response to radiotherapy.

To measure radiosensitivity, several endpoints have been used including the surviving fraction after 2 Gy (SF_2) and the initial slope of the survival curve (α) [13]; the surviving fraction after 3.5 Gy ($SF_{3.5}$) [14]; the inverse of the final slope of the survival curve (D_0) and the dose required to reduce the survival to 10% (D_{10}) [53]; and the dose required to reduce survival to 1% (D_1) [11]. These parameters have been measured after both high dose rate irradiation (HDR) and low dose rate irradiation (LDR). There has been much debate whether the SF_2 model, or models based on low levels of survival are

relevant predictors of radiosensitivity [54-57] It is argued that survival after a single 2 Gy fraction does not have the resolution to predict what will happen in the clinic after 25-35 daily fractions of 2 Gy

Although several studies have found a correlation between SF_2 of tumour cells and treatment outcome [3-5] there has been much discussion about the reliability of these results The tumour response is dependent on many factors including: microenvironment (hypoxia, pH etc.), cell contact effect and tumour cell heterogeneity along with host responses such as immune response and vascular response to radiation. Tumour cell heterogeneity, has led to some debate about what cell type is actually being measured. Recent studies have found that a large population of cells grown from tumour biopsies are actually fibroblasts and not tumour cells One study went on to find that the response of the fibroblasts population better represented the overall response than did the tumour cells [58]

Since radiotherapy is limited by the tolerance of the normal tissue in the treatment field, and due to the problems associated with measuring the response of tumour cells, it may be preferable to evaluate normal tissue responses to predict radiosensitivity Of normal cells, fibroblasts are the most amenable to assay, being readily available from skin punch biopsies and growing well in tissue culture conditions. Although SF_2 may not have the resolution to predict patient response, some studies have found a wide distribution of SF_2 values in normal tissue grown in culture. These SF_2 values have been shown to correlate with late reactions to radiotherapy [13,16,51], although no correlation to early reactions has been seen [12].

Another argument against using SF_2 as a predictor of radiation response is the fact that repair capacity is thought to be an important component of radiosensitivity [59]. SF_2 measurements do not allow any time for repair so prediction is being made solely on initial cell kill measurements. Therefore, assays which involve radiation protocols allowing repair time should be better predictors of radiation response.

1.3 Asymmetric Field Inversion Gel Electrophoresis

1.3.1 Theory of gel electrophoresis

Gel electrophoresis is a method which can be used to separate charged biological molecules of different sizes and different charge. This method can be used for proteins, RNA and DNA which are all charged and will migrate in an electric field. More recently, electrophoresis has been used to measure the amount of damage to DNA by measuring the broken fragments of DNA which migrate into the gel.

In conventional gel electrophoresis, DNA samples are placed in a solid gel matrix with an electric field applied across it. The DNA, being negatively charged, migrates towards the anode with a velocity which is dependent on the coefficient of viscosity of the gel, the radius of the molecule and the net force on the molecule [60]. The net force is determined from the product of the electric field and the net charge on the particle. The separation of the DNA molecules of different sizes depends predominantly on the sieving properties of the gel matrix: smaller molecules move more easily than larger molecules. However, other factors also affect the mobility of the DNA through the gel including composition and concentration of the gel, the buffer, the temperature and the magnitude

of the electric field

Using a static electric field, DNA molecules as large as 30 kilobase pairs (kb) can be separated. Molecules larger than 30 kb migrate at approximately the same rate because they elongate and align with the field to migrate towards the anode by a method termed "reptation" [61]. This means that the head of the DNA selects a path and the rest of the molecule follows and its cross sectional radius is, therefore, independent of molecule size. Several attempts were made to separate larger molecules by reducing the concentration of the gel and using low voltage gradients but these were mostly unsuccessful [62].

In the early 1970's it was found that after the removal of the electric field, the elongated molecules relaxed back into their unperturbed state [63]. The rate of relaxation depended on the length of the DNA. This concept was used to separate larger DNA molecules by periodically changing the orientation of the electric field to force the DNA to reorient in the gel. This method allowed yeast chromosomes of several hundred kilobase pairs to be separated.

Pulsed field gel electrophoresis (PFGE) was developed from this observation. When the electric field was initially applied to the gel, the DNA elongated in the direction of the field. If this field was removed and another applied at some angle to the first field, the DNA reoriented before it could migrate in the direction of the new field. This reorientation time was highly dependent on the length of the DNA molecule: large DNA took more time to realign than small DNA because of the physical constraint of the gel matrix. This meant that large DNA spent more time reorienting and less time migrating than small DNA and, therefore, travelled a shorter distance [64].

Carle *et al.* (1986) found that large DNA could be separated by simply applying an electric field 180° to the original field direction which could be easily achieved by reversing the polarity of the electric field. This process was termed field inversion gel electrophoresis (FIGE). Forward migration was achieved by keeping the product of the voltage gradient and the switch time in the forward direction larger than in the reverse direction [65]. By varying the time of the pulses in each direction, different amounts of separation could be obtained.

This method was taken one step further by applying an asymmetric field to the gel (asymmetric field inversion gel electrophoresis or AFIGE) which means that, along with different pulse times, the strength of the voltage in the forward and reverse directions are different. This method allowed separation of DNA up to 9 megabase pairs (Mbp) with a conventional gel box, a single power supply and a simple switching unit [66,67]

This AFIGE method was applied to the detection of DNA dsbs in mammalian cells in 1990 by Stamato and Denko. Mammalian chromosomes are 50-250 Mbp and cannot migrate through the gel. If, however, the DNA is damaged, smaller pieces of DNA caused by dsbs will move through the gel under an electric field. The fraction of DNA which moves out of the cell sample and through the gel is therefore, a measure of the induced DNA dsbs [67].

1 3.2 Measurement of DNA dsbs as a predictive assay

Much work has been done on correlating DNA dsbs with cell kill and, therefore, with cell radiosensitivity. DNA dsbs were originally measured in 1968 by neutral velocity

sedimentation after high radiation doses [68]. More recently, other assays have been developed including the modified neutral velocity sedimentation procedure [69], the neutral filter elution assay [32], clamped homogenous electric field gel electrophoresis (CHEF) [70], and asymmetric field inversion gel electrophoresis (AFIGE) [30], all of which are sensitive down to a few Gray of radiation. Several reviews exist on these methods [35,71-75] and each method has some advantages and disadvantages. CHEF and AFIGE have been found to be sensitive down to 2 Gy and have the advantage of the cells being lysed in agarose, reducing any extra damage to the DNA during sample manipulation. Although CHEF may better separate the different DNA fragment sizes, AFIGE is more economical to set up in the laboratory and is equally capable of measuring the total DNA that has migrated out of the cell sample and through the gel.

These assays have been used to try to acquire more information on the induction and repair of dsbs and how they relate to the radiation response of the cells to radiation. To date, no straightforward answers have been found. There is evidence that the number of dsbs immediately after acute irradiation may play an important role in radiosensitivity [76]. There is also evidence that the rate and amount of repair of these dsbs may correlate with radiosensitivity [30,77-81]. In some studies both initial damage and repair of DNA dsbs have been correlated with sensitivity [82-84], while others have shown that only residual damage after a given repair time is related to radiosensitivity, either after high dose rate irradiation (HDRI) [85] or low dose rate irradiation (LDRI) [86]. It is hoped that with increasing sensitivity of assays being developed, more details about DNA damage and repair will be elucidated in the future.

There is much optimism that the gel assays will be further developed to provide more sensitivity. At the present, it is already the most sensitive assay available for measuring DNA dsbs and may also be fast enough to be useful as a clinical predictor of a patient's response to radiation.

1.4 Hypothesis and specific aims

The hypothesis of this thesis is that differences in radiosensitivity of patients can be measured *in vitro* using normal skin fibroblasts taken from the patients as a measurement system. To measure these differences, levels of radiation greater than 2 Gy must be administered and cells must be allowed a repair interval in order to allow all mechanisms of cell killing to occur. The clonogenic assay can provide useful information, however, a faster assay is required for clinical use. AFIGE appears to be the best assay available at this time.

Specific aims are:

- to design and implement a low dose rate irradiator which can be run at various temperatures to mimic physiological and hyperthermic conditions
- to determine whether survival levels measured after fractionation on two established melanoma cell lines with very different radiosensitivities would be a better predictor of radiation response than single dose HDR or LDR
- to investigate whether survival levels measured on normal skin fibroblasts after fractionated radiation would be a better predictor than single dose HDR or LDR

This irradiation protocol mimics the external beam therapy which is the cause of

most skin reactions.

- to investigate the relationship between cell kill as measured by the clonogenic assay and DNA dsbs as measured by asymmetric field inversion gel electrophoresis using the two melanoma cell lines.
- to determine whether the AFIGE assay would be useful in measuring the cellular radiosensitivity of normal skin fibroblasts and therefore in predicting the response of patients to radiotherapy.
- to measure the inpatient variation in radiosensitivity and to determine whether the above assays can resolve interpatient variation in radiosensitivity.
- to determine whether the AFIGE assay could be developed for direct measurement of dsbs from tissue samples in order to be fast enough to be clinically useful.

2.0 METHODS AND MATERIALS

2.1 Fibroblast growth

The fibroblasts used in these experiments were grown from punch biopsies, 4 mm in diameter, taken from the forearm (unless otherwise stated) of volunteers who gave informed consent after the nature of the procedure was fully explained. The tissue was transported in saline solution and immediately soaked two times for 10 minutes in 5 mL of a mixture of DMEM/F-12 (Gibco) containing 0.1 mM non-essential amino acids (Gibco), 20 mM Hepes (Boehringer Mannheim) and 10 mM NaHCO₃ (Sigma). Antibiotics were added to this media at 10x concentration (1000 IU/mL Penicillin G sodium, 1000 µg/mL streptomycin sulphate, 2.5 µg/mL amphotericin B). The tissue was transferred to a petri dish where it was minced into small pieces using two sterile scalpels. The pieces were pressed to the bottom of 25 cm² flasks (T-25s) with 4-5 pieces in each of four flasks. Carefully, 2 mL of the above medium containing 20% fetal bovine serum (FBS) (Gibco BRL) and 1x antibiotics (100 IU/mL Penicillin G sodium, 100 µg/mL streptomycin sulphate, 25 µg/mL amphotericin B) (Gibco BRL) were added to each flask. The flasks were observed daily until cell growth could be seen at which time the volume of medium was increased to 4 mL. After the culture was established, the serum levels were changed to 7.5% FBS and 7.5% newborn calf serum (NCS) (Gibco BRL). Experiments were done on passage numbers less than 15. Extra cells were frozen for future use at -115°C at a concentration of 5×10^5 cells/mL in 1 mL aliquots in complete medium with 10% Dimethyl Sulphoxide (DMSO).

The two cell lines used throughout most of this study were the OMB1 (Ottawa Medical Biophysics #1) and S11358 cell lines. The OMB1 cell line was grown from a biopsy from a 46 year old male volunteer who had no known history of radiosensitivity. This cell line was found to be less radiosensitive than the S11358 cell line although it is not known whether this volunteer would have had a normal response to radiation therapy. Due to the ethical difficulties of obtaining a tissue sample from a patient with no complications due to radiation treatment, the OMB1 cell line was used as a normal control.

The S11358 cell line was grown from a biopsy from a 33 year old female patient with cervical cancer who had severe chronic normal tissue injury following conventional irradiation [1]. She had a large (6 cm) tumour on the posterior lip of the cervix which involved the right broad ligament and extended to the pelvic side wall. Biopsy showed invasive squamous cell cancer. The clinical findings of a stage IIIB cancer of the cervix were confirmed by examination under anesthesia. The patient had been on birth control pills for 10 years, had a history of smoking and alcohol abuse, appendectomy, and non-insulin dependent diabetes. She had a family history of cancer.

From March 23 to May 5, 1982, she received external cobalt irradiation to the pelvis, using an anterior-posterior opposed pair of fields (17 x 17 cm), to a dose of 4000 cGy in 20 daily fractions. Both fields were irradiated five days per week.

One month later, there was no visible tumour. A Fletcher cesium insertion was used to deliver an additional 3800 cGy. She tolerated the treatment extremely well with no evidence of recurrence or side-effects during her first year of follow-up.

In March 1983, she was admitted with severe late radiation effects in her abdominal area, however there was no evidence of tumour recurrence. Her condition gradually deteriorated and she became paraplegic. She died in July 1984, sixteen months after her admission to hospital.

One month prior to her death, tissue was taken from the forearm for fibroblast growth and radiosensitivity testing. The cells were obtained from Atomic Energy of Canada Limited, Chalk River already in tissue culture at passage 11. The cells were maintained and experimented upon under the same conditions as for the OMB1 cell line.

Other cell lines have been grown from patients exhibiting different degrees of normal tissue reactions after radiation therapy from several hospitals in Ottawa, Kingston and Montreal. The details of their sensitive responses will be discussed in the results.

2.2 Melanoma Cell Lines

Two melanoma cell lines were used. The SKMEL3 cell line is an adherent, fibroblast-like, pigmented, radioresistant melanoma cell line established from a 41-year-old female (American Type Culture Collection (ATCC)). The HT144 cell line is a non-pigmented, radiosensitive melanoma cell line established from a 29-year-old male (ATCC). These cell lines were tested regularly for mycoplasma contamination.

2.3 Clonogenic Methods

2.3.1 General tissue culture methods

All cell manipulation was carried out under sterile conditions in a laminar flow

hood. Cells were grown in the same medium as above with 7.5% FBS and 7.5% NCS, without antibiotics and were kept at 37°C in an incubator with 97.5% air, 2.5% CO₂ and 100% humidity.

Cells were grown in either T-25s or 80 cm² flasks (T-80s). While the cells were still in exponential phase, they were subcultured by rinsing with isotonic citrate saline (134 mM KCl (BDH), 17.6 mM citric acid (Sigma)) to remove any trypsin inhibiting substances in the medium and cellular debris. 0.5 mL of trypsin (0.2% w/v in citrate saline) (Gibco BRL) was added and rinsed over the cells. The excess trypsin was removed and the flasks were incubated at 37°C for 5 min. The cells were resuspended in 5 mL of medium and a 0.5 mL aliquot was removed for cell counting on an automatic cell counter (Particle Data Elzone 80, calibrated by haemocytometer). Growth curves for the melanoma cell lines have been previously done [87]. The growth curves for two of the fibroblasts, OMB1 and S11358, are shown in Figure 2.3.1. These growth curves were used to determine the optimal feeding schedule and initial cell concentration to reach confluence. For experiments, the melanoma cell lines were seeded at 5×10^5 cells/T-25, fed on day 5 and reached confluence (plateau phase) on day 8. The fibroblasts were seeded at 1×10^5 cells/T-25, fed on day 5 and reached confluence on day 10. Both cell lines were seeded with 4 mL of complete medium. All experiments were conducted on plateau phase cell cultures.

Figure 2.3.1 Growth curves of OMB1 and S11358 with cells fed on day 5. Error bars represent SEM of three experiments.

2.3.2 Colony forming assay

The survival of the cells was measured by the colony forming assay. This assay measures the proportion of cells which are capable of continuously reproducing (at least 5 divisions) after experimental treatment. Following treatment, the medium on the plateau phase cells was removed, the cells were rinsed with citrate saline to rinse away the remaining medium, and trypsin was added. The excess trypsin was removed and the cells were incubated at 37°C for 5 min until the cells loosened from the growing surface. The cells were resuspended in 5 mL of complete medium and counted as above. The cells were diluted to the desired concentration and plated such that 50 surviving cells would grow in each plate. For low concentrations of cells, 60 mm plates were used with 4 mL of complete medium. For low survival, where more cells were needed to be plated ($>4 \times 10^4$ cells) or in the case of the fibroblasts which are large cells and form large colonies, 100 mm plates were used with 10 mL of complete medium per plate. The cells were allowed to grow to form colonies for either 11 days (SKMEL3) or 14 days (HT144 and fibroblasts) before they were stained with crystal violet (0.2% w/v in 70% ethanol). Surviving cells were counted as those which produced a colony of greater than 50 cells. The colonies were counted under a Fisher Stereomaster II 10X stereoscopic microscope.

Typically, three dishes of cells were plated for each experimental dose plus three for a control point which was used to calculate the plating efficiency (PE). The mean and the standard deviation was calculated for each set of three plates. The surviving fraction (SF) was calculated according to the following formulas:

$$PE = \frac{\text{number of colonies per control plate}}{\text{number of cells seeded per control plate}} \quad (2-1)$$

$$SF = \frac{\text{number of colonies per plate}}{(\text{number of cells seeded per experimental plate}) \times (PE)} \quad (2-2)$$

Each experiment was repeated at least two times and usually three or four times. The SF was plotted versus the dose with the error bars representing the standard error of the mean (SEM) between the experiments.

2.3.3 Curve Fitting

Survival curves were fit to the linear quadratic model by taking the natural logarithm of eq. 1-1 and plotting the $\ln(S)$ vs dose. This was then fit to a second order polynomial using the fitting routine in the Grapher version 1.24 (Golden Software Inc) plotting program. The data was then normalized to the zeroth order coefficient which had the effect of setting the point which received zero dose to a surviving fraction of 1. The first and second order polynomial coefficients were taken to be α and β respectively.

Recovery ratio curves were fit to the mono-exponential repair equation

$$S(t) = S_0 + (S_{\infty} - S_0)(1 - e^{-kt}) \quad (2-3)$$

where k is the repair constant such that the half-time of repair ($T_{1/2}$) is equal to $\ln 2/k$. S_0 is the initial survival, S_{∞} is the final survival after repair

2.4 AFIGE Methods

The AFIGE method used was developed by Stamato and Denko [66,67,88]. Cells used for the asymmetric field inversion gel electrophoresis (AFIGE) assay were grown to confluence in media containing 0.02 $\mu\text{Ci/mL}$ ^{14}C -thymidine (Dupont NEN Products). The dose acquired by the cells from the ^{14}C was too low to induce any adaptive response. The cells were either irradiated and then suspended in agarose or vice versa, depending on the experiment. Control experiments were done which determined the difference between cells irradiated in monolayers or in plugs (Figure 2.4.1). It was found that, although the slopes of the two lines were significantly different ($p=.002$), the results from these two methods would never be compared directly. This comparison did show that the two methods gave a generally similar dose response. To suspend the cells in agarose, they were washed twice with Dulbecco's phosphate buffered saline (PBS) (Gibco BRL) and then treated with 0.02% trypsin. They were incubated at 37°C for 5 minutes and then resuspended in PBS. The 0.5 mL aliquot of the cells were counted using an automatic cell counter to determine the number of cells in the sample. The remaining cells were centrifuged for 5 minutes at 1200 rpm at 4°C. The supernatant was removed, leaving a pellet of cells in the bottom of the tube. These cells were resuspended at a concentration of 1×10^6 cells/mL in 1% low melting point agarose (Gibco BRL) in PBS at 37°C. Using a 1 mL syringe, the liquid agarose containing cells was drawn up into 3 mm (internal diameter) plastic tubing and allowed to solidify on ice for 5 minutes. Using the same syringe, air pressure was used to extrude the solid agarose. The agarose was then cut into 5 mm lengths. The previously unirradiated samples were irradiated and lysed in 0.5 mL of

Figure 2.4.1 Comparison of FAR of SKMEL3 cells after HDRI between cells irradiated in plugs or in flasks. The experiment was repeated 2 times with the error bars representing the SEM and the errors on the slope being the standard deviation between the two experiments.

lysis buffer (5 mM EDTA (Sigma), 1.0% w/v N-lauryl sarcosine (Sigma), 1.0 µg/mL Proteinase K (Gibco BRL)) and heated at 50°C in a Fisher Scientific dry bath incubator overnight. The cells that were irradiated before imbedding in agarose plugs were put directly into the lysis buffer. After lysing, the plugs were dialysed two times against 0.5 mL of TE buffer (10mM Tris base (Gibco BRL), 1 mM EDTA) for 1 h each. The RNA was then removed from the samples by digesting the plugs in 0.5 mL TE buffer with 0.1 µg/mL ribonuclease A (RNAase A) (Sigma) for 2 h at 37°C. The plugs were then ready to be put in the gel. They could be stored in TE buffer at 4°C for several weeks.

The gel was made of 0.8% electrophoresis grade agarose (Gibco BRL) in 200 mL of 0.5 x TBE buffer (0.9 M Tris base, 0.9 M Boric Acid (BDH), 25 mM EDTA). The agarose was heated in a microwave oven until it dissolved. It was cooled to 50°C and poured into a levelled HE97X 15 x 20 cm casting tray (Hoeffer). The bubbles were removed using the capillary action of a glass Pasteur pipette and the well combs were put in place. The gel was allowed to cool at room temperature until it was solid and it was transferred to 4°C until use (< 1 h). The combs were then removed and one plug was placed in each well. DNA standards *Schizosaccharomyces pombe* (*S. pombe*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) (Gibco BRL), were placed at the beginning and end of each row as controls for the gel. *S. pombe* has a chromosome size range between 3 and 6 Mbp and *S. cerevisiae* has a chromosome size range between 200 and 2000 kbp. Each well, whether empty or containing a plug, or standard, was covered with low melting point agarose.

The gel was placed in the HE99X MAX horizontal agarose submarine unit

(Hoeffer) and covered with 1.5 L of 0.5 x TBE buffer. The gel was run with 1.25 V/cm in the forward direction for 900 s and 5 V/cm in the reverse direction for 70 s using a Pulsewave 760 electrophoretic field switcher and a Pulsewave 760 programming block (Biorad) with a Fisher Biotech FB570 power supply (Figure 2.4.2). A diode box was added to the system to control the pulse heights in each direction independently (Figure 2.4.3). The total run time was 40 h. During the running of the gel, the buffer was circulated through a water cooling bath (Neslab) set at 12°C which kept the gel at 20°C.

When the electrophoresis was finished, the gel was removed from the buffer into a staining container with 1 L of 0.5 x TBE buffer containing 100 µL of 10 mg/mL ethidium bromide (EtBr) (Sigma) and agitated gently for 1 h on a rotating plate. The EtBr attaches to the DNA in the gel but can also stain the gel. The EtBr buffer was removed and the gel was covered with warm distilled water and agitated gently for another 15 min to destain the gel while the DNA remained stained. The gel was placed on a 312 nm variable intensity transilluminator (Fisher Biotech) which fluoresces the EtBr so that the stained DNA can be viewed and photographed. A typical gel is shown in Figure 2.5.

To quantify the DNA that had migrated out of the gel, the gel was cut into lanes, separating the wells from the rest of the lane. The gel pieces were placed in glass scintillation vials and 50 µL of 10 M HCl was added. Each vial was placed on a hot plate until the gel melted. The HCl stopped the gel from solidifying as it cooled. Once melted, 10 mL of Ecolite scintillation fluid (ICN Radiochemicals) was added and shaken vigorously to mix the fluid with the melted gel. The samples were placed in a Tri Carb 1900 TR liquid scintillation analyzer (Packard) and each sample was counted for ^{14}C for

Figure 2.4.2 AFIGE apparatus

Figure 2.4.3 Diode box circuit diagram

Figure 2.4.4 Sample of an asymmetric field inversion gel electrophoresis run on plateau phase SKMEL3 cells after high dose rate irradiation. The cells were at a concentration of 1×10^6 cells/mL. The first and last lanes are DNA size markers

4 minutes. The count per minute (cpm) for each sample was determined and the fraction of activity released from the well was calculated by:

$$FAR = \frac{cpm \text{ in lane}}{cpm \text{ in lane} + cpm \text{ in well}} \quad (2-4)$$

The FAR was plotted versus the dose and the plot was fit with a straight line using weighted linear regression with SPSS version 6.1 (SPSS Inc.). The fit was weighted by the SEM of each point. The data was normalized to the intercept of the FAR axis so that points that received zero dose had a FAR of zero.

2.5 Irradiations

2.5.1 High Dose Rate

The high dose rate (HDR), fractionated and residual damage experiments were irradiated with X-rays (150 kVp, 1 mm Al filter) at a dose rate of 112 cGy/min. The cells were plated in T-25s and grown to confluence. The HDR irradiations (HDRI) were carried out on ice (~ 4°C) in order to minimize the amount of repair occurring during the irradiation procedure. Fractionated irradiation was given in 2 Gy fractions, irradiated on ice, and incubated for at least 6 h at 37°C between the irradiations. Three fractions were given each day. For the residual damage experiments, HDRI was given to the cells on ice and they were subsequently incubated at 37°C for 6 h before being assayed. For the AFIGE assay, the fraction of rejoined damage (FRD) was determined as:

$$FRD = \frac{\text{slope of initial damage} - \text{slope of residual damage}}{\text{slope of initial damage}} \quad (2-5)$$

where the *slope of the initial damage* is the slope of the line of the FAR vs. dose plot from the initial HDRI and the *slope of the residual damage* is the slope after 6 h of rejoining time was allowed.

2.5.2 Low Dose Rate

For the low dose rate irradiations (LDRI), cells were irradiated either in a radium irradiator at a dose rate of 0.882 cGy/min [89] or in a cesium irradiator at a dose rate of 0.75 cGy/min both in temperature controlled incubators. The temperature was monitored to +/- 0.1°C using thermocouples placed in the irradiation chamber. This cesium irradiator was designed to use 15 decommissioned gynaecological cesium sources from the Ottawa General Hospital Division of the Ottawa Regional Cancer Centre. The sources were placed in a planar array to obtain a flat dose distribution at a distance of 2.5 cm from the plane. The dose rate was determined using TLD dosimetry relative to a ⁶⁰Co reference protocol. The sources were shielded on all sides by 5 cm of lead. See appendix for details of design and radiation protection calculations.

The cells were grown in 1.3 cm² glass vials, at the same cell concentration as the flasks, to confluence. The irradiations were carried out at 37°C allowing repair to occur during the irradiation.

2.6 Flow Cytometry

For the fractionation experiments, where the duration of the experiment allowed possible cell cycling, the progression of the cells through the cell cycle was monitored. Replicate samples from the fractionation experiments were trypsinized as for the clonogenic assay. After trypsinization, they were resuspended in 2 mL PBS and a sample of cells was counted. The remaining cells were resuspended in 5 mL of 70% ice cold ethanol, vigorously vortexing the cells as the ethanol was added dropwise to prevent sudden changes in concentration and cell clumping. These samples could be stored at -20°C for several weeks. On the day of analysis, the cells were centrifuged at 2000 rpm for 10 min and the ethanol removed. The cells were resuspended in 1 mL of PBS containing 5 µg/mL propidium iodide, which stains the DNA and allows the amount of DNA in the nucleus to be measured. 100 µL of RNAase was also added to breakdown the RNA in the cells. The amount of DNA in a cell is related to the phase of the cell cycle at the time of treatment. Cells in G₂ or mitosis (M) will have twice as much DNA as those in G₁. Those cells in the phase between G₁ and G₂ where DNA is synthesized (S-phase) will have an amount of DNA between the amounts in G₁ and G₂/M.

The samples were then analyzed on a Coulter Epics XL flow cytometer at the Health Sciences Centre, University of Ottawa. The cell cycle distribution was analyzed using the Multicycle program (Phoenix Flow Systems) which determines the percent of cells in each phase of the cell cycle.

3.0 RESULTS

3.1 Clonogenic Results

The aim of these experiments was to determine whether differences in radiosensitivity could be measured *in vitro* between two cell lines and which radiation protocol would best distinguish these differences. Ratios of surviving fractions were examined at different dose levels along with dose ratios for different survival levels to determine which measurement would be the best predictor of the differences between the two cell lines. The two melanoma cell lines were used as a model of two cell lines of extremely different radiosensitivities. From the results of the melanoma experiments the hypothesis that larger doses and fractionated doses would be better predictors of radiosensitivity was tested. The results of these experiments were then applied to the fibroblast pair of sensitive and normal cell lines to determine whether these protocols would be able to distinguish between two cell lines with a smaller difference in radiosensitivity.

3.1.1 Melanoma cell lines

The two melanoma cell lines, SKMEL3 and HT144, have previously been shown to be radioresistant and radiosensitive, respectively [87,90]. Figure 3.1.1 shows their survival curves after HDR irradiation (HDI). These curves were fit to the linear quadratic model with the SKMEL3 cell line having an α value of -.0434 and a β value of .0400 and the HT144 cell line having an α value of 1.69 and a β value of .00022. The

SKMEL3 survival curve has a large shoulder region, which actually initially has a slightly positive slope (negative α), indicating that, at low doses, sublethal damage is accumulated and the survival remains high. At higher doses, the damage is lethal due to more sublethal damage accumulation and the survival decreases more rapidly. The HT144 survival curve has no shoulder region (very small β) indicating that, even at low doses, there is little sublethal damage accumulated before the damage becomes lethal. The large difference in their radiation responses is evident by their different survival levels, even after low doses of irradiation. This difference after HDRI, along with other radiation protocols, is quantified in Table 3.1.1 which shows the surviving fractions (SF) of the resistant and sensitive cells after various doses along with the ratio of the SF of the resistant to the sensitive cell line. This data is a measure of radiosensitivity at isodose levels where a lower SF indicates a more radiosensitive cell line. A large ratio between the two cell lines (column 4) indicates a large difference in their radiosensitivities. Table 3.1.2 shows the dose of each modality of radiation required to obtain 50% (SF_{50}) and 10% (SF_{10}) survival. This table compares the radiosensitivity of the two cell lines at isosurvival levels where the smaller the dose required to reach a survival level, the more sensitive the cell line and a large ratio indicates a large difference in radiosensitivity. The values in both tables are taken from the fitted linear quadratic curve of the data. The errors, which for Table 3.1.1 are equal to the error bars on the survival curve, are calculated from the SEM between the experiments. The P value, calculated from a two-tailed student's t-test using the Instat biostatistics program (GraphPad Software Inc.), indicates the significance of the differences of the SF's between the two cell lines. For the HT144 cell line, the 6 Gy points

from both the HDR and residual damage curves were extrapolated since survival could only be measured to 5 Gy due to a low plating efficiency and few surviving colonies. Since the survival of the SKMEL3 cell line is very high after fractionation, the SF_{10} data point was also extrapolated. From these two tables it is obvious that, after HDR and at isodose and isosurvival levels, these two cell lines are significantly different in their radiation responses at all doses and make a good model for preliminary testing of a predictive assay.

Table 3.1.1 Comparison between the surviving fractions of SKMEL and HT144 after various doses and irradiation protocols

Dose Rate		SKMEL3	HT144	Ratio
HDR	2 Gy	.93 ± .16	.0340 ± .0004	27 (P=.005)
	6 Gy	.31 ± .02	(3.9 ± .5) × 10 ⁻⁵	7800 (P=.0007)
LDR	2 Gy	.71 ± .03	.117 ± .007	6.1 (P<.0001)
	6 Gy	.35 ± .04	(8.6 ± .4) × 10 ⁻⁴	410 (P=.0007)
2 Gy/fraction	2 Gy	.93 ± .16	.034 ± .0004	27 (P=.005)
	6 Gy	.59 ± .04	(3.8 ± .6) × 10 ⁻⁴	1580 (P=.0001)
	8 Gy	.41 ± .01	(1.1 ± .5) × 10 ⁻⁵	38800 (P<.0001)
Residual Damage	2 Gy	.88 ± .07	.053 ± .008	16 (P=.007)
	6 Gy	.47 ± .01	(3.78 ± .5) × 10 ⁻⁴	1200 (P=.0003)

Table 3.1.2 Radiation doses (Gy) which reduce cell survival to 50 % and 10 % for SKMEL3 and HT144

Dose Rate		SKMEL3	HT144	Ratio
HDR	SF ₅₀	4.7 ± .4	.41 ± .03	11 (P=.005)
	SF ₁₀	8.1 ± .8	1.36 ± .01	6.0 (P=.001)
LDR	SF ₅₀	4.0 ± .3	.7 ± .1	6.0 (P=.0005)
	SF ₁₀	13 ± 2	2.1 ± .2	6.1 (P=.006)
2 Gy/fraction	SF ₅₀	7.0 ± .7	.44 ± .07	16 (P<.0001)
	SF ₁₀	13 ± 1	1.5 ± .2	8.7 (P<.0001)
Residual Damage	SF ₅₀	5.38 ± .09	.425 ± .04	13 (P=.0004)
	SF ₁₀	9.5 ± .2	1.37 ± .1	6.9 (P=.0008)

Figure 3.1.2 shows the fitted HDRI survival curves for SKMEL3 and HT144 as dashed lines along with the LDRI curves. Both cell lines show a dose rate sparing effect (DRSE). This means that when the irradiation is delivered over a longer time period, the cells have the opportunity to repair damage during the irradiation and subsequently have a higher survival. The shoulder of the SKMEL3 survival curve virtually disappears after LDRI showing that the sublethal damage is being repaired before a subsequent damaging event can make it lethal. This produces the effect of greatly changing the survival at high doses (>8 Gy) while having no or little effect at the low doses. At doses below 8 Gy, it appears that the HDRI survival curve is higher than after LDRI. This may be due to

several factors: 1) the small doses of acute radiation may induce a repair mechanism that is not turned on by LDRI; 2) it may be an artefact of fitting the curve to the linear quadratic model which may not exactly fit the data; or 3) after HDRI, the SF at 2 Gy actually goes above 1 which may indicate that the irradiated cells have a higher plating efficiency than unirradiated cells.

When the HT144 cells are given LDRI, the slope of the survival curve changes so that the survival is increased as compared to HDRI even at low doses. These changes can be quantified by taking the ratio of survival at various doses after LDRI to that after HDRI or taking the ratio of the dose required to achieve SF_{50} and SF_{10} after LDRI to HDRI. Both methods are a measure of the DRSE, the first at isodose levels and the second at isosurvival levels. This method will be used in evaluating all radiation protocols and the results are shown in Tables 3.1.3 and 3.1.4. It should be noted that these ratios are less sensitive at the isosurvival levels than at the isodose levels and small differences are harder to detect. The data from Table 3.1.3 confirms how the DRSE increases with dose for the SKMEL3 cells, only having a large effect at doses greater than 8 Gy. On the other hand, the HT144 cells show the sparing effect at much lower doses, already showing some sparing by 2 Gy. Table 3.1.4 shows however that, at isosurvival levels, the DRSE is similar for both cell lines. At 50% survival, the DRSE is still larger for the HT144 cells but at 10% survival, the DRSE is the same for both cell lines. Therefore, except for at low doses, these two cell lines have similar DRSE's after LDRI

Table 3.1.3 Ratios of SF after various radiation protocols to SF after HDR1 for SKMEL3 and HT144

Dose Rate		SKMEL3	HT144
LDR	2 Gy	$.8 \pm .1$	$3.4 \pm .2$
	6 Gy	$1.1 \pm .1$	22 ± 3
	8 Gy	$2.2 \pm .6$	-
	12 Gy	23 ± 5	-
2 Gy/fraction	2 Gy	$1.0 \pm .2$	$1.00 \pm .001$
	6 Gy	$1.9 \pm .2$	10 ± 2
	8 Gy	$3.8 \pm .1$	-
Residual Damage	2 Gy	$.9 \pm .2$	$1.6 \pm .2$
	6 Gy	$1.5 \pm .09$	10 ± 2
	8 Gy	$1.8 \pm .6$	-

Table 3.1.4 Ratio of doses (Gy) which reduce cell survival to 50% and 10% for different radiation protocols to those for HDR1, for SKMEL3 and HT144

Dose Rate		SKMEL3	HT144
LDR	SF ₅₀	$.8 \pm .1$	$1.7 \pm .3$
	SF ₁₀	$1.6 \pm .3$	$1.5 \pm .1$
2 Gy/fraction	SF ₅₀	$1.5 \pm .2$	$1.1 \pm .2$
	SF ₁₀	$1.6 \pm .2$	$1.1 \pm .1$
Residual Damage	SF ₅₀	$1.1 \pm .1$	$1.01 \pm .09$
	SF ₁₀	$1.2 \pm .1$	$1.01 \pm .07$

The survival curves after fractionated radiation are shown in Figure 3.1.3. Again, a DRSE is observed. The fractionation is delivered in 2 Gy fractions, 3 fractions each day and therefore has an average dose rate of .42 cGy/min, which is less than the LDR of .88 cGy/min. The survival after fractionation for the SKMEL3 cell line is slightly higher than after LDRI. This result is not what is predicted by the standard linear quadratic model (LQM) which predicts that fractionation should have a lower survival than LDRI [28]. The LQM predicts that survival after fractionated radiation falls in between survival after HDRI and survival after LDRI. With this cell line, however, it has already been shown that there is no DRSE at the 2 Gy level so the survival after 2 Gy of HDRI is similar to that after 2 Gy of LDRI. This means that when the fractions are given in 2 Gy acute doses the survival after the first dose would be the same as after 2 Gy of LDRI. In other words, survival after 2 Gy is independent of the dose rate. However, due to the overall average dose rate being lower in fractionation, more damage could be repaired and the survival at higher doses would increase as compared to LDRI. This situation would only arise if survival after 2 Gy is independent of dose rate. If survival after 2 Gy is dependent on dose rate, ie. there is a measurable DRSE after 2 Gy, survival after 2 Gy of fractionation would be less than after 2 Gy of LDRI and the increased repair due to a lower dose rate in fractionation may or may not be enough to result in an overall higher survival. Another explanation for higher survival after fractionation than LDRI is that the 2 Gy acute doses could induce a repair mechanism that is not induced by LDRI. This was also seen after 2 Gy of HDRI where the survival was higher than after 2 Gy of LDRI. The data in Table 3.1.3 confirms that the DRSE is higher for fractionated irradiation than after LDRI at all

doses. At SF_{10} for the two radiation protocols, the DRSE is the same but at SF_{50} fractionation still has a higher DRSE than LDRI.

The HT144 fractionation curve agrees with conventional fractionation theory in that the survival curve falls between the survival curves of HDRI and LDRI. The DRSE is less for fractionation than LDRI at both the isodose levels (Table 3.1.3) and at isosurvival levels (Table 3.1.4).

In Table 3.1.1, the ratio of the survival of the SKMEL3 to HT144 cells at a dose of 6 Gy was higher after fractionation than LDRI but less than after HDRI showing no advantage as a predictive assay at this dose. The advantage of fractionation is, however, that survival can be measured after higher doses of radiation than with HDRI and after 8 Gy of fractionated irradiation, the ratio of the surviving fractions is greater than after 6 Gy of HDRI. At isosurvival levels, the ratio of SKMEL3 to HT144 is similar for all dose rates, however at SF_{50} the ratio after HDRI is higher than after fractionation which is higher than after LDRI. This is in agreement with the ratios at isodose level. This method, however, is limited by the high survival of the SKMEL3 so that only the low dose region of the HT144 survival curve is taken into account.

Figure 3.1.5 shows the survival of these two cell lines after HDRI followed by 6 h of repair time at 37°C. This protocol has been termed "residual damage" measuring the survival with any damage that is residual after a 6 h incubation. There is still a large difference between the survival of these two cell lines but smaller than after HDRI at 6 Gy. Both cell lines show evidence of repair but repair different amounts as shown in Table 3.1.3. After low doses, however, the same effect is observed as with the other radiation

protocols in that the HT144 cell line repairs more than the SKMEL3 cell line. At isosurvival levels, these two cell lines have previously been shown to have similar repair ratios (RR) of potentially lethal damage (PLD) although SKMEL3 repairs slightly more [90]. The repair ratio in that study was defined to be the survival 8 h after the irradiation divided by the survival immediately after irradiation when the cells were irradiated with isosurvival levels of 10 Gy and 2 Gy for SKMEL3 and HT144 respectively. Under these conditions, the RR's were $2.1 \pm .3$ and $2.4 \pm .3$ for SKMEL3 and HT144 respectively measured from 6 independent experiments. In this case, however, after 6 h the RR's were $2.8 \pm .7$ (extrapolated to 10 Gy) and $1.6 \pm .2$ for SKMEL3 and HT144 respectively. These RR's are not the same ($P < .05$) and show that the SKMEL3 cells have a higher recovery ratio than HT144.

Figure 3.1.1 Survival curves of SKMEL3 and HT144 after high dose rate irradiation on ice. The error bars represent the SEM of three independent experiments.

Figure 3.1.2 Survival curves of SKMEL3 and HT144 cell lines after high and low dose rate irradiation. The HDR experiment was conducted on ice and the LDR experiment at 37°C. Error bars represent the SEM of three independent experiments.

Figure 3.1.3 Survival curves of SKMEL3 and HT144 after high dose rate, low dose rate and fractionated irradiation. The HDR and fractionated experiments were irradiated on ice and the LDR irradiations at 37°C. In the fractionation experiments the flasks were incubated at 37°C between irradiations. Error bars represent the SEM of three independent experiments.

Figure 3.1.4 Cell cycle analysis of SKMEL3 and HT144 cell lines during fractionated irradiation using flow cytometry. Cells were in plateau phase throughout the experiment.

Figure 3.1.5 Survival curves of SKMEL3 and HT144 after high dose rate irradiation, before and after 6 hours of repair time. The HDR irradiation was conducted on ice and the cells were incubated at 37°C. The error bars represent the SEM of three independent experiments.

3.1.2 Fibroblast cell lines

The same methods as were used for the melanoma cell lines were used to evaluate the differences in radiosensitivity between the two human fibroblast lines cultured from skin biopsies. These two cell lines, OMB1 and S11358, came from a normal volunteer with no known pathological radiation syndromes, and a radiosensitive patient, respectively. The radiation survival curves of the cell lines after HDRI are shown in Figure 3.1.6. These curves were fit to the linear quadratic model with the OMB1 cell line having an α value of .428 and a β value of .0316 and the S11358 cell line having an α value of .392 and a β value of .0554. The differences in their radiosensitivities *in vitro* are much smaller than with the melanoma cell line pair. At 2 Gy, no difference can be seen between the two cell lines and separation of the curves becomes apparent only above 6 Gy. The survival levels measured for the S11358 cell line were limited by a very low plating efficiency of less than 10% while the OMB1 cell line had a plating efficiency of 15-25%. In Table 3.1.5, the ratio of the SF of the two cell lines after 2 Gy is 1 while after 6 Gy of HDR, the ratio is 1.9 with a P value that indicates the difference is approaching significance. At isosurvival levels, however, the ratios of the doses required to reduce the survival to either 50% or 10% by LDRI to HDRI are both 1 (Table 3.1.6).

Table 3.1.5 Comparison between the survival of OMB1 and S11358 after various doses and irradiation protocols

Dose Rate		OMB1	S11358	Ratio
HDR	2 Gy	.37 ± .09	.37 ± .03	1.0 (P=.9)
	6 Gy	.025 ± .005	.013 ± .003	1.9 (P=.07)
LDR	2 Gy	.40 ± .02	.422 ± .001	1.0 (P=.4)
	6 Gy	.11 ± .03	.10 ± .02	1.1 (P=.8)
2 Gy/fraction	2 Gy	.55 ± .05	.53 ± .03	1.0 (P=.8)
	6 Gy	.22 ± .03	.15 ± .01	1.4 (P=.1)
	12 Gy	.11 ± .02	.026 ± .004	4.0 (P=.02)
Residual Damage	2 Gy	.495 ± .005	.49 ± .05	1.0 (P=.9)
	6 Gy	.0934 ± .0005	.07 ± .02	1.3 (P=.3)

Table 3.1.6 Radiation doses (Gy) which reduce cell survival to 50% and 10% after HDRI, LDRI, fractionation and 6 h after HDRI.

Dose Rate		OMB1	S11358	Ratio
HDR	SF ₅₀	1.5 ± .3	1.5 ± .1	1 (P=1)
	SF ₁₀	4 ± 1	4 ± 1	1 (P=1)
LDR	SF ₅₀	1.5 ± .2	1.8 ± .2	.8 (P=.4)
	SF ₁₀	6 ± 2	6 ± 1	1 (P=1)
2 Gy/fraction	SF ₅₀	2.3 ± .5	2.2 ± .2	1.1 (P=.8)
	SF ₁₀	14.0 ± .1	7.4 ± .7	1.9 (P=.02)
Residual Damage	SF ₅₀	2.12 ± .02	2.2 ± .2	.96 (P=.6)
	SF ₁₀	5.76 ± .03	5 ± 1	1.2 (P=.5)

In Figure 3.1.7, the survival curves after LDRI are shown, along with the fitted HDRI curves (dashed lines). Both cell lines have a DRSE, however, the S11358 cell line has a larger effect since after HDRI it has a lower survival than OMB1 but the same survival as OMB1 after LDRI up to 6 Gy. After 6 Gy of LDRI, however, the survival curves of the two cell lines start to diverge as the OMB1 cell line plot starts to curve upwards. This suggests either an induced resistance for the OMB1 cell line or an inability to induce resistance in the S11358 cell line during LDRI. In Table 3.1.7, it can be seen that the ratio of survival after LDRI to survival after HDRI for S11358 is double that of OMB1 after 6 Gy while after 2 Gy there is no difference. This ratio increases substantially for the OMB1 cells after 8 Gy but, unfortunately, can not be measured at this dose for S11358 cells due to low plating efficiency. In Table 3.1.8, where the DRSE is quantified at isosurvival levels, no difference exists between the two cell lines.

Table 3.1.7 Ratios of SF after various radiation protocols to SF after HDRI for OMB1 and S11358

Dose Rate		OMB1	S11358
LDR	2 Gy	$1.1 \pm .3$	$1.14 \pm .08$
	6 Gy	4 ± 1	8 ± 2
	8 Gy	16 ± 8	-
2 Gy/fraction	2 Gy	$1.5 \pm .4$	$1.4 \pm .1$
	6 Gy	9 ± 2	11 ± 3
	8 Gy	40 ± 10	-
Residual Damage	2 Gy	$1.3 \pm .3$	$1.3 \pm .2$
	6 Gy	$3.7 \pm .8$	5 ± 2
	8 Gy	6 ± 3	-

Table 3.1.8 Ratio of doses which reduce cell survival to 50% and 10% for different radiation protocols to those for HDRI for OMB1 and S11358

Dose Rate		OMB1	S11358
LDR	SF ₅₀	$1 \pm .2$	$1.2 \pm .1$
	SF ₁₀	$1.5 \pm .6$	$1.5 \pm .4$
2 Gy/fraction	SF ₅₀	$1.5 \pm .7$	$1.5 \pm .4$
	SF ₁₀	$3.5 \pm .9$	$1.8 \pm .5$
Residual Damage	SF ₅₀	$1.4 \pm .3$	$1.5 \pm .4$
	SF ₁₀	$1.4 \pm .3$	$1.2 \pm .4$

The survival curves after fractionated radiation (Figure 3.1.8) are more separated than both the HDRI and LDRI survival curves. These results are similar to the melanoma cell lines where after 6 Gy the ratio between the surviving fractions had actually decreased compared to HDRI because of higher survival levels. However, in this case, fractionation allowed survival to be measured up to 12 Gy after which the ratio of survival between the cell lines increased to 4.0 with $P < .05$ indicating a significant difference between their surviving fractions. In Table 3.1.6 the ratios for HDRI and LDRI are all around 1 except for SF_{10} after fractionated radiation when the ratio is 1.9 with $P < .05$. This shows that, even at isosurvival levels, fractionated radiation is a better predictor of radiosensitivity but only after higher doses of radiation.

From Table 3.1.7, it can be seen that after 2 Gy and 6 Gy of fractionated radiation, the DRSE is the same for both cell lines. The DRSE for the S11358 cell line after fractionation is similar to that after LDRI, however, after fractionation, the DRSE of the OMB1 cell line is greater than that after LDRI. This is similar to what was found with the SKMEL3 cell line and may also be due to an induced repair mechanism from acute 2 Gy doses which is not induced after LDRI and does not seem to be induced in the S11358 cells. This effect is also seen when looking at iso-survival levels: the ratio of the fractionated dose required to reduce survival to 10% to the HDR dose required is larger for OMB1 than S11358. This ratio is also larger than the equivalent ratio for LDRI.

The survival curves for both fibroblast cell lines after fractionation have a similar upward curve as the HT144 cell line, with OMB1 curving more than S11358. This

indicates an increasing resistance of the cells as the treatment progresses. To determine whether cell cycling was causing this increased resistance, flow cytometry was used to measure the cell cycle distribution throughout fractionation (Figure 3.1.9). This fractionated radiation was administered over 32 hours. The changes in the cell cycle distribution for both cell lines are minimal and not enough to explain the increase in radioresistance. The OMB1 cell had a slight increase in S phase at 30 hours which subsequently dropped. The S1358 cells slightly increased in S phase but only by a few percent, not enough to explain the upward curve.

Figure 3.1.10 shows the survival of these cells after HDR and 6 h of repair time. Both cell lines repaired some damage during the 6 hours. The rate of repair after 6 Gy can be seen in Figure 3.1.11. The RR's calculated from the residual damage experiment (Figure 3.1.10) were 5 ± 2 for S11358 and $3.7 \pm .8$ for OMB1 (Table 3.1.8). From the repair experiment (Figure 3.1.11) the RR's were calculated to be 5 ± 1 for S11358 and $4.2 \pm .6$ for OMB1. Combining the results of the two experiments, the RR's are 5 ± 2 for the S11358 cell line and 4 ± 1 for the OMB1 cell line. These RR's are not significantly different, however, the OMB1 cell line does have a slightly shorter half-time of repair ($T_{1/2}$) than S11358 ($1.3 \pm .2$ h vs. $1.7 \pm .1$ h) suggesting that damage is repaired faster in the OMB1 cell line which may have an affect on the survival after fractionated radiation if all damage is not repaired within 6 h.

Figure 3.1.6 Survival curves of OMB1 and S11358 after high dose rate irradiation on ice. The error bars represent the SEM of three independent experiments

Figure 3.1.7 Survival curves of OMB1 and S11358 cell lines after high and low dose rate irradiation. The HDR experiment was conducted on ice and the LDR experiment at 37°C. Error bars represent the SEM of three independent experiments.

Figure 3.1.8 Survival curves of OMB1 and S11358 after high dose rate, low dose rate and fractionated irradiation. The HDR and fractionated experiments were irradiated on ice and the LDR irradiations at 37°C. In the fractionation experiments the flasks were incubated at 37°C. between irradiations. Error bars represent the SEM of three independent experiments.

Figure 3.1.9 Cell cycle analysis of OMB1 and S11358 cell lines during fractionated irradiation using flow cytometry. Cells were in plateau phase throughout the experiment.

Figure 3.1.10 Survival curves of OMB1 and S11358 after high dose rate irradiation, before and after 6 hours of repair time. The HDR irradiation was conducted on ice and the cells were incubated at 37°C. The error bars represent the SEM of three independent experiments.

Figure 3.1.11 Recovery ratios as a function of time after 6 Gy of acute irradiation for the OMB1 and S11358 cell lines. The flasks were incubated at 37°C for time, t, after irradiation. The error bars represent the SEM of three independent experiments. The k value was calculated from $\ln(2)/T_{1/2}$.

3.2 AFIGE results

The clonogenic results determined that differences in radiosensitivity could be measured *in vitro* and that fractionation was the best predictor of these differences. The clonogenic assay, although a useful tool for measuring survival, requires several weeks to determine the results. For a clinical predictor to be useful, a faster assay is required. Since DNA dsbs are thought to be the lesion leading to cell lethality, an assay that measures dsbs might be useful as a predictor. The AFIGE assay has been used for this purpose and requires 4-5 days for results. From the results of the clonogenic assay, it was decided to repeat the same radiation protocols, first with the melanoma cell lines to determine if the differences measured by the clonogenic assay could be detected using the AFIGE assay. These experiments were also conducted on the fibroblast cell lines to determine whether smaller differences in radiosensitivity could be detected.

3.2.1 Melanoma results

The results of the clonogenic assay after HDRI, LDRI, fractionation and after 6 h of repair showed the HT144 cell line had a much lower survival than the SKMEL3 cell line in all cases. Both cell lines repaired damage within 6 h after HDRI but the HT144 still had a much lower survival than the SKMEL3 cells.

Figure 3.2.1 shows the fraction of activity released (FAR) from the well as a function of dose after HDRI. The data has been normalized so that the fitted line intercepts the FAR axis at zero. Before normalization, the zero dose point typically had a FAR of 0% to 10%. This can be explained by background DNA damage which existed in

the cell prior to irradiation or due to DNA dsbs induced by manipulation of the cells, DNA replication sites, cell turnover or apoptosis. Since the FAR is a measure of the DNA dsbs in the cells at the time of the assay, an increased FAR/Gy indicates increased DNA dsbs which should correlate with radiosensitivity, i.e. the more sensitive cells should have more dsbs per Gy and therefore higher FAR/Gy. It has also been found in previous studies that the amount of DNA that can migrate out of the well will reach a limit at some dose of radiation after which the curves are no longer linear [91-93]. These studies showed linearity of the graph up to 40 Gy but this range of linearity seems to be dependent on the cell type and the exact conditions used. Both the melanoma cell lines start to flatten out above 30 Gy therefore the data were fitted only up to this dose.

In Figure 3.2.1, it can be seen that the more sensitive HT144 cells have a slightly steeper slope than the resistant SKMEL3 cells indicating more DNA released per Gy. Table 3.2.1 shows the slopes of all the AFIGE plots of FAR versus dose for different radiation protocols. The regression coefficient, r , was calculated from a weighted linear regression fit with SPSS using the SEM for the weighting of each point. The P values quoted are a measure of the difference between the slopes of the curves of the two cell lines and were calculated from a two-tailed student's t -test. In this table it can be seen that after HDRI, the difference in the slopes is highly significant.

Table 3.2.1 Slopes of lines from AFIGE assay for SKMEL3 and HT144 in FAR/Gy

Dose Rate		SKMEL3	HT144	P value
LDR	slope	.0000 ± .0004	.0000 ± .0008	1
	r	.02	.54	
HDR	slope	.0092 ± .0005	.0131 ± .0003	<.0001
	r	.981	.997	
2 Gy/fraction	slope	.0000 ± .0007	.0030 ± .0007	.02
	r	.07	.8	
Residual Damage	slope	.00257 ± .00008	.0020 ± .0002	<.0001
	r	.99	.99	

After LDRI (Figure 3.2.2) the FAR versus dose curve is flat even though the clonogenic survival of the HT144 cells was much less than the SKMEL3 cells. This may indicate that all DNA dsb damage is rejoined as neither cell line shows any DNA released at any dose as the slopes of both the SKMEL3 and HT144 lines are zero. Another explanation is that most of the DNA dsb damage is repaired and any remaining damage is too small to be measured by this assay. Other studies have shown the limit of resolution of PFGE to be on the order of 200 dsbs/cell [92] which would be enough dsbs to kill the cell. The FRD calculation shown in the methods can also be used as a measure of the DRSE after LDRI and fractionation. This data is shown in Table 3.2.2. After LDRI, all damage appears to be rejoined and the FRD is 1 for both cell lines. These results emphasize the difference between what is being measured by each assay. The clonogenic assay measures survival while the AFIGE assay measures DNA dsbs. Survival is

dependent on correct dsb repairing while DNA dsbs which are rejoined, whether correctly or incorrectly, are no longer detected using the AFIGE assay. After LDRI, all measurable damage appears to be rejoined but, since this is not reflected in the survival levels, clearly not all damage is repaired correctly.

Table 3.2.2. FRD as calculated by equation 2-4 after various radiation protocols for SKMEL3 and HT144

Dose Rate	SKMEL3	HT144	P value
LDR	1.00 $\pm$.08	1.00 $\pm$.03	1
2 Gy/fraction	1.00 $\pm$.08	.77 $\pm$.06	.004
Residual Damage	.72 $\pm$.08	.85 $\pm$.03	.02

After fractionated irradiation (Figure 3.2.3) there was a significant difference in the FAR/Gy of the two cell lines with the HT144 cell line having a greater slope than the SKMEL3 cell line ($p=0.01$). This trend is in agreement with the clonogenic results where the HT144 cell line had lower survival than the SKMEL3 cell line, however the difference between the two cell lines is much smaller with the AFIGE assay than with the clonogenic assay. Table 3.2.2 shows that all the damage in the SKMEL3 cell line appears to be rejoined while only 77% of the damage in the HT144 cell line is rejoined as compared to HDRI.

When the cells were given a HDRI and were incubated for 6 h (Figure 3.2.4), there was little residual DNA damage in both cell lines although some damage still remained as the slopes were not zero. The difference between the residual damage of the two cell lines is very small, however, due to the small error bars and high r values, these slopes were determined to be significantly different (Table 3.2.1). The FRD after HDRI and incubation for 6 h, calculated as shown in the methods, was determined to be $0.72 \pm .08$ for the SKMEL3 cells and $0.85 \pm .03$ for the HT144 cells. The clonogenic results under these same conditions showed RR's of $2.8 \pm .7$ and $1.6 \pm .2$ for SKMEL3 and HT144 respectively at isosurvival levels. These ratios show that both cell lines have similar abilities to recover from damage which would otherwise kill the cells, with the SKMEL3 cell line repairing slightly more than the HT144 cell line as measured by the clonogenic assay. The HT144 cell line, however, is rejoining more DNA damage than the SKMEL3 cell line as measured by the AFIGE assay, but may be rejoining incorrectly which leads to lower survival as measured by the clonogenic assay.

Figure 3.2.1 Fraction of activity released as a function of dose for high dose rate irradiation for SKMEL3 and HT144 cell lines. The lines were fit up to 30 Gy above which the curves appear to reach a saturation level. The error bars represent the SEM of n independent experiments. Cell concentration of 1×10^6 cells/mL.

Figure 3.2.2 Fraction of activity released as a function of dose for low dose rate and high dose rate irradiation for SKMEL3 and HT144 cell lines. The error bars represent the SEM of n independent experiments. Cell concentration of 1×10^6 cells/mL

Figure 3.2 3 Fraction of activity released as a function of dose for fractionated and high dose rate irradiation for SKMEL3 cells and HT144 cell lines. Error bars represent the SEM of n independent experiments. Cell concentration of 1×10^6 cells/mL

Figure 3 2 4 Fraction of activity released as a function of dose after high dose rate irradiation and after 6 h of incubation at 37°C. Error bars represent the SEM of n independent experiments. Cell concentration 1×10^6 cells/mL.

3 2 3 Fibroblasts results

The results from the melanoma DNA dsb AFIGE assay showed that it was more difficult to determine differences between two cell lines of different radiosensitivity than with the clonogenic assay. Initial DNA damage was not as significantly different between the two cell lines as was the clonogenic survival, and rejoining of DNA dsbs made differences after other radiation protocols virtually disappear. Knowing that the clonogenic results of the fibroblasts showed smaller differences than the melanoma cell lines, it seemed unlikely that any differences would be detectable in the fibroblasts cell lines using the AFIGE assay. Nevertheless, the same experiments were repeated in hopes of gaining some insight into the mechanisms of DNA dsb damage and its relationship to clonogenic survival.

Figure 3.2.5 shows the FAR as a function of dose of the OMB1 and S11358 cell lines after HDRI. As with the melanoma cell lines, there is evidence of both the cell lines reaching saturation, but with the fibroblasts this occurs after 25 Gy. Therefore, the points above 25 Gy on the graph are omitted from the fit and these slopes are shown in Table 3 2.3. The sensitive S11358 cell line has a significantly higher amount of DNA dsb produced per Gy than the OMB1 cell line as was found with the melanoma cell lines.

Table 3.2.3 Slopes of lines from AFIGE assay for OMB1 and S11358 in FAR/Gy

Dose Rate		OMB1	S11358	P value
LDR	slope	0008 $\pm$ 0005	0000 $\pm$ 0007	4
	r	0.34	0.012	
HDR	slope	.0145 $\pm$.0006	.0180 $\pm$.0009	.002
	r	.99	.99	
2 Gy/fraction	slope	.000 $\pm$.001	- .0021 $\pm$.0004	2
	r	0.14	0.75	
Residual Damage	slope	.0006 $\pm$.0006	.0027 $\pm$.0006	.04
	r	.25	.68	

Figure 3.2.6 shows the FAR as a function of dose following LDRI. The S11358 cell line, like the melanoma cell lines, appears to have rejoined all the DNA dsbs and has a zero slope. The OMB1 cell line has a slope very close to zero signifying that essentially all of the dsbs have also been rejoined. There is no significant difference between the slopes of these two lines. Using the FRD as a measure of the DRSE, it can be seen that the S11358 appears to have repaired all its damage while 94% of the OMB1 damage is repaired. These differences, however, are not significant.

Table 3.2.4. FRD as calculated by equation 2-4 after various radiation protocols for OMB1 and S11358

Dose Rate	OMB1	S11358	P value
LDR	.94 ± .07	1.00 ± .03	.22
2 Gy/fraction	1.00 ± .06	1.12 ± .04	.005
Residual Damage	.96 ± .07	.85 ± .05	.02

After fractionated irradiation (Figure 3.2.7) a more complicated situation exists. While the graph of FAR as a function of dose for the OMB1 cell lines exhibited a zero slope indicating complete rejoining of any DNA dsbs, that of the S11358 cell line has a negative slope. Remembering that these graphs have been normalized to zero and that, before normalization, even the zero dose point possessed a small amount of FAR (0-10%), this negative slope may indicate a rejoining of the background DNA dsb damage along with any radiation induced damage. Calculating the FRD, it is found that the OMB1 cells rejoin 100% of the damage while the S11358 cells rejoining 112% of the damage, 12% of which may be a reduction in the background damage. This might indicate that the S11358 cell line has an overactive rejoining mechanism that allows the DNA to rejoin easily but incorrectly, even rejoining breaks that existed prior to radiation. This mechanism may preempt some of the correct rejoining and therefore result in misrepaired DNA dsbs.

This overactive mechanism is not apparent when the cells are left to rejoin damage for 6 h after HDR1 (Figure 3.2.8). In this case, the OMB1 cells have a lower slope indicating that they have rejoined more of the initial damage obtained after HDR1. The

difference between the slopes of the residual damage graphs of the two fibroblasts cell lines is statistically significant as shown in Table 3.2.3. The FRD's of the two cell lines are also significantly different with the OMB1 cell line rejoining 96% of its initial damage while the S11358 cell line rejoins only 85%. This is contrary to the clonogenic findings where the S11358 cell line had a higher RR for the same dose level. The apparent absence of an overactive rejoining mechanism after HDR1 may indicate that this mechanism requires repeated assaults on the cells to be induced and may only show up after fractionated irradiation. It should also be noted that the dose levels administered in the AFIGE and clonogenic assay are very different and it may be that after high doses (40 Gy) used in the AFIGE assay, repair may be saturated. Another important consideration is that the clonogenic assay is measuring survival of only the cells that attach to the plate while the AFIGE assay measures the DNA dsbs of all the cells, whether viable or not. Since the fibroblasts have a very low plating efficiency (0.5-4%) the populations of cells being examined in each assay is very different.

Figure 3.2.5 Fraction of activity released as a function of dose for high dose rate irradiation for OMB1 and S11358 cell lines. The lines were fit up to 25 Gy above which the curves appear to reach a saturation level. The error bars represent the SEM of n independent experiments. The cell concentration is 1×10^6 cells/mL.

Figure 3.2.6 Fraction of activity released as a function of dose for high dose rate and low dose rate irradiation for OMB1 and S11358 cell lines. The error bars represent the SEM of n independent experiments. The cell concentration is 1×10^6 cells/mL.

Figure 3.2.7 Fraction of activity released as a function of dose for high dose rate and fractionated irradiation for OMB1 and S11358 cell lines. The error bars represent the SEM of n independent experiments. The cell concentration is 1×10^6 cells/mL

Figure 3.1.8 Survival curves of OMB1 and S11358 after high dose rate, low dose rate and fractionated irradiation. The HDR and fractionated experiments were irradiated on ice and the LDR irradiations at 37°C. In the fractionation experiments the flasks were incubated at 37°C between irradiations. Error bars represent the SEM of three independent experiments.

3.3 Clonogenic vs. AFIGE

In the cases when the slope of the line of the FAR vs dose graph is greater than zero, the surviving fraction versus the FAR was plotted. This was only possible after HDRI for both cell line pairs and after HDRI with 6 h of incubation at 37°C for the melanoma cell lines. The residual damage data for the fibroblasts was not plotted in this manner because the scatter of the data around the fitted line and the error bars were both too large to make the comparison meaningful. This data shows the relationship between the survival of the cells and the amount of DNA dsb damage. In the case of residual damage, it can give some insight into the amount of damage that is being misrepaired.

Figures 3.3.1 and 3.3.2 show the four cell lines, SKMEL3, HT144, OMB1 and S11358 after HDRI. In Figure 3.3.1, it can be seen that to achieve the same survival levels, the SKMEL3 cell line requires more DNA dsbs than the HT144 cell line. This suggests that the SKMEL3 are more resistant to DNA dsbs, accumulating more dsbs before the damage becomes lethal. In the case of the fibroblast cell lines (Figure 3.3.2), both cell lines have the same survival for the same amount of dsbs indicating that they have the same ability to accumulate DNA dsbs before the damage becomes lethal.

Figures 3.3.3 shows the melanoma cell lines after HDRI but with a 6 h incubation at 37°C allowing for repair and/or rejoining of DNA damage. In this case, the more sensitive HT144 cell line still requires less DNA dsbs to achieve the same level of survival as the SKMEL3 cell line but the separation between the two lines is greater. The separation between the two lines can be measured as the ratio of the FAR required to achieve 10% survival of the resistant cell line to the sensitive cell line. These data are

shown in Tables 3.3.1 and 3.3.2. No errors are shown in these tables since the values are calculated from the fitted curves. From Table 3.3.1, it can be seen that the ratio between the SKMEL3 and HT144 after HDR is 3.1 while after allowing repair and/or rejoining to occur, the ratio increases to 8.9. This increase reinforces the hypothesis that the HT144 cell line is misrepairing damage and that this increase in the ratio is due to a higher amount of rejoining of DNA dsbs in the HT144 cell line.

Table 3.3.1 FAR required to reduce the surviving fraction to 10% for SKMEL3 and HT144 cell lines after HDR and after HDR with a 6 h incubation at 37° C

Dose Rate	SKMEL3	HT144	Ratio
HDR	.075	.024	3.1
Residual Damage	.024	.0027	8.9

Table 3.3.2 FAR required to reduce the surviving fraction to 10% for OMB1 and S11358 cell lines after HDR.

Dose Rate	OMB1	S11358	Ratio
HDR	0.06	0.064	.92

Figure 3 3.1 Surviving fraction versus FAR for SKMEL3 and HT144 cell lines after high dose rate irradiation. The points are calculated from the fitted curves from the surviving fraction versus dose and FAR versus dose graphs.

Figure 3 3.2 Surviving fraction versus FAR for OMB1 and S11358 cell lines after high dose rate irradiation. The points are calculated from the fitted curves from the surviving fraction versus dose and FAR versus dose graphs.

Figure 3.3.3 Surviving fraction versus FAR for SKMEL3 and HT144 cell lines after high dose rate irradiation and 6 hours of incubation at 37 °C. The points are calculated from the fitted curves from the surviving fraction versus dose and FAR versus dose graphs.

3.4 Other patients

Over the course of a few years, skin samples from patients who showed a sensitive response to radiation were acquired through collaborations with radiation oncologists. These samples were grown into cell culture and frozen at -115°C until the results from the S11358 and OMB1 cell lines were completed. From those results, it was decided that the clonogenic assay after fractionated treatment provided the best test for radiosensitivity *in vitro*. In this group of radiosensitive patients, there were various responses to radiation from reddening of the skin in the treatment field to extreme normal tissue complications leading to patient death. This section will discuss each patient's response and the results of their clonogenic assays. Some of the samples also had survival curves after HDR1 or LDR1 performed and these will also be presented. The order of presentation is in chronological order of when the sample was received. The cells are labelled in order using the same notation as OMB1 (Ottawa Medical Biophysics #1). Some cell numbers are missing due to complications in growing the cell lines or difficulty in thawing the cell stock due to age of the cell line and problems with the freezer.

The first sample successfully grown, frozen and thawed was the OMB7 cell line. In 1987 this 52 year old male patient presented with squamous cell carcinoma of the anal canal which involved the skin. He was treated with 4400 cGy in 22 fractions to the pelvis in two opposing fields (anterior-posterior and posterior-anterior (AP/PA)) from August 26th to September 25th 1987 with an 18 MV photon beam. In November 1987 he was given an additional 2940 cGy over 42 h with an LDR interstitial implant. During his external beam therapy he developed moist desquamation and later showed signs of

telangiectasia which is a late effect. Three years later he showed some late effects including swelling of the groin and lower abdomen and cellulitis in the area of radiation. In February 1993, a skin sample was taken and grown into culture. This patient was also overweight at the start of his treatment and had mild hypertension. After therapy he developed diabetes which may have affected his skin reactions. The results from the fractionated treatment on this cell line (2 Gy/fraction, 3 fractions/day, 12 Gy total) are shown in Figure 3.4.1 along with the survival curves of the OMB1 and S11358 cell lines. The OMB7 cell line showed an extremely sensitive response in comparison to the two other cell lines. Of all the cell lines tested, this cell line showed the most sensitive response to radiation.

In Figures 3.4.2 and 3.4.3 the survival curves of the OMB8 cell line are shown. This sample was from a 32 year old female who had a sensitive response to radiation in May 1993. She was diagnosed with cervical carcinoma and was treated with 4860 cGy in 27 fractions external beam plus 3500 cGy with a low dose rate radium implant, both to the pelvic region. The treatment resulted in fibrosis along the lumbar vertebrae and a mass in her pelvis which was not cancer. This reaction is considered a severe late reaction. As of May 1995, she had no further signs of cancer but had a slow recovery from the radiation effects. In Figure 3.4.2, the HDR1 survival curve is shown along with OMB1 and S11358. After HDR1, these cells showed a more sensitive response than the two other cell lines used for comparison. After fractionated irradiation (Figure 3.4.3), the OMB8 cell lines had a response between the OMB1 and S11358 cell lines but closer to the more sensitive S11358 cell line. Therefore, this cell line seems to be more sensitive than normal which

would be expected in a patient with a severe late reaction to radiotherapy

The OMB9 and OMB10 cell lines came from the same arm of the same patient. This female patient had non small cell lung carcinoma which was treated with 20 Gy in 5 fractions to the midplane. She had prolonged toxicity with substantial dysphagia over several weeks and skin changes consisting of moist desquamation which healed over several weeks. She was later treated for brain metastasis with a reduced dose of 14 Gy in 5 fractions which she tolerated well and had a good clinical response for 26 months. She was retreated with a full dose of 20 Gy in 5 fractions which she tolerated well and her metastasis has been in control for a year. There has been no sign of any late reactions. The cells were treated with HDRI, LDRI and fractionated irradiation (Figures 3.4.4, 3.4.5 and 3.4.6). In all cases, the two cell lines had similar radiation response to each other which was a useful test of intra-patient variation. After HDRI, these cells showed a similar response to the two other cell lines used for comparison. At higher doses, they responded more like the normal cell line, OMB1. After LDRI, they exhibited a more resistant response than either OMB1 or S11358 which would indicate a larger dose-rate sparing effect in this cell line. After fractionated irradiation, the OMB9 and OMB10 cell lines have a response between the OMB1 and S11358 cell lines but closer to the more sensitive S11358 cell line. Therefore, this cell line seems to be more sensitive than normal to fractionated radiation but not to either HDRI or LDRI.

The OMB13 cell line was from a 56 year old male patient who, in the fall of 1993, was diagnosed with local advanced and metastatic prostate cancer. In January 1994, the metastases was in his bones and he was given 8 Gy of radiation in one fraction for

palliation. The treatment was delivered to the pelvis in a 32 cm x 24 cm field AP/PA with a 6 MV beam. In early March he was dehydrated and generally unwell. On March 9th he was hospitalized and deteriorated quickly over the next 24 h. Under surgical investigation it was found that his bowel had become perforated and he had extensive infection in his abdomen. He died shortly afterwards. The cells were treated with HDRI, LDRI and fractionated treatment. The HDRI results (Figure 3.4.7) show that this cell line responded similarly to the sensitive cell line and by 8 Gy appeared to be much more sensitive than the normal cell line. The LDRI results (Figure 3.4.8) show that the OMB13 cell line falls within the same range as the other two cell lines which have similar responses to LDR. After fractionation (Figure 3.4.9), OMB13 had a response in between the sensitive and normal cell lines. These results indicate that this patient is sensitive to HDR irradiation, especially after higher doses. After fractionation, there is a slight sensitivity, however, after LDR, no radiation sensitivity has been seen.

The OMB14 cell line was grown from a female patient who had systemic sclerosis and lung cancer who had not yet received any radiation therapy. There have been several case reports which described exaggerated fibrosis in patients with systemic sclerosis following radiation therapy [94-96]. There was concern that radiation would accelerate the growth of her fibroblasts so growth curves were done on these fibroblasts with and without an initial 2 Gy dose. These two growth curves are shown in Figure 3.4.10 along with those of OMB1 and S11358 for comparison. These cells grew to a higher density than the other two cell lines but no difference was seen between the irradiated and unirradiated cells. The cells were also treated with HDRI and fractionated irradiation.

After HDR (Figure 3.4.11), these cells showed a similar response to the two other cell lines used for comparison after doses up to 4 Gy. At higher doses, they responded more like the sensitive cell line, S11358. After fractionated irradiation (Figure 3.4.12), the OMB14 cell line again had a response between the OMB1 and S11358 cell lines and closer to the more sensitive S11358 cell line. Therefore, this cell line seems to be more sensitive than normal to fractionated radiation and HDR and grows to a higher density than other fibroblasts but their growth rate does not seem to be affected by radiation. This patient was subsequently given 30 Gy in 10 fractions to the lungs with 6 MV photons in December 1995. In February 1996 she had a chest X-ray which showed less than 50% shrinkage of the tumour but no sign of a sensitive reaction. She died later that month before any fibrosis would have become apparent.

Table 3.4.1 Summary of details of patients showing sensitive response to radiation therapy

Cell Line	Gender	Age	Disease	Treatment
OMB7	male	52	squamous cell anal ca	44 Gy 22 fx (18 MV) 29 Gy 42 h (HDR)
OMB8	female	32	cervical ca	49 Gy 27 fx 35 Gy (1 D)
OMB9 & OMB10	female	45	small lung cell ca - brain mets	20 Gy 5 fx to midplane
OMB13	male	56	palliative care for bone mets	8 Gy 1 fx (6 MV)
OMB14	female	53	lung ca	30 Gy 10 fx (6 MV)

2 OF 2

PM-1 3" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

Table 3 4 2 Summary of responses of radiosensitive patients to radiation therapy and of their cells to radiation administered *in vitro*

Cell Line	Protocol	Severity	Reaction type	<i>in vitro</i> sensitivity *
OMB7	fractionation	mild	early and late	--
OMB8	HDRI fractionation	severe	late	-- -
OMB9 & OMB10	HDRI LDRI fractionation	mild	early	+ + -
OMB13	HDRI LDRI fractionation	severe	early	- + -
OMB14	HDRI fractionation	N/A	N/A	- -

* + = more resistant or same as OMB1 response

- + more sensitive than OMB1 and more resistant than S11358

-- = more sensitive than S11358

Figure 3 4 1 Survival curves of OMB1, OMB7 and S11358 after fractionated irradiation. The cells were irradiated on ice and incubated at 37°C between irradiations. Error bars represent SEM of three independent experiments.

Figure 3.4.2 Survival curves of OMB1, OMB8 and S11358 after high dose rate irradiation. The cells were irradiated on ice. The error bars represent the SEM of three independent experiments.

Figure 3 4 3 Survival curves of OMB1, OMB8 and S11358 after fractionated irradiation. The cells were irradiated on ice and incubated at 37°C between irradiations. The error bars represent the SEM of three independent experiments.

Figure 3.4.4 High dose rate survival curves of OMB1, OMB9, OMB10 and S11358. The cells were irradiated on ice. The error bars represent the SEM of three independent experiments.

Figure 3.4.5 Low dose rate survival curves of OMB1, OMB9, OMB10 and S11358. The cells were irradiated at 37°C. The error bars represent the SEM of three independent experiments.

Figure 3.4.6 Survival curves of OMB1, OMB9, OMB10 and S11358 after fractionated irradiation. The cells were irradiated on ice and incubated at 37°C between irradiations. The error bars represent the SEM of three independent experiments.

Figure 3 4 7 Survival curves of OMB1, OMB13 and S1358 after high dose rate irradiation. The cells were irradiated on ice. The error bars represent the SEM of three independent experiments.

Figure 3.4.8 Survival of OMB1, OMB13 and S11358 after low dose rate irradiation. The cells were irradiated at 37°C. The error bars represent the SEM of three independent experiments.

Figure 3.4.9 Survival curves of OMB1, OMB13 and S11358 after fractionated irradiation. The cells were irradiated on ice and incubated at 37°C between irradiations. The error bars represent the SEM of three independent experiments.

Figure 3.4.10 Growth curves of OMB1, OMB14 and S11358. OMB1 and S11358 were fed on days 5 and 10. OMB14 was fed on days 6, 12, and 19. The feeding days are shown with arrows.

Figure 3.4.11 Survival curves of OMB1, OMB14 and S11358 after high dose rate irradiation. The cells were irradiated on ice. The error bars represent the SEM of three independent experiments.

Figure 3.4.12 Survival curves of OMB1, OMB14 and S11358 after fractionated irradiation. The cells were irradiated on ice and incubated at 37°C between irradiations. The error bars represent the SEM of three independent experiments.

3.5 Intra-patient variation

For a predictive assay to be useful, it must not only be able to detect differences between cell lines derived from skin samples of different patients, but also have reproducible results within the same patient. To test whether the clonogenic assay and the AFIGE assay would have the same results if several samples were taken from the same patient, a large tissue sample was obtained by a surgeon from a non-radiotherapy patient who was undergoing thoracic surgery. From this piece of tissue, 10 separate cell lines were started. These were labelled OMB12A-J. OMB12E and OMB12J became contaminated and the experiments were done on the remaining 8 cell lines.

Growth curves were performed on OMB12A and OMB12G as shown in Figure 3.5.1. These curves show similar growth characteristics for the two cell lines and also similar to the OMB1 and S11358 cell lines. It was assumed that the other 6 OMB12 cell lines would have the same growth and the the same feeding schedule used for all other fibroblast cell lines would be appropriate since the cells were in plateau phase on day 10 after being fed on day 5.

The survival curves after HDRI are shown in Figure 3.5.2. From this graph it can be seen that the results from different samples from the same patient are quite reproducible. The average survivals after 2, 6 and 8 Gy are $2.5 \pm .03$, $.009 \pm .003$ and $.0009 \pm .0003$. These errors are on the same order of magnitude as those found from inter-experiment variation of a single cell line. The variance of the survival at each of these dose levels was analysed using ANOVA and in each case, the variance of the means was not significantly greater than expected by chance. This method has the disadvantage

of all samples being taken from the same anatomical location, however, it would be unrealistic to ask a volunteer to donate samples from different locations

These cell lines were also given fractionated irradiation (Figure 3.5.3). Again, the survival curves are closely grouped. The average survivals after 6 and 12 Gy are $.18 \pm .05$ and $.06 \pm .01$. These errors are also on the same order of magnitude as those found from inter-experiment variation of a single cell line. The variance of the survival at 6 and 12 Gy was also found to be not significantly greater than expected by chance.

These data suggest that the clonogenic measurement of survival after HDRI and fractionated irradiation is reproducible from one cell line to another taken from the same patient.

The AFIGE assay was also tested for intra-patient variability by the same method with the same 8 cell lines but only after HDRI (Figure 3.5.4). The average slope of the lines was calculated to be $.020 \pm .002$ FAR/Gy. The standard deviation of the slopes is greater than the inter-experiment error determined for the other cell lines. The data was also analysed using ANOVA and the variation was found to be significantly greater than expected by chance. This indicates that the reproducibility of the AFIGE assay is not good enough to be a predictive assay when determining small differences in radiosensitivity. It can, however, still be a useful tool for gaining better understanding of how DNA dsbs are related to the survival of a cell line

Figure 3.5.1 Growth curves of OMB12A and OMB12G. Cells were seeded at 4×10^3 cells/cm² and either unfed or fed on day 5. Plateau phase was reached by day 10.

Figure 3.5.2 Survival curves of OMB12 cell lines after high dose rate irradiation. The cells were irradiated on ice. Curves were not fit to the linear quadratic equation since only four data points were measured. Error bars represent the SEM of three independent experiments.

Figure 3.5.3 Survival curves of OMB12 cell lines after fractionated irradiation. The cells were irradiated on ice and incubated at 37°C between irradiations. Curves were not fit to the linear quadratic equation since only three data points were measured. Error bars represent the SEM of three independent experiments.

Figure 3.5.4 Fraction of activity released after high dose rate irradiation for OMB12 cell lines. The cells were irradiated on ice. The error bars represent the SEM of three independent experiments.

3.6 Time scale of the AFIGE assay

The AFIGE assay is still undergoing much development and there are similar gel assays such as those using a clamped homogeneous electric field (CHEF) which have been producing more reproducible results. If, however, cells still need to be grown into tissue culture before being tested, the process, no matter how reliable, would still be too slow to be clinically practical. The samples need to be run quickly and, therefore, a method needs to be developed which would allow the tissue to be assayed directly or for the cells to be harvested directly from the tissue and assayed immediately. A preliminary experiment was run to determine whether this might be possible with the AFIGE assay.

Tissue samples that were remaining from the OMB12 tissue had been frozen at -80°C for future use. These samples were thawed, minced into small pieces, rinsed three times in citrate saline and trypsinized in 30 mL of 0.25% trypsin with 1mM EDTA at 37°C . While trypsinizing, the cells were agitated by stirring with a magnetic stirring bar at ~ 180 rpm. After 30 min of stirring, the supernatant was removed and centrifuged at 1500 rpm (500 g) for 5 min. More trypsin was added to the remaining tissue and the process was repeated twice. The pellet of cells resulting from centrifugation was resuspended in ice cold PBS and left on ice until all the cells were collected. These cells were counted and centrifuged again. They were resuspended in 1% low melting agarose and made into plugs at a concentration of 5×10^5 cells/mL as described in the methods section. A lower concentration of cells than usual was used due to the low number of cells collected. The plugs were irradiated with up to 40 Gy.

Figure 3.6.1 shows the results of this preliminary experiment. There is definitely a

dose response with the slope of the line being 0.09 ± 0.01 with a regression coefficient of .96. This slope is slightly smaller than that found when the same tissue was grown into culture and assayed, however, this tissue had been frozen for nearly two years. A fresh tissue sample may have given better results. More aggressive methods are also available for disaggregating tissue into single cells which may be able to produce a higher yield of cells and a better dose response. These data indicate, however, that it would be possible to assay cells directly from the tissue sample using an electrophoresis method. This would make the time period required to run this assay on the order of a few days which would be useful in a clinic.

Figure 3 6 1 FAR versus dose for human skin cells trypsinized from a previously frozen tissue sample. The cells were not grown into tissue culture but immediately suspended in agarose plugs and irradiated at a high dose rate. The cell concentration is 5×10^5 cells/mL. The error bars represent the SEM of two independent experiments.

4.0 DISCUSSION

4.1 Clonogenic Assay

The clonogenic assay is a standard assay for measuring the survival of cells. It is a very useful tool in measuring the ability of the cells to reproduce, which has particular relevance to cancer therapy since halting indefinite proliferation is the goal of cancer treatment. The clonogenic assay, however, provides no information on the molecular mechanisms affecting cell survival. It is also a relatively slow assay, taking several weeks to perform and, therefore, is not very useful as a predictive assay in the clinic since most tumours need to be treated as quickly as possible in order to get a good response. In this study, however, the clonogenic assay was used as a benchmark to determine whether *in vitro* survival levels of cells of differing radiosensitivities could be distinguished regardless of the molecular mechanism responsible for their response. This approach has been used by many groups and there is still much controversy over the results. Previous findings will be discussed where comparisons can be made to this work.

4.1.1 Melanoma cell lines

The melanoma cell lines were chosen for the initial experiments to establish the feasibility of measuring differences in radiosensitivity. The SK-MEL-3 cell line is extremely resistant to radiation while the HT144 cell line is extremely sensitive. These two cell lines differ in sensitivity and are suitable to test the hypothesis that differences in radiosensitivity can be measured *in vitro* and to examine which radiation protocol optimizes this.

difference

The survival curves of the melanoma cell lines confirmed the large difference in radiosensitivity. After all radiation protocols, the survival values between the two cell lines were large, even at low doses. In this case, radiosensitivity can be predicted after an acute dose of 2 Gy (SF_2). These two cell lines are, however extremely different and not representative of typical radiosensitivities found in tumour cells. Other studies have found the range of SF_2 to be 0.13-0.93 for cervical carcinoma [5], 0.16-0.59 for head and neck carcinoma [8], 0.19-0.076 for glioma blastoma multiforme [97] and 0.50 with a CV of 32% for melanoma [25]. The SF_2 values for SKMEL3 and HT144 are 0.93 ± 0.16 and 0.034 ± 0.004 respectively which are at the upper and lower limits of any other measurements on tumour cell lines. Therefore, these measurements, although useful for initial testing of a predictive assay, do not represent clinical data.

The shape of the HDRI and LDRI curves for the SKMEL3 cells were interesting in that the survival after 2 Gy of HDRI was actually higher than after LDRI. Other investigators have found that low doses of X-rays are more effective per gray than higher doses in both rodent [98], and a radioresistant human tumour cell line [52,99]. This effect, which in human tumour cells has only been seen at doses lower than 1.5 Gy and in rodent cells at doses lower than 0.5 Gy [98], has been attributed to a repair system or other radioprotective mechanisms which are only triggered at higher doses after a threshold of damage is induced [52,99]. It may, however, also be due to increased induction of damage at doses lower than 1.5 Gy through unrecognised mechanisms. A similar effect may be appearing here where the HDRI induces this repair mechanism while

the LDRI irradiation does not cause enough damage at these low doses to activate this mechanism, although in this case the LDRI survival is less than the HDRI survival up to about 6 Gy. As stated in the results, the higher survival after HDRI than LDRI may also be due to curve fitting artefacts, changes in plating efficiency or statistical variation. The experiments were repeated at least three times with the same results each time so statistical variation is probably not the cause of this effect.

It has been found in some cell types that radiosensitivity is due to a deficiency in repair [100]. If this is the case, radiation protocols which allow time for repair to occur should be better predictors of radiation response. It may, therefore, be expected that survival after LDRI should show a larger separation than after HDRI since repair is also occurring during the irradiation. With the SKMEL3 and HT144 cells there is little difference in the PLDR capacity, however, a difference in the sublethal damage repair (SLDR) has been measured [90] and SKMEL3 repairs about 3 times more SLD than HT144. Also the separation does not appear to increase after LDRI as the ratio of the surviving fractions is greater after HDRI than LDRI. This may be an artefact of higher survival levels after LDRI since the ratios of isosurvival doses calculated at SF_{10} are the same. Conversely, isosurvival ratio is still greater after HDRI at SF_{50} . Based on the melanoma cell lines, it would seem that the best predictor, or highest ratio of survivals is found after high total doses of fractionated irradiation. Although the difference between the survival of the two cell lines is smaller after the same dose, increased survival allows higher doses to be used where the difference between the two cell lines increases. At isosurvival levels there is no difference between the HDRI ratios and the fractionation

ratios but these levels do not represent the highest doses used for the HT144 cell line and therefore do not exploit the advantage of fractionation. During fractionated irradiation, repair is also allowed to occur so that both cell kill by HDR and repair capacity can contribute to the outcome. A previous study showed that survival after single 2 Gy doses predicted the response after fractionation in human tumour cells [101]. This agrees with these melanoma results, however it should be noted that this may only be true when large differences in radiosensitivity exist between the cell lines being examined. Another study on human tumour cells showed that a more precise value of SF₂ could be determined using a multifraction assay than using a single dose survival curve [102]. This would indicate that even if SF₂ was a useful predictor, better measurements of this value can be made using a fractionated radiation protocol.

The results from the residual damage experiment did not contribute to the search for a better predictive assay but did provide some information about the repair capacity of the cell lines. Contrary to previous studies on these two cell lines [90], it was found that the more resistant SKME-3 cell line had a slightly higher, but significantly different, repair ratio for PLDR than the more sensitive HT144 cell line.

Much work has been done on tumour tissues grown *in vitro* as a predictor of radiosensitivity and tumour response [4-9]. Some trends have been seen between tumour sensitivity *in vitro* and patient response to radiotherapy. It is, however, the response of the normal tissue that limits the dose that can be safely administered to the patient. The goal is to give the tumour as much dose as possible with minimal damage to normal tissue in the treatment field, so it is normal tissue sensitivity which is required for

treatment planning. The tumour is also a heterogeneous environment with many factors which might affect the tumour response. This makes it difficult to choose any one factor on which to base a predictive assay for intrinsic radiosensitivity of the tumour [84].

4.1.2 Fibroblast cell lines

Similar results to the melanoma cell lines were found with the fibroblast cell lines although the difference in radiosensitivity between the two cell lines is much less, resulting survival curves that have a smaller separation as was observed between the OMB1 and S11358 cell lines. The survival after HDR irradiation predicted that differences between the more sensitive S11358 cell line and the normal OMB1 cell line could be predicted *in vitro*. This difference could only be realized at higher doses of 6 Gy or above. At the 2 Gy level, there was no measurable difference between the two cell lines. Many other laboratories have claimed that differences between sensitive and resistant cell lines, both tumour cells [3-7,9] and normal cells [13,14,16] can be measured at low doses. In the case of tumour cells, the measurement of cell survival after low doses has been correlated to patient outcome [3-5] or local control [6,7,9]. Other studies have shown no correlation to cell survival in malignant gliomas [97,103-104]. Normal human fibroblast survival after low doses has been shown to correlate with skin fibrosis [14] and telangiectasia [16] which are both late reactions, or to all late skin reactions combined [13] however another study found no correlation between skin fibrosis and telangiectasia [19]. No correlation has been found between the survival of fibroblasts after low doses and early reactions [12,13]. Regardless of these results, there has been much debate on whether models

based on low levels of survival are relevant predictors of radiosensitivity [54-57]. The results of this work support the argument that small differences in radiosensitivity can not be measured after single acute low doses (~ 2 Gy) and higher doses (≥ 6 Gy) are required.

The results after LDRI also agreed with the results from the melanoma cell lines in that the separation between the two cell lines was less than after HDRI. In fact, all evidence of a difference between the two cell lines disappeared after LDRI. Geara et. al. also found that LDRI did not amplify differences in the parameters of the survival curves [10] but attributed their results to their LDR being higher than normally used (2.85 cGy/min)

As with the melanoma cell lines, the fractionated radiation showed the greatest separation which was not apparent until after high doses (> 8 Gy) were administered. Survival after these doses could not be measured after HDRI due to high levels of cell killing. The results show that these differences are greater after fractionated radiation even at equivalent survival levels.

No advantage was gained by allowing the cells to repair for 6 h after HDRI. Both cell lines showed evidence of repair, however the more sensitive S11358 cell line had a slightly higher RR after 6 Gy, although this was not statistically significantly different. At isosurvival levels, the RR was the same.

Although previous studies have shown correlations between patient response and survival following a single acute HDR irradiation [13,14], these results clearly show that, when trying to detect small differences in radiosensitivity, survival after a single 2 Gy dose can not predict any differences. The differences in the normal tissue responses that will be

seen between patients will not appear until much higher doses have been administered or after fractionated irradiation. Using fractionated irradiation as an *in vitro* predictor allows higher doses to be given to the cells and allows repair between doses. This radiation protocol is also a better model of clinical irradiation.

4.2 AFGE assay

A linear relationship of FAR with dose would be expected if the FAR is directly proportional to DNA dsbs induced (or remaining after rejoining) and if DNA dsb induction is directly proportional to dose. This was seen up to 25 or 30 Gy with the cell lines in this study but above these doses, a reduced FAR was seen. This resulted in curve saturation which has been seen in previous studies [91-93]. This shape is inevitable due to the FAR being expressed as a fraction. The FAR is constrained by a maximum of 1 which will never be reached because of the small but consistent fraction of DNA released from the unirradiated controls which has been subtracted from all the data points [93]. Regardless of the controls, the curve will also reach saturation because most DNA fragments would be degraded to sizes which can move through the gel by this saturation dose [105]. It has also been suggested that the shape of the curve after higher doses may be due to an increase in DNA crosslink formation which may limit movement of the DNA [67].

For these reasons, only the initial portion of the dose range, where the response is linear, was considered so that comparisons could be made between the slopes of the lines which would represent the dsbs induced (or remaining after rejoining). Some other

researchers have also fit the results to curvilinear lines [106].

4.2.1 Melanoma cell lines

The results of the AFIGE assay raise many questions. After HDRI, the more radiosensitive HT144 cells have greater slope indicating more DNA dsbs induced per Gy ($p < .0001$). These results agree with other findings using the neutral filter elution assay [76,107] and with pulsed field gel electrophoresis [108], all on rodent cells. One study on melanoma cell lines, however, found no difference in the DNA dsbs induced after HDRI between the HT144 cell lines and three more resistant melanoma cell lines although none of the resistant melanoma cell lines in the study were as resistant as the SKMEL3 cell line [109]. Another study on human tumour cells agreed with this study finding that the initial damage is a major determinant of intrinsic cell radiosensitivity [110]. If one considers only the physical interaction of radiation with cells, the same amount of ionizational energy is being deposited in each cell target. This would suggest that the same amount of DNA dsbs would be expected for all cell lines [30]. There are several explanations for differences in the initial DNA dsbs induced. The response may be affected by different chromatin structure which could make DNA more susceptible to higher numbers of dsb [30,83] or by ploidy, which has been shown to have an effect on the radiation response [111]. The difference between the two cell lines might also be explained by a series of chemical reactions which occur immediately after the irradiation event and may alter the biological effects [112]. These reactions may be affected by factors such as oxygen concentration [113] or glutathione levels [114] or to a very fast

component of repair during manipulation of the samples. The radiosensitivity of cells is also cell cycle dependent [115,116], but these cell lines were both in plateau phase for these experiments and had similar cell cycle distribution.

Other groups have found that initial DNA dsbs do not correlate with radiosensitivity [79,80,85,117,118]. All this evidence, along with the results from this study, indicate that there are likely many different factors affecting the response of cells to radiation, some of which are exhibited as differences in induction of DNA dsbs and some of which become apparent when examining other endpoints such as DNA repair or repair rates.

In the case of LDRI, the FAR/Gy is too small to be measured in both cell lines. This may be due to differences in misrepair between the two cell lines. The AFIGE assay measures only the DNA dsbs that are rejoined but not necessarily repaired correctly. The fidelity of the repair is unknown, whereas the clonogenic assay measures the survival which is less tolerant to error prone repair. Both cell lines may be misrepairing to some extent but the HT144 cells seem to be misrepairing to a greater extent since they have a much lower clonogenic survival than the SKMEL3 cells while apparently having the same DNA dsbs as measured by AFIGE. Since the LDRI is given over a long period of time, the cells have time to repair and/or rejoin dsbs during the irradiation. It should also be noted that the AFIGE assay measures the DNA dsbs of all cells while the clonogenic assay measures only surviving cells which adhere to the plate. Differences in plating efficiency (60 % for SKMEL3 and 10% for HT144) may explain some of the differences between the two cell types. Another complication in measuring DNA dsbs after LDRI is that the

sensitivity of the dsb measurement may not be sufficient to measure differences at low levels of damage. This means that if differences exist between the two cell lines after LDRI, the levels may be too small to be detected using the AFIGE assay. There are very few studies on LDRI using pulsed field electrophoresis on human tumour cells, however other studies have measured DNA dsbs after LDRI using the neutral filter elution assay. One such study found that the majority of the DNA dsbs were repaired and more damage was repaired in radioresistant cell lines [86]. The same group examined the response of a human tumour cell line to LDRI using pulsed field gel electrophoresis and found a strong dependence of DNA extracted from the well on dose rate which decreased with dose rate down to 1 cGy/min [119]. The slope of the line, in their case, never reached zero although their lowest dose rate was higher than used in this study. The gel apparatus used in their study could also have better resolution at low levels of DNA damage.

When the cells are given fractionated irradiation the HT144 cells showed significantly more FAR/Gy than the SKMEL3 cells. With the clonogenic assay there is a large difference in survival between the two cell lines. During this radiation protocol, the cells have time to repair and/or rejoin between the doses but no repair/rejoining occurs after the final dose since the cells are assayed immediately after this dose. Some of the FAR may be due to the damage from the last 2 Gy dose. These repeated 2 Gy irradiations may also make it more difficult for the cells to rejoin or repair the damage than during LDRI which is delivered at a much lower dose rate. There may still be some misrepair occurring in the HT144 cells with this protocol since the difference in the FAR/Gy is much less statistically significant than the difference in the survival of the two cell lines.

The amount of repair after 6 h was determined in order to observe repair after different doses of radiation. It has been previously shown that clonogenic repair is largely complete by 6 h [90] and DNA rejoining as measured by neutral filter elution is complete in 4 h even for the most radiosensitive human tumour cells [80]. It would also be interesting to compare repair rates with these two assays. When the cells were irradiated with a HDRI and allowed a 6 h repair time, the residual damage was slightly higher for the HT144 cell line ($p < 0.0001$). With the clonogenic assay, the survival of the two cell lines was very different. One other study that examined residual damage in human tumour cells also found no correlation between clonogenic survival and the final level of unrejoined dsbs [120]. The ability of both these cell lines to recover from PLD, as measured by the clonogenic assay, was the same: the cell lines had similar recovery ratios [90], however when the FRD is determined, the HT144 cells rejoined a greater fraction of the initial DNA dsbs than the SKMEL3 cells.

After all radiation protocols, there is evidence that the HT144 cells rejoin DNA damage but may not repair it correctly. Since the HT144 cells are the more radiosensitive cells, perhaps they are sensitive because their repair mechanisms are defective, allowing the DNA to rejoin but incorrectly. It appears that the HT144 cells misrepair dsbs to a greater extent than the SKMEL3 cells. It would be useful to examine the DNA repair fidelity more closely using the plasmid assay as described by Powell and McMillan [121]. This would allow misrepaired lesions to be analyzed to determine whether the HT144 cells are defective in their repair.

4.2.2 Fibroblasts

The differences in radiosensitivity between the fibroblast cell lines was more difficult to measure using the AFGE assay. After LDRI, there was a significant difference in their responses with the more sensitive S11358 cell line having a greater FAR/Gy than the OMB1 cell line. Although other studies have shown little difference in initial strand breaks between fibroblasts cell lines except when the cell lines were derived from AT patients [122], differences in rodent cell lines have been measured. The slopes of the lines found by this research group were similar to those in the study using fibroblasts with values ranging from 0.0149 to 0.0212 FAR/Gy for the normal fibroblast lines while the OMB1 cells had a slope of 0.0145 and the S11358 had a slope of 0.0169 FAR/Gy. The explanation for different initial DNA dsbs would be the same as for the melanoma cell lines.

After LDRI, both cell lines had slopes close to, or equal to, zero indicating that the amount of DNA dsbs remaining after irradiation were too small to be measured by this assay. The OMB1 cell line had a slightly higher slope, however this was not significantly different from the slope of the S11358 cell line response. With both cell lines, most of the DNA dsb damage was rejoined during the irradiation. A study comparing AT fibroblasts, which are characterized by their hypersensitivity to radiation, and normal fibroblasts after LDRI irradiated at 37°C using pulsed field gel electrophoresis showed greatly reduced FAR for both cell lines than when irradiated at 4°C where no repair would occur. When the cells were irradiated at 37°C, the AT cells had a higher FAR/Gy while no difference was observed between the two cell lines when they were irradiated at 4°C. At a dose rate

of 1 cGy/min, there was a measurable amount of damage remaining [106]. As with the melanoma cell lines and the LDRI experiments, this was a slightly higher dose rate than used in this study and with an apparatus that might have higher resolution for measuring low levels of DNA damage.

After fractionated irradiation, the graph of FAR vs. dose of the OMB1 cell line had a zero slope indicating that most of the dsbs had been rejoined during irradiation. The slope of the graph of the S11358 cell line was negative although not significantly different from zero. One way to explain this is to remember that the plots have been normalized to have zero FAR after zero Gy. Before normalization, even the zero Gy point had some amount of DNA released into the gel indicating that some DNA is broken at the time of irradiation or during the cell manipulation or breaks due to replication sites or cell turnover. This amount of DNA could be rejoined to DNA which is broken during the irradiation and have the effect of reducing the total DNA dsbs during irradiation. The initial damage (prior to normalization) after zero dose was the same for both fibroblasts cell lines (0.16) but this amount was slightly higher than for the melanoma cell line (0.13). This would provide the fibroblasts cell lines with more pre-existing DNA dsbs to repair having the effect of lowering the slope of the line. The fact that the two fibroblast cell lines had the same zero dose damage may indicate that the S11358 cell line has an overactive rejoining mechanism which repairs very quickly but incorrectly, not allowing correct repair to occur. This would have the effect of reducing the number of DNA dsbs but not increasing the survival.

When the cells were incubated for 6 h after HDRI, there was a small but significant

difference between the slopes of their FAR vs dose response curves with the more sensitive S11358 cell lines having more DNA dsbs/Gy. Another study which looked at the residual damage after a repair period found that the differences between cell lines of differing sensitivities were greater if a repair period was allowed [122]. They looked at a normal fibroblast cell line along with fibroblast cell line taken from an AT patient and found a higher ratio between the slopes of the two cell lines after 4 h of repair than immediately after HDRI irradiation. The same comparison would hold true for this data (1.2 for initial damage vs 4.5 for residual damage), but it should be emphasized that the difference between the slopes is more significant immediately after HDRI than if a 6 h repair period is allowed ($P = 0.01$ vs. $P = 0.4$). This is due to the residual damage data having larger errors. From these comparisons, these data still support the hypothesis that initial damage is a better predictor of radiosensitivity than residual damage which is in agreement with the work carried out by Radford with the neutral filter elution assay on rodent cells [76,107,118].

4.3 Clonogenic vs. AFGE

Plotting the clonogenic survival vs. FAR shows how DNA dsbs are related to survival. The curves are of the linear quadratic form since FAR is linear with dose over the range of dose used in these experiments. With the melanoma cell lines after HDRI, the SKMEL3 requires more DNA dsbs than HT144 to reach the same level of survival showing that the SKMEL3 cells are more resistant to DNA dsbs. With the fibroblast curves, however, the curves were the same after HDRI indicating that the same number of

initial DNA dsbs causes the same amount of cell kill in these two cell lines

The graph of the residual damage for the melanoma cell lines show a greater separation after 6 h of repair time than immediately after HDR1 indicating that the SKMEL3 are even more resistant to residual dsbs than HT144 as compared to initial dsbs. This is evidence that the HT144 cells may be misrepairing their damage because there was a much larger decrease in FAR for the HT144 cells than the SKMEL3 cells during the same repair time at the 10% survival level.

There are a few important points to remember when comparing the clonogenic assay to the AFIGE assay. The populations being examined in each assay may be very different since the clonogenic assay only measures the cells that survive and adhere to the plate. The AFIGE assay, on the other hand, measures the DNA dsbs of all the cells, dead or alive. The doses used in each assay are also quite different with the clonogenic assay limited by the number of colonies formed and therefore to low doses (< 12 Gy) while the AFIGE assay is limited by saturation at about 30 Gy. Another important point is that the resolution of the AFIGE assay is on the order of a few hundred DNA dsbs per cell [92] which could be more than enough to kill a cell. This means that even at DNA dsb levels less than resolvable by the AFIGE assay, cell death could be occurring. For these reasons, comparisons between the two assays must be done carefully with all the considerations in mind.

4.4 Other patients

Of all the other patients whose cells were grown into tissue culture, the most sensitive cell line came from a patient who showed both early and late reactions to radiotherapy although the reactions were mild (OMB7). The OMB8 cell line which came from a patient who had a severe late reaction to radiation also showed a sensitive reaction *in vitro*. All the patients who had shown some sensitive response to radiotherapy had some degree of sensitivity after fractionated treatment *in vitro* although the patients who showed only an early response had a very slight decrease in their clonogenic survival. This was true regardless of the severity of the patient's reaction to radiation. One patient (OMB14) who showed a slight increase in their *in vitro* radiosensitivity died from the cancer before any sign of a reaction to their radiation therapy could be seen. This patient had presented with systemic sclerosis which has been shown to become more severe with radiation. Although her cells *in vitro* did grow to a higher density than other fibroblast cell cultures, an initial dose of 2 Gy did not increase this plateau phase cell density. No sign of increased fibrosis was seen in the patient, although fibrosis is a late reaction which would not have had time to develop.

From these few cell lines, there is a trend that the cells of patients who have a late reaction to radiation show more sensitivity *in vitro*. The cell lines from patients who showed only an early reaction were not as sensitive to radiation *in vitro*. This is in agreement with several other studies that have shown correlation between late reactions and clonogenic survival [13,14,19] but no correlation between early reactions and clonogenic survival [12]. Many more cell lines, however, need to be examined to confirm

these results

4.5 Inter-patient variability

From the experiments conducted with several cell lines taken from the same patient, it was found that the clonogenic results after HDRI were quite reproducible with the standard deviation of the data being on the same order of magnitude as the inter-experiment variation of a single cell line. The same results were found after fractionated irradiation using the clonogenic assay. This data suggests that the clonogenic assay is sufficiently reproducible from one sample to another to be a useful way of measuring radiosensitivity.

The results from the AFIGE assay were not as promising. The standard deviation of the slopes of the FAR vs. dose graphs after HDRI was greater than the inter-experiment error from a single cell line. This indicates that the reproducibility of this assay is not adequate for use as a predictive assay when determining small differences in radiosensitivity. It should be noted here that the gel apparatus used in these experiments was not the best available on the market due to cost constraints. As described in the methods, this unit was assembled from some commercially available components with some home made components added. There has been and still is much development on gel apparatus with one of the best available units on the market being the CHEF DRII (Bio-Rad) unit. With such a unit, more reproducible results may be possible making the gel electrophoresis assay more feasible for the clinic.

4.6 Time scale of AFIGE assay

For an assay to be useful in the clinic, it needs to be able to be conducted in a few days. This means that there is no time to grow tissue samples into cell culture in order to run the assay. This rules out the clonogenic assay as a useful predictive assay in the clinic but the AFIGE assay still has potential since cells can be run in the gel without having been grown into culture. In the preliminary experiment with a skin sample being digested into single cells and run directly on the gel, the results were promising. A linear dose response was found when the cells were irradiated up to 30 Gy. The slope of this line was less than those of fibroblasts which had been grown in tissue culture, however this tissue had been frozen for several years. The method for digesting the cells was not optimized and with further investigation, using fresh tissue samples, better digestion and hence higher cell numbers, a steeper dose response curve may be obtained.

An advantage of this method is that all cells present in the tissue sample are tested for DNA dsb induction. When cells are grown in tissue culture, only the cells selected (in this study, fibroblasts) are tested. It is likely that the response of the skin to radiation is a result of many cell types in the skin reacting together.

5.0 CONCLUSIONS

There has been much discussion over the years on the future of predictive assays for radiotherapy [123-125]. Some success has been found with correlating *in vitro* tumour cell sensitivity to patient outcome and with correlating normal skin fibroblasts sensitivity *in vitro* with late reactions. Both these correlations are important, however, predicting normal tissue sensitivity is of primary concern since the reaction of the skin to radiation in the treatment field can be the dose limiting factor in radiotherapy. Some positive results have been found in this study confirming that differences in radiation sensitivity between cell lines are measurable with the clonogenic assay. It was found that with both the melanoma cell lines and the fibroblast cell lines, the clonogenic assay was able to separate differences in radiosensitivity. These differences were largest when a fractionated irradiation protocol was used, however, this was true only after high doses (>8 Gy) were administered. After a single HDRI, survival at these high doses could not be measured because of high levels of cell kill.

To measure differences in radiosensitivity two factors seem to be of importance. The first is that a radiation protocol which allows repair to occur is essential since repair undoubtedly plays a part in radiosensitivity. The second is that giving acute doses could induce an increased repair mechanism that does not occur after LDRI. In order to include these two factors in a predictive assay a fractionated irradiation protocol is needed and it is not surprising that this protocol best predicted the differences in radiosensitivity. This protocol also has the advantage of mimicking the protocols used in the clinic.

The clonogenic assay is also reproducible since the variation of the survival of samples taken from one patient was on the same order of magnitude as the variation of the survival of several experiments using a single cell line.

The drawback of the clonogenic assay, however, is that it takes too long to get results to be clinically feasible. A faster assay is required that can be completed in the few days after diagnosis of the disease and before treatment begins. The AFIGE assay was chosen as a potential predictor of radiosensitivity since it measures DNA dsbs which are thought to be the critical cellular lesion leading to cell death. The AFIGE assay was able to differentiate between the radiosensitivity of the melanoma cell lines, however, this worked best after HDR. DNA dsbs were almost all rejoined in the other radiation protocols resulting in low levels of residual damage which were difficult to measure with the resolution of this assay. With the fibroblast cell lines, differences in radiosensitivity were very difficult to detect with AFIGE even after HDR. Neither radiosensitive cell lines were deficient in rejoining DNA dsbs but both may have been misrepairing the dsbs to some extent. It is possible that it is the amount of correct repair which distinguishes the degree of radiosensitivity in the cell lines.

The AFIGE assay was not very reproducible as the variation in the slopes of the FAR versus dose lines from several samples taken from the same patient was greater than that of several experiments on the same cell line. The variation of the clonogenic survival curves of the same cell lines were reproducible, indicating that the samples were of similar radiosensitivity.

This AFIGE assay apparatus is, however, one of many which are used for gel

electrophoresis. Much work is being done on the development of this assay and the resolution is continuing to increase. The gel assay has an advantage over the clonogenic assay because the cells can be digested directly from the tissue and run in the gel instead of having to be grown into tissue culture. The feasibility of this was shown when a dose response was found after HDRI when such a method was used.

The results from the AFIGE assay indicated that the melanoma cell lines and the fibroblasts cell lines had different radiosensitivities for different reasons. Cell lines that were more sensitive to radiation after all radiation protocols, as measured by the clonogenic assay, did not always have more DNA dsbs/Gy, as measured by the AFIGE assay. After HDRI, both the sensitive HT144 cell line and the S11358 cell line had more initial DNA dsbs than their resistant partners but after the other radiation protocols, their induced dsbs relative to their associated resistant cell line were not comparable. This would indicate that different radiosensitive cell lines are so for different reasons. They may be sensitive to initial damage, deficient in repair, deficient in correct repair, sensitive to misrepaired damage, over actively misrepairing dsbs, have different repair rates or a host of other reasons. If this is true, it may be necessary to use several assays to determine radiosensitivity. Other assays that might be useful include fluorescent in-situ hybridization (FISH) to observe chromosome aberrations along with premature chromosome condensation [126], the comet assay to measure DNA dsbs in individual cells [127] or apoptosis [128], and repair fidelity assays [39].

There is still much work to be done before a predictive assay could be clinically feasible. Measurement of any one predictive factor will likely not be sufficient. A panel of

predictive factors may need to be measured for each patient and the treatment could be tailored from a multifactorial base. Investigation is needed into many predictive assays which could be combined to provide a reliable method of determining patient radiosensitivity which could be used routinely in determining optimum radiotherapy treatments

5.1 Future Directions

The work done in this thesis has given rise to many questions about the molecular mechanisms controlling radiosensitivity. The most important of these questions is that of misrepair. Is misrepair occurring in cell lines? to what extent? and how does misrepair relate to radiosensitivity? Repair fidelity assays exist which can determine whether the cells are undergoing correct or incorrect repair. One of these assays is a plasmid assay developed by Debenham [129] which tests for correct repair of DNA dsbs. With such an assay, the repair fidelity of the melanoma and fibroblast cell lines could be measured to determine whether correct repair is correlated with radiosensitivity and whether some cell lines can deal with misrepaired DNA better than others.

Another question which has arisen is whether or not repair mechanisms can be induced by acute doses of radiation. This can be tested by delivering a priming dose to the cells to determine how different priming doses affect subsequent survival curves. It would be interesting to look at both the amount of priming dose and the time between when it is delivered and the survival levels are assessed.

Once some of these questions have been answered, many samples need to be taken

from patients who have shown sensitive responses to radiation in order to obtain statistically significant results. These samples should be tested by several of the assays and ranked on a multifactorial basis and correlated with the clinical observations of radiation sensitivity. Although this is a significant undertaking which will require collaboration between many research groups, establishing a practical predictive assay in the clinic requires an understanding of the mechanisms of radiation sensitivity which can only be accomplished by a careful and thorough investigation.

REFERENCES

- 1 Brown, R.C , Raaphorst, G P , Danjoux, C.E., and Drouin, P. Multi-organ fatal chronic complications following radiation treatment for cancer of the cervix - results of fibroblast assay *Gynecologic Oncology*, **61**: 116-121, 1996.
- 2 Dunst, J and Gebhart, E Unexpected tumor response, radiation myelitis and increased *invitro*-radiosensitivity of lymphocytes in a patient with non-small lung cancer. *Radiother. Oncol.* **36**: 158, 1995
- 3 Davidson, S E , West, C.M.L , Roberts, S A , Hendry, J.H., and Hunter, R.D. Radiosensitivity testing of primary cervical carcinoma. evaluation of intra- and inter-tumour heterogeneity *Radiother. Oncol.* **18**: 349-356, 1990.
- 4 West, C M.L , Davidson, S E , Roberts, S A , and Hunter, R.D. Intrinsic radiosensitivity and prediction of patient response to radiotherapy for carcinoma of the cervix *Br. J. Cancer*, **68**: 819-823, 1993.
- 5 West, C M.L , Davidson, S.E., Burt, P.A , and Hunter, R D. The intrinsic radiosensitivity of cervical carcinoma: correlations with clinical data. *Int. J. Radiation Oncology Biol. Phys.* **31**: 841-846, 1995.
- 6 Brock, W A , Baker, F.L , and Peters, L.J. Radiosensitivity of human head and neck squamous cell carcinomas in primary culture and its potential as a predictive assay of tumor radiocurability. *Int. J. Radiat. Biol.* **56**: 751-760, 1989.
- 7 Brock, W A , Baker, F.L., Wike, J.L , Sivon, S.L., and Peters, L.J. Cellular radiosensitivity of primary head and neck squamous cell carcinomas and local tumor control *Int. J. Radiation Oncology Biol. Phys.* **18**: 1283-1286, 1990.
- 8 Weichselbaum, R.R., Beckett, M A , Vijayakumar, S., Simon, M.A., Awan, A.M., Nachman, J., Panje, W.R , Goldman, M.E., Tybor, A.G., Moran, W.J., Everett, E.V., Ahmed-Swan, S , and Farhangi, E Radiobiological characterization of head and neck and sarcoma cells derived from patients prior to radiotherapy. *Int. J. Radiation Oncology Biol. Phys.* **19**: 313-319, 1990
- 9 Girinsky, T., Lubin, R., Pignan, J P , Chavaudra, N., Gazeau, J., Dubray, B.M., Cosset, J M , Socie, G , and Fertil, B. Predictive value of *in vitro* radiosensitivity parameters in head and neck cancers and cervical carcinomas: preliminary correlations with local control and overall survival *Int. J. Radiation Oncology Biol. Phys.* **25**: 3-7, 1992

10. Geara, F.B., Peters, L.J., Ang, K.K., Wike, J.L., Sivon, S.L., Gutterberger, R., Callender, D.L., Malaise, E.P., and Brock, W.A. Intrinsic radiosensitivity of normal human fibroblasts and lymphocytes after high- and low-dose-rate irradiation *Cancer Res.* **52**: 6348-6352, 1992
11. Burnet, N.G., Nyman, J., Turesson, I., Wurm, R., Yarnold, J.R., and Peacock, J.H. Prediction of normal-tissue tolerance to radiotherapy from *in-vitro* cellular radiation sensitivity. *The Lancet*, **339**: 1570-1571, 1992
12. Begg, A.C., Russell, N.S., Knaken, H., and Lebesque, J.V. Lack of correlation of human fibroblast radiosensitivity *in vitro* with early skin reactions in patients undergoing radiotherapy. *Int. J. Radiat. Biol.* **64**: 393-405, 1993
13. Geara, F.B., Peters, L.J., Ang, K.K., Wike, J.L., and Brock, W.A. Prospective comparison of *in vitro* normal cell radiosensitivity and normal tissue reactions in radiotherapy patients. *Int. J. Radiation Oncology Biol. Phys.* **27**: 1173-1179, 1993
14. Johansen, J., Bentzen, S.M., Overgaard, J., and Overgaard, M. Evidence for a positive correlation between *in vitro* radiosensitivity of normal human skin fibroblasts and the occurrence of subcutaneous fibrosis after radiotherapy. *Int. J. Radiat. Biol.* **66**: 407-412, 1994.
15. Burnet, N.G., Nyman, J., Turesson, I., Wurm, R., Yarnold, J.R., and Peacock, J.H. The relationship between cellular radiation sensitivity and tissue response may provide the basis for individualising radiotherapy schedules. *Radiother. Oncol.* **33**: 228-238, 1994
16. Brock, W.A., Tucker, S.L., Geara, F.B., Turesson, I., Wike, J.L., Nyman, J., and Peters, L.J. Fibroblast radiosensitivity versus acute and late normal skin responses in patients treated for breast cancer. *Int. J. Radiation Oncology Biol. Phys.* **32**: 1371-1379, 1995.
17. Emami, B., Lyman, J., Brown, A., Coia, L., Goitein, M., Munzenrider, J.E., Shank, B., Solin, L.J., and Wesson, M. Tolerance of normal tissue to therapeutic irradiation. *Int. J. Radiation Oncology Biol. Phys.* **21**: 109-115, 1991
18. Nyman, J., Burnet, N.G., Turesson, I., Wurm, R., and Peacock, J.H. Correlation between *in vitro* cell radiosensitivity and normal tissue response. Part I - normal tissue response (Meeting abstract). *Br. J. Cancer*, **66(suppl XVII)**: 6, 1992
19. Bentzen, S.M., Overgaard, M., and Overgaard, J. Clinical correlations between late normal tissue endpoints after radiotherapy: implications for predictive assay of radiosensitivity. *Eur. J. Cancer*, **29A**: 1373-1376, 1993

20. Murnane, J.P. and Kapp, L.N. A critical look at the association of human genetic syndromes with sensitivity to ionizing radiation. *Seminars in Cancer Biology*, 4: 93-104, 1993.
21. Taylor, A.M.R., Harnden, D.G., Arlett, C.F., Harcourt, S.A., Lehman, A.R., Stevens, S., and Bridges, B.A. Ataxia-telangiectasia, a human mutation with abnormal radiation sensitivity. *Nature*, 258: 427-429, 1975.
22. Cole, J., Arlett, C.F., Green, M.H.L., Harcourt, S.A., Priestley, A., Henderson, L., Cole, H., James, S.E., and Richmond, F. Comparative human cellular radiosensitivity: II. The survival following gamma-irradiation of unstimulated (G_0) T-lymphocytes, T-lymphocyte lines, lymphoblastoid cell lines and fibroblasts from normal donors, from ataxia-telangiectasia patients and from ataxia-telangiectasia heterozygotes. *Int. J. Radiat. Biol.* 54: 929-943, 1988.
23. Turesson, I. Individual variation and dose dependency in the progression rate of skin telangiectasia. *Int. J. Radiation Oncology Biol. Phys.* 19: 1569-1574, 1990.
24. Tucker, S.L., Turesson, I., and Thames, H.D. Evidence for individual differences in the radiosensitivity of human skin. *Eur. J. Cancer*, 28A: 1783-1791, 1992.
25. Deschavanne, P.J. and Fertil, B. A review of human cell radiosensitivity *in vitro*. *Int. J. Radiation Oncology Biol. Phys.* 34: 251-266, 1996.
26. Chapman, J.D. and Gillespie, C.J. Radiation induced events and their time scale in mammalian cells. *Advances in Radiation Biology*, 9: 143-199, 1981.
27. Powell, S.N. and McMillan, T.J. DNA damage and repair following treatment with ionizing radiation. *Radiother. Oncol.* 19: 95-108, 1990.
28. Hall, E.J. Radiobiology for the radiologist. J B Lippincott Company, Philadelphia, 1994.
29. von Sonntag, C. *The chemical basis of radiation biology*, Taylor & Francis, London, 1987.
30. Ward, J.F. The yield of DNA double-strand breaks produced intracellularly by ionizing radiation. a review. *Int. J. Radiat. Biol.* 57: 1141-1150, 1990.
31. Cassarett, A.P. Radiation Chemistry In: *Radiation Biology*, 57-77. Prentice-Hall Inc. Englewood Cliffs, New Jersey, 1968.

32. Bradley, M.O. and Kohn, K.W. X-ray induced DNA double strand break production and repair in mammalian cells as measured by neutral filter elution. *Nucleic Acid Res.* **7**: 793-804, 1979.
33. Ward, J.F. Biochemistry of DNA lesions. *Radiat Res.* **104**: S103-S111, 1985.
34. Ward, J.F. DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation and repairability. *Progress in Nucleic Acids and Molecular Biology*, **35**: 95-125, 1988.
35. Whitaker, S.J. DNA damage by drugs and radiation: what is important and how is it measured? *Eur. J. Cancer*, **28**: 273-276, 1992.
36. Bryant, P.E. Enzymatic restriction of mammalian DNA using *Pvu* II and *Bam* HI: evidence for double strand break origin of chromosomal aberrations. *Int. J. Radiat. Biol.* **46**: 57-65, 1984.
37. Bryant, P.E. Enzymatic restriction of mammalian cell DNA: evidence for double-strand breaks as potentially lethal lesions. *Int. J. Radiat. Biol.* 1985.
38. Dewey, W.C., Ling, C.C., and Meyn, R.E. Radiation-induced apoptosis: relevance to radiotherapy. *Int. J. Radiation Oncology Biol. Phys.* **33**: 781-796, 1995.
39. Powell, S.N. and McMillan, T.J. The repair fidelity of restriction enzyme-induced double strand breaks in plasmid DNA correlates with radioresistance in human tumor cell lines. *Int. J. Radiation Oncology Biol. Phys.* **29**: 1035-1040, 1994.
40. Powell, S.N., Whitaker, S.J., Edwards, S.M., and McMillan, T.J. A DNA repair defect in a radiation-sensitive clone of a human bladder carcinoma cell line. *Br. J. Cancer*, **65**: 798-802, 1992.
41. Puck, T.T., Morkovin, D., Marcus, P.I., and Cieciura, S.J. Action of X-rays on mammalian cells. II. Survival curves of cells from normal human tissues. *J Exp Med*, **106**: 485-497, 1957.
42. Freshney, R.I. Culture of animal cells: A manual of basic technique. Wiley/Liss, New York, 1994.
43. Leenhouts, H.P. and Chadwick, K.H. Advances in radiation biology. 55-101. Academic Press, New York, 1978.
44. Chadwick, K.H. and Leenhouts, H.P. The molecular theory of radiation biology. Springer Verlag, Heidelberg, 1981.

- 45 Chadwick, K.H. and Leenhouts, H.P. A molecular theory of cell survival. *Phys. Med. Biol.* **18**: 78-87, 1973.
- 46 Hall, E.J. Radiobiology for the radiologist. J.B. Lippincott Co. Philadelphia, 1988.
- 47 Hall, E.J. Molecular biology in radiation therapy: the potential impact of recombinant technology on clinical practice. *Int. J. Radiation Oncology Biol. Phys.* **30**: 1019-1028, 1994.
- 48 Kellerer, A.M. and Rossi, H.H. The theory of dual radiation action. *Curr Top Radiat Res*, **Q.8**: 85-158, 1972.
- 49 Kellerer, A.M. and Rossi, H.H. A generalized formulation of dual radiation action. *Radiat Res*, **75**: 471-488, 1978.
- 50 Little, J.B., Nove, J., Strong, L.C., and Nichols, W.W. Survival of human diploid skin fibroblasts from normal individuals after X-irradiation. *Int. J. Radiat. Biol.* **54**: 899-910, 1988.
- 51 Geara, F.B., Peters, L.J., Ang, K.K., Wike, J.L., and Brock, W.A. Radiosensitivity measurement of keratinocytes and fibroblasts from radiotherapy patients. *Int. J. Radiation Oncology Biol. Phys.* **24**: 287-293, 1992.
- 52 Lambin, P., Fertil, B., Malaise, E.P., and Joiner, M.C. Multiphasic survival curves for cells of human tumor cell lines: induced repair or hypersensitive subpopulation? *Radiat Res*, **138**: S32-S36, 1994.
- 53 Loeffler, J.S., Harris, J.R., Dahlberg, W.K., and Little, J.B. *In vitro* radiosensitivity of human diploid fibroblasts derived from women with unusually sensitive clinical responses to definitive radiation therapy for breast cancer. *Radiat Res*, **121**: 227-231, 1990.
- 54 Raaphorst, G.P. Prediction of radiotherapy response using SF₂: is it methodology or mythology? *Radiother. Oncol.* **28**: 187-188, 1993.
- 55 Bentzen, S.M. Prediction of radiotherapy response using SF₂: Why it may work after all. *Radiother. Oncol.* **31**: 85-86, 1994.
- 56 West, C.M.L. and Hendry, J.H. Prediction of radiotherapy response using SF₂: is it methodology or mythology? *Radiother. Oncol.* **31**: 86-88, 1994.
- 57 Raaphorst, G.P., Ng, C.E., and Bichay, T.J. Further on prediction of radiotherapy response using SF₂. Is it methodology or mythology? *Radiother. Oncol.* **31**: 88-89, 1994.

58. Stausbol-Gron, B., Nielsen, O.S., Bentzen, S.M., and Overgaard, J. Predictive assays in radiotherapy. *In vitro* radiosensitivity in SCC of the head and neck. 1996.(Abstract)
59. McMillan, T.J., Cassoni, A.M., Eady, J.J., Powell, S.N., and Steel, G.G. DNA damage induction and repair in human tumour cell lines (Meeting Abstract). *Br. J. Cancer*, **62** (suppl 11): 7, 1990.
60. Lorrain, P. and Corson, D. Electrostatic fields II. In: *Electromagnetic fields and waves*, 91-137. W. H. Freeman and Company, New York, 1970.
61. Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular cloning. A laboratory manual. Cold Spring Harbour Laboratory Press, Plainview, N.Y. 1989
62. Birren, B. and Lai, E. Pulsed field gel electrophoresis. A practical guide. Academic Press, Inc. San Diego, 1993.
63. Klotz, L.C. and Zimm, B.H. Retardation times of deoxyribonucleic acid solutions. II. Improvements in apparatus and theory. *Macromolecules*, **5**: 471-481, 1972.
64. Gurrieri, S., Rizzarelli, E., Beach, D., and Bustamante, C. Imaging of kinked configurations of DNA molecules undergoing orthogonal field alternating gel electrophoresis by fluorescence microscopy. *Biochemistry*, **29**: 3396-3401, 1990.
65. Carle, G.F., Frank, M., and Olson, M.V. Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. *Science*, **232**: 65-68, 1986.
66. Denko, N., Giaccia, A., Peters, B., and Stamato, T.D. An asymmetric field inversion gel electrophoresis method for the separation of large DNA molecules. *Analytical Biochemistry*, **178**: 172-176, 1989.
67. Stamato, T.D. and Denko, N. Asymmetric field inversion gel electrophoresis: a new method for detecting DNA double-strand breaks in mammalian cells. *Radiat Res*, **121**: 196-205, 1990.
68. Corry, P.M. and Cole, A. Radiation induced double strand scission of the DNA of mammalian metaphase chromosomes. *Radiat Res*, **36**: 528-543, 1968.
69. Blocher, D. DNA double strand breaks in Ehrlich ascites tumor cells at low doses of X-rays. I. Determination of induced breaks by centrifugation at reduced speed. *Int. J. Radiat. Biol.* **42**: 317-328, 1982.

- 70 Blocher, D., Einspinner, M., and Zajackowski, J. CHEF electrophoresis, a sensitive technique for the determination of DNA double-strand breaks. *Int. J. Radiat. Biol.* **56**: 437-448, 1989.
- 71 Radford, I.R. The dose-response for low-LET radiation-induced DNA double-strand breakage: methods of measurement and implications for radiation action models. *Int. J. Radiat. Biol.* **54**: 1-11, 1988.
- 72 Iliakis, G., Blocher, D., Metzger, L., and Pantelias, G. Comparison of DNA double-strand break rejoining as measured by pulsed field gel electrophoresis, neutral sucrose gradient centrifugation and non-unwinding filter elution in irradiated plateau-phase CHO cells. *Int. J. Radiat. Biol.* **59**: 927-939, 1991.
- 73 McMillan, T.J., O'Neill, P., Peacock, J.H., and Prise, K. Workshop report. 1st LH Gray workshop. Measurement of radiation-induced damage. *Int. J. Radiat. Biol.* **58**: 391-396, 1990.
- 74 Whitaker, S.J., Powell, S.N., and McMillan, T.J. Molecular assays of radiation-induced DNA damage. *Eur. J. Cancer*, **27**: 922-928, 1991.
- 75 Olive, P.L., Chan, A.P.S., and Cu, C.S. Comparison between the DNA precipitation and alkali unwinding assays for detecting DNA strand breaks and cross-links. *Cancer Res.* **48**: 6444-6449, 1988.
- 76 Radford, I.R. The level of induced DNA double-strand breakage correlates with cell killing after X-irradiation. *Int. J. Radiat. Biol.* **48**: 45-54, 1985.
- 77 Giaccia, A., Weinstein, R., Hu, J., and Stamato, T.D. Cell cycle-dependent repair of double-strand DNA breaks in a gamma-ray-sensitive Chinese hamster cell. *Somatic Cell and Molecular Genetics*, **11**: 485-491, 1985.
- 78 Kemp, L.M., Sedgwick, S.G., and Jeggo, P.A. X-ray sensitive mutants of Chinese hamster ovary cells defective in double-strand break rejoining. *Mutat. Res.* **132**: 189-196, 1984.
- 79 Giaccia, A., Schwartz, J., Shieh, J., and Brown, J.M. The use of asymmetric-field inversion gel electrophoresis to predict tumor cell radiosensitivity. *Radiother. Oncol.* **24**: 231-238, 1992.
- 80 Schwartz, J.L., Rotmensch, J., Giovannazzi, S., Cohen, M.B., and Weichselbaum, R.R. Faster repair of DNA double-strand breaks in radioresistant human tumor cells. *Int. J. Radiation Oncology Biol. Phys.* **15**: 907-912, 1988.

81. Schwartz, J L , Giovanazzi, S , and Weichselbaum, R R Recovery from sublethal and potentially lethal damage in an x-ray-sensitive CHO cell *Radiat Res*, **111**: 58-67, 1987.
82. Whitaker, S J , Ung, Y C , and McMillan, T J DNA double-strand break induction and rejoining as determinants of human tumour cell radiosensitivity A pulsed-field gel electrophoresis study. *Int. J. Radiat. Biol.* **67**: 7-18, 1995
83. McMillan, T J , Cassoni, A M , Edwards, S M , Holmes, A , and Peacock, J H The relationship of DNA double-strand break induction to radiosensitivity in human tumour cell lines. *Int. J. Radiat. Biol.* **58**: 427-438, 1990
84. Wlodek, D and Hittelman, W N The repair of double-strand DNA breaks correlates with radiosensitivity of L5178Y-S and L5178Y-R cells *Radiat Res*, **112**: 146-155, 1987
85. Badie, C., Iliakis, G , Foray, N , Alsbeih, G , Cedervall, B , Chavaudra, N , Pantelias, G., Arlett, C., and Malaise, E.P Induction and rejoining of DNA double-strand breaks and interphase chromosome breaks after exposure to x rays in one normal and two hypersensitive human fibroblast cell lines. *Radiat Res*, **114**: 26-35, 1995
86. Cassoni, A.M., McMillan, T.J., Peacock, J H , and Steel, G.G Differences in the level of DNA double-strand breaks in human tumour cell lines following low dose-rate irradiation. *Eur. J. Cancer*, **28A**: 1610-1614, 1992.
87. Bichay, T.J., Feeley, M M., and Raaphorst, G P A comparison of heat sensitivity, radiosensitivity and PLDR in four human melanoma cell lines *Melanoma Research*, **2**: 63-69, 1992.
88. Stamato, T.D., Guerriero, S., and Denko, N Two methods for assaying DNA double-strand break repair in mammalian cells by asymmetric field inversion gel electrophoresis. *Radiat Res*, **133**: 60-66, 1993
89. Heller, D P Radiobiological aspects of cellular recovery following high and low dose-rate irradiation with/without mild hyperthermia in a human glioma cell model 152-156, 1993.(Abstract)
90. Ng, C E., Bussey, A M , and Raaphorst, G P Inhibition of potentially lethal and sublethal damage repair by camptothecin and etoposide in human melanoma cell lines *Int. J. Radiat. Biol.* **66**: 49-57, 1994
91. Iliakis, G., Metzger, L , Denko, N , and Stamato, T D Detection of DNA double-strand breaks in synchronous cultures of CHO cells by means of asymmetric field inversion gel electrophoresis *Int. J. Radiat. Biol.* **59**: 321-341, 1991

- 92 Ruiz de Almodovar, J M , Steel, G G , Whitaker, S J , and McMillan, T J A comparison of methods for calculating DNA double-strand break induction frequency in mammalian cells by pulsed-field gel electrophoresis *Int. J. Radiat. Biol.* **65**: 641-649, 1994
- 93 Whitaker, S J and McMillan, T J Oxygen effect for DNA double-strand break induction determined by pulsed-field gel electrophoresis *Int. J. Radiat. Biol.* **61**: 29-41, 1992
- 94 Abu-Shakra, M and Lee, P Exaggerated fibrosis in patients with systemic sclerosis (scleroderma) following radiation therapy *J Rheumatol*, **20**: 1601-1603, 1993
- 95 Cooper, S , G and Denham, J , W Progressive systemic sclerosis (diffuse scleroderma) and radiotherapy *Br J Radiol*, **63**: 804-805, 1990
- 96 Varga, J , Haustein, U F , Crecch, R H , Dwyer, J P , and Jimenez, S A Exaggerated radiation-induced fibrosis in patients with systemic sclerosis *JAMA*, **265**: 3292-3295, 1991
- 97 Taghian, A , Suit, H , Pardo, F , Gioiso, D , Tomkinson, K , DuBois, W , and Gerweck, L E *In vitro* intrinsic radiation sensitivity of glioblastoma multiforme *Int. J. Radiation Oncology Biol. Phys.* **23**: 55-62, 1992
- 98 Marples, B and Joiner, M C The response of Chinese hamster V79 cells to low radiation doses Evidence of enhanced sensitivity of the whole cell population *Radiat Res*, **133**: 41-51, 1993
- 99 Lambin, P , Marples, B , Malaise, E P , Fertil, B , and Joiner, M C Hypersensitivity of a human tumour cell line to very low radiation doses. *Int. J. Radiat. Biol.* **63**: 639-650, 1993
- 100 Thacker, J and Wilkinson, R E The genetic basis of cellular recovery from radiation damage-response of radiosensitive IRS to low-dose-rate irradiation *Radiat Res*, **144**: 294-300, 1995
- 101 Matthews, J H L , Meeker, B E , and Chapman, J D Response of human tumor cell lines *in vitro* to fractionated irradiation *Int. J. Radiation Oncology Biol. Phys.* **16**: 133-138, 1989
- 102 Gerweck, L E and Zaidi, S T The reproducibility and relationship between single and fractionated dose estimates of the surviving fraction at 2 Gy *Int. J. Radiation Oncology Biol. Phys.* **35**: 81-87, 1996

- 103 Taghian, A , Ramsay, J , Allalunis-Turner, J , Budach, W , Gioiso, D , Pardo, F , Okhunieff, P , Bleehen, N M , Urtasun, R , and Suit, H Intrinsic radiation sensitivity may not be the major determinant of poor clinical outcome of glioblastoma multiforme *Int. J. Radiation Oncology Biol. Phys.* **25**: 243-249, 1993
- 104 Ramsay, J , Ward, J F , and Bleehen, N M Radiosensitivity testing of human malignant gliomas *Int. J. Radiation Oncology Biol. Phys.* **24**: 675-680, 1992
- 105 Blocher, D In CHEF electrophoresis a linear induction of dsb corresponds to a nonlinear fraction of extracted DNA with dose *Int. J. Radiat. Biol.* **57**: 7-12, 1990
- 106 Foray, N , Fertil, B , Alsbeih, G , Badie, C , Chavaudra, N , Iliakis, G , and Malaise, E P Dose-rate effect on radiation-induced DNA double-strand breaks in the human fibroblast HF19 cell line *Int. J. Radiat. Biol.* **62**: 241-249, 1996
- 107 Radford, I R Evidence for a general relationship between the induced level of DNA double-strand breakage and cell-killing after X-irradiation of mammalian cells *Int. J. Radiat. Biol.* **49**: 611-620, 1986
- 108 Murray, D , Simpson, R , Rosenberg, E , Carraway, A , and Britten, R Correlation between gamma-ray-induced DNA double-strand breakage and cell killing after biologically relevant doses: analysis by pulsed-field gel electrophoresis *Int. J. Radiat. Biol.* **65**: 419-426, 1994
- 109 McKay, M J and Kefford, R F The spectrum of *in vitro* radiosensitivity in four human melanoma cell lines is not accounted for by differential induction or rejoining of DNA double strand breaks *Int. J. Radiation Oncology Biol. Phys.* **31**: 345-353, 1994
- 110 Ruiz de Almodovar, J M , Nunez, M I , McMillan, T J , Olea, N , Mort, C , Villalobos, M , Pedraza, V , and Steel, G G Initial DNA damage is a determinant of intrinsic cellular radiosensitivity *Br. J. Cancer*, **69**: 457-462, 1994
- 111 Radford, I R and Hodgson, G S Effect of ploidy in the response of V79 cells to ionizing radiation *Int. J. Radiat. Biol.* **51**: 765-778, 1987
- 112 McMillan, T J and Peacock, J H Molecular determinants of radiosensitivity in mammalian cells *Int. J. Radiat. Biol.* **65**: 49-55, 1994
- 113 Whitaker, S J and McMillan, T J Pulsed-field gel electrophoresis in the measurement of DNA double-strand break repair in xrs-6 and CHO cell lines: DNA degradation under some conditions interferes with the assessment of double-strand break rejoining *Radiat. Res.* **130**: 389-392, 1992

114. Carmichael, J., Park, J.G., Degraff, W.G., Gamson, J., Gazdar, A.F., and Mitchell, J.B. Radiation sensitivity and study of glutathione and related enzymes in human colorectal cancer cell lines. *Eur. J. Cancer Clin. Oncol.* **24**: 1219-1224, 1988.
115. Sinclair, W.K. and Morton, R.A. Survival and recovery in X-irradiated synchronized chinese hamster cells. In: *Cellular radiation biology*, 418-422. Williams and Wilkins, Baltimore. 1965
116. Dewey, W.C. and Humphrey, R.M. Radiosensitivity and recovery of radiation damage in relation to the cell cycle. In: *Cellular radiation biology*, 340-375. Williams and Wilkins, Baltimore, 1965.
117. Peacock, J.H., Eady, J.J., Edwards, S.M., Holmes, A., McMillan, T.J., and Steel, G.G. Initial damage or repair as the major determinant of cellular radiosensitivity. *Int. J. Radiat. Biol.* **56**: 543-547, 1989.
118. Radford, I.R. and Hodgson, G.S. A comparison of the induction of DNA double-strand breakage and lethal lesions by x-irradiation in Ataxia Telangiectasia and normal fibroblasts. *Radiat Res.* **124**: 334-335, 1990.
119. Ruiz de Almodovar, J.M., Bush, C., Peacock, J.H., Steel, G.G., Whitaker, S.J., and McMillan, T.J. Dose-rate effect for DNA damage induced by ionizing radiation in human tumor cells. *Radiat Res.* **138**: S93-S96, 1994.
120. Nunez, M.I., Villalobos, M., Olea, N., Valenzuela, M.T., Pedraza, V., McMillan, T.J., and Ruiz de Almodovar, J.M. Radiation-induced DNA double-strand break rejoining in human tumour cells. *Br. J. Cancer*, **71**: 311-316, 1995.
121. Powell, S.N. and McMillan, T.J. Clonal variation in DNA repair in a human glioma cell line. *Radiother. Oncol.* **21**: 225-232, 1991.
122. Wurm, R., Burnet, N.G., Duggal, N., Yarnold, J.R., and Peacock, J.H. Cellular radiosensitivity and DNA damage in primary human fibroblasts. *Int. J. Radiation Oncology Biol. Phys.* **30**: 625-633, 1994.
123. Terry, N.H.A., Brock, W.A., and Hendry, J.H. Workshop report: Future directions for predictive assays, 25-27 March 1993, round top, Texas. *Int. J. Radiat. Biol.* **64**: 335-338, 1993.
124. Peters, L.J. Inherent radiosensitivity of tumor and normal tissue cells as a predictor of human tumor response. *Radiother. Oncol.* **17**: 177-190, 1990.

125. Peters, L.J. and Brock, W A. Cellular radiosensitivity as predictors of treatment outcome: where do we stand? *Int. J. Radiation Oncology Biol. Phys.* **25**: 147-148, 1992
126. Brown, J.M., Evans, J., and Kovacs, M S. The prediction of human tumor radiosensitivity *in situ*: an approach using chromosome aberrations detected by fluorescence *in situ* hybridization *Int. J. Radiation Oncology Biol. Phys.* **24**: 279-286, 1992.
127. Olive, P.L., Wlodek, D., and Banath, J.P. DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. *Cancer Res.* **51**: 4671-4676, 1991
128. Olive, P.L., Frazer, G., and Banath, J.P. Radiation-induced apoptosis measured in TK6 human B lymphoblast cells using the comet assay *Cancer Metastasis Rev.* **11**: 179-195, 1993.
129. Debenham, P.G., Webb, M.B.T., Stretch, A., and Thacker, J. Examination of vectors with two dominant selectable genes for DNA repair and mutation studies in mammalian cells. *Mutat. Res.* **199**: 145-158, 1988.
130. Johns, H.E. and Cunningham, J.R. The physics of radiology Charles C Thomas, Springfield, Illinois, 1983.
131. Atomic Energy Control Board Proposed amendments to the *Atomic Energy Control Regulations* for reduced radiation dose limits based on the 1991 recommendations of the International Commission on Radiological Protection Consultative Document, C-122, 1991.(Abstract)

APPENDIX I

Low Dose Rate Irradiator

The Civic Division of the Ottawa Regional Cancer Centre received 15 decommissioned ^{137}Cs gynecological sources (7 uterine tube, 8 ovoid sources) from the General Division on June 27, 1992. Each uterine tube had an overall length (OL) of 20.9 mm, a diameter of 3.0 mm with the actual source length (AL) being 15.0 mm. Each ovoid source had an OL of 15.9 mm, a diameter of 3.0 mm and an AL of 10.0 mm. The source identification numbers and activities, as measured with a Capintec Radioisotope Calibrator (CRC-12R, serial# 12856), are shown in Table A-1.

Table A-1. Cesium source identification and activity measured on June 28, 1992.

Source Identification	Activity (GBq)
M3	0.643
M5	0.651
M7	0.652
AA1	0.663
AA2	0.646
M8	0.974
M9	0.982
AA7	1.01
AA8	0.982
N2	1.29
N4	1.281
BB3	1.29
BB4	1.3
BB7	1.52
BB8	1.54
Total Activity	15.425

The uterine tube identification numbers are represented by two characters and the ovoid sources are denoted with three characters.

The half life ($T_{1/2}$) of cesium is 30.0 years with a γ ray energy of 0.662 MeV. The half value layer(HVL) is 5.5 mm Pb.

Initially, the dose rate was approximated by calculating the radium equivalent (Ra_{eq}) by:

$$Ra_{eq} = A \frac{\Gamma_{Cs}}{\Gamma_{Ra}} \quad (A-1)$$

where A is the activity of cesium and Γ is the exposure rate constant of each isotope Γ_{Ra} equals $0.825 \text{ Rm}^2\text{hr}^{-1}\text{Ci}^{-1}$ and Γ_{Cs} equals $0.328 \text{ Rm}^2\text{hr}^{-1}\text{Ci}^{-1}$. Converting the activity from Table A-1 to 0.416 Ci, the activity of cesium was determined to be 166 mg Ra_{eq} .

To estimate the distance away from the source plane required for the desired dose rate, data was taken from Table 13-4 in Johns and Cunningham [130] which gives the number of milligram hours (mg hr) for radioactivity arranged over an area in tissue equivalent material. The largest area available on the table (80 cm^2) was used.

Table A-2 Dose rate for varying distances from source plane

Distance (cm)	mg hr for 10 Gy	Dose rate (cGy/min)
1	1567	1.77
2	2726	1.05
3	3891	.710
4	5213	.531
5	6756	.410

Sample calculation for 1 cm:

$$\begin{aligned}
 \text{hr for 10 Gy} &= \frac{\text{mg hr eq for 10 Gy}}{\text{mg eq}} \\
 &= \frac{1567 \text{ mg hr eq for 10 Gy}}{166 \text{ mg Ra eq}} \\
 &= 9.44 \text{ hr for 10 Gy} \\
 &= 1.06 \text{ Gy/hr} \\
 &= 1.77 \text{ cGy/min}
 \end{aligned}$$

To estimate the best source configuration to obtain an iso-dose-rate plane, the Manchester system was used [130]. To get the desired dose rate, the source plane had to be about 2 cm from the plane of the irradiated samples. The sources were placed in a 9 cm x 9 cm square arrangement and since the length of one side of this square was to be greater than twice the distance of the source to the plane of irradiation, two parallel lines of sources were placed inside the square spaced by 3 cm (Figure A1.1).

Once the source configuration was estimated, the source strengths and positions

Figure A1.1 Cesium sources before Theraplan

Figure A1.2 Cesium sources after theraplan with shielding

were entered into the treatment planning computer (TP-11 Theraplan, Theratronics) in the dosimetry lab and the dose-rate at 2.5 cm from the source plane was calculated using subroutine QISODO for linear sources. An iterative process of adjusting the source positions and recalculating the dose-rate was carried out until the flattest isodose-rate lines were found (Figure A1.2). The shielding shown was added later to flatten the dose distribution. The cesium sources were then sandwiched between two pieces of 0.5 cm acrylic with grooves carved in them to hold the sources in place.

The required shielding was calculated based on a person sitting at the closest desk in the lab as follows

$$\text{Exposure Rate without shielding} = \frac{X}{t} = \frac{\Gamma A}{d^2} \quad (\text{A-2})$$

where X is the exposure, t is time, Γ is the exposure rate constant which is defined as the exposure rate in R/hr in free air at a point 1 m from a 1 Ci source, A is the activity of the source and d is the distance from the source [130]. Using the distance to the closest desk to be 4 m, the exposure rate was calculated to be $5.46 \times 10^{-3} \text{ Rhr}^{-1}$.

To convert the exposure rate to dose rate the exposure rate was multiplied by f_{med} where

$$f_{\text{med}} = \frac{\left(0.00873 \frac{\text{Gy}}{\text{R}} \right) \left(\frac{\mu_{\text{ab}}}{\rho} \right)_{\text{med}}}{\left(\frac{\mu_{\text{ab}}}{\rho} \right)_{\text{air}}} \quad (\text{A-3})$$

and μ_{at}/ρ is the mass attenuation coefficient for either the medium or air. From this equation, the dose-rate 5 m from the source without shielding was found to be 5.30×10^{-5} Gy/hr and using a quality factor of 1 for cesium, to convert to dose equivalent, this was equal to 5.30×10^{-5} Sv/hr.

Assuming this desk was occupied 8hr/day, 5 day/wk and 50 wk/yr, the occupancy was calculated to be 2000 hr/yr which would give a total dose of 106 mSv/yr if the source was unshielded. According to the AECB C-122, the limit of radiation allowed for a radiation worker is 20 mSv/yr and for a non radiation worker is 1 mSv/yr [131]. This cesium irradiator was being placed next to a pre-existing radium irradiator. The dose from the radium irradiator at the same desk was 1.2 mSv/yr. It was decided that it would be reasonable to raise this amount by 0.1 mSv/yr to 1.3 mSv/yr.

The amount of lead needed to shield the ^{137}Cs irradiator down to this amount was calculated as follows:

Attenuation factor required	Unshielded dose rate shielded dose rate
	106 mSv/yr 0.1 mSv/yr
	1.06×10^3
Tenth value layer (TVL)	$\log_{10}(\text{attenuation factor})$ 3.02 TVL 9.68 half value layers (HVL)

The HVL for lead shielding cesium is 5.5 mm therefore 5.3 cm of lead were required

Since lead comes in 5 cm thick blocks, this was considered to be adequate given the conservative estimates made in the calculations

The shielding was designed to encase the sources while allowing access to load and unload the cell samples. To reduce exposure to the experimenter during sample loading/unloading, the access was made through a sliding lead door on the side of the irradiator so that the experimenter would not be directly exposed to the sources at any time. The samples were loaded with a long rod which allowed the experimenter to stay at least 1 m from the irradiator at all times.

Another consideration for the design of the irradiator included the size of container in which the cells would be grown. For the radium irradiator already in use, the cells were grown in 1.3 cm³ glass vials which were again used for the cesium irradiator for consistency. A 12 cm x 12 cm square held 36 vials (Figure A1.3 and A1.4).

The final design of the irradiator is shown in Figure A1.5.

Once the sources were placed into the incubator, the dose-rates at various distances from the incubator were measured with a survey meter and compared to the dose-rate that existed before the installation of this second LDR irradiator. The dose-rates are summarized in Table A-3.

$.75 \pm .04$ cGy/min

$.65 \pm .03$ cGy/min

Fig A1-3 Vial holder from above

Figure A1 4 Vial holder from the side

Figure A1.5 Final design of incubator and irradiator

Table A-3. Dose-rates before and after installation of Cesium irradiator

Area Monitored	Measured Dose Rate (mSv/yr)	
	Existing	With Cs irradiator
outside Cs irradiator at floor	40	100
outside room, door knob	4	6
outside door, eye height	4	8
closest desk	1.2	1.4
in dark room		10

Once constructed and tested for radiation protection, the dose was measured in each of the vials using TLDs and the dose-rate in vials in the centre was found to be much higher than at the edges. A 1 mm shield was added (Figure A1.2) to try to reduce this discrepancy. With the shielding added, the dose rate of the centre 16 vials was 75 ± 0.4 cGy/min. The vials along the edges (excluding corners) had a dose-rate of 0.65 ± 0.3 cGy/min and the corners had a dose-rate of 49 ± 0.2 cGy/min. In most experiments only the central 16 vials were used with the edges being used only if necessary and exposure times being adjusted accordingly.

END

1 7 - 0 6 - 9 7

FIN