THE INFLUENCE OF DNA DAMAGE, DNA REPAIR AND CHROMATIN STRUCTURE ON RADIOSENSITIVITY

By

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature   Date

ABSTRACT

The factors which control radiosensitivity are of vital importance for the understanding of cell inactivation and for cancer therapy. Cell cycle blocks, total induced DNA damage, DNA repair, apoptosis and chromatin structure are likely to play a role in the responses leading to cell death.

I have examined aspects of irradiation-induced G2/M blocks in DNA damage and repair. In HT29, L132 and ATs4 cells the total amount of induced DNA damage by isodoses of 4.5 Gy, 5 Gy and 2 Gy was found to be 14 %, 14 % and 12 % respectively. Most of the DNA repair was completed before the G2/M maximum and only 3 % of DNA damage remains to be restored in the G2/M block.

The radiosensitivity in eleven cell lines was found to range from SF2 of 0.02 to 0.61. By FADU assay the undamaged DNA at 5 Gy was found to range from 56% to 93%. The initial DNA damage and radiosensitivity were highly correlated (r^2=0.81). After 5 Gy irradiation and 12 hours repair two groups of cell lines emerged. The group 1 cell lines restored undamaged DNA to a level ranging from 94 % to 98 %. The group 2 cell lines restored the undamaged DNA to a level ranging from 77 % to 82 %. No correlation was seen between residual DNA damage remaining after 12 hours repair and radiosensitivity.

In CHO-K1 cells chromatin condensation induced by Nocodazole was found to marginally increase the radiosensitivity as shown by the change of the mean inactivation dose ($\overline{D}$) from 4.446 to 4.376 Gy. Nocodazole also increased the

initial DNA damage, induced by 5 Gy, from 7 % to 13 %. In xrs1 cells these conditions increased the radiosensitivity from $\overline{D}$ of 1.209 to 0.7836 Gy and the initial DNA damage from 43 % to 57 %. Disruption of chromatin structure with a hypertonic medium was found to increase radiosensitivity in CHO-K1 cells from $\overline{D}$ of 4.446 to 3.092 Gy and the initial DNA damage from 7 % to 15 %. In xrs1 cells these conditions caused radiosensitivity to decrease from $\overline{D}$ of 1.209 to 1.609 Gy and the initial DNA damage from 43 % to 36 %.

Repair inhibition by Wortmannin increased the radiosensitivity in CHO-K1 from a $\overline{D}$ of 5.914 Gy in DMSO controls to a $\overline{D}$ 3.043 Gy. In xrs1 cells repair inhibition had no effect on radiosensitivity. Significant inhibition of repair was seen in CHO-K1 at 2 hours (p<0.0001) and at 20 hours (p=0.0095). No inhibition of repair was seen in xrs1 cells at 2 hours (p=0.6082) or 20 hours (p=0.6069).

While DNA repair must be allocated to the post-irradiation period, the G2/M block seen in p53 mutants reaches a maximum only 12 hours post-irradiation when most of the repair is completed. As the G2/M block resolves and cells re-enter cycle 28 hours after the G2 maximum it appears that repair processes cannot be the only reason for the G2/M cell cycle arrest. At low doses of irradiation initial DNA damage correlates with radiosensitivity. This suggests that the initial DNA damage is a determinant for radiosensitivity. Repair of DNA double-strand breaks by the non-homologous end joining (NHEJ) mechanism, identified by inhibition with Wortmannin, was shown to influence residual DNA damage and cell survival. Both the initial DNA damage and DNA repair were found to be influenced by chromatin structure. Chromatin structure was

modulated by high salt and by Nocodazole, and has heen identified as a parameter which influences radiosensitivity.

OPSOMMING

Die faktore wat betrokke is in die meganisme van stralings-sensitisering is van hoogs belang vir die begrip van sel inaktiveering en kanker terapie. Sel siklus blokke, totale geïnduseerde DNS skade, DNS herstel, apoptose en chromatien struktuur is moontlike rol vertolkers in die sellulêre response wat ly tot seldood. Ek het die aspekte van stralings-geïnduseerde G2/M blokke in DNS skade en DNS herstel ondersoek. Die hoeveelheid geïnduseerde DNS skade, deur ooreenstemmende stralings-dosisse, in HT29, L132 en ATs4 selle is 14 %, 14 % en 12 %. Meeste van die DNS herstel is klaar voordat die G2/M maksimum beryk word en net 3 % DNS skade bly oor om herstel te word in die G2/M blok. Die stralings-sensitiwiteit in elf sel lyne varieer tussen 'n SF2 van 0.02 en 0.61. Deur die gebruik van die FADU metode is gevind dat die onbeskadigde DNS na 5 Gy bestraling varieer tussen 56 % en 93 %. Die totale geïnduseerde DNS skade en stralings-sensitiwiteit was hoogs gekorreleer (r^2=0.81). Na 5 Gy bestraling en 12 ure herstel kan die sel lyne in twee groepe gegroepeer word. Die groep 1 sel lyne herstel die onbeskadigde DNS terug na 'n vlak wat varieer tussen 94 % en 98 %. Die groep 2 sel lyne herstel die onbeskadigde DNS terug tot op 'n vlak wat varieer tussen 77 % en 82 %. Geen korrelasie is gesien tussen oorblywende DNS skade en stralings-sensitiwiteit na 12 ure herstel nie. In die CHO-K1 sel lyn, chromatien kompaksie geïnduseer deur Nocodazole, vererger die stralings- sensitiwiteit soos gesien deur die gemiddelde inaktiveerings dosis ($\overline{D}$) wat verlaag het van 4.446 tot 4.376. Nocodazole het ook die totale DNS skade verhoog van 7 % tot 13 %. Onder dieselfde kondisies,

in die xrs1 sel lyn, is 'n verergering van stralings-sensitiwiteit ($\overline{D}$) gesien van 1.209 tot 0.7836 en verhoog DNS skade van 43 % tot 57 %. Die ontwrigting van die chromatien struktuur deur die gebruik van hipertoniese medium het die stralings-sensitiwiteit ($\overline{D}$) vererger in CHO-K1 selle van 4.446 tot 3.092. Die totale DNS skade is verhoog van 7 % tot 15 %. Onder dieselfde kondisies, in die xrs1 sel lyn, verbeter die stralings-sensitiwiteit ($\overline{D}$) van 1.209 tot 1.609 en die totale DNS skade verminder van 43 % tot 36 %. DNS herstel inaktiveering in die teenwoordigheid van Wortmannin het die stralings-sensitiwiteit ($\overline{D}$) in CHO-K1 selle vererger van 5.914 in DMSO verwysings kondisies tot 3.043. Die DNS herstel inaktiveering in xrs1 selle het geen uitwerking gehaat op stralings-sensitiwiteit nie. Noemenswaardige inaktiveering van DNS herstel is gesien in CHO-K1 selle na 2 ure (p<0.0001) en na 20 ure (p=0.0095). Geen inaktiveering is gesien in xrs1 selle na 2 ure (p=0.6082) of na 20 ure (p=0.6069) nie.

Terwyl DNS herstel moet plaasvind na die bestralings periode, beryk die G2/M blok in p53 gemuteerde selle sy maksimum 12 ure na bestraling terwyl meeste van die DNS herstel alreeds voltooi is. Aangesien die G2/M blok eers 28 ure later begin sirkuleer moet die G2/M blok nog 'n funksie vervul anders as DNS herstel. By lae dosisse van bestraling korreleer die totale geïnduseerde DNS skade met stralings-sensitiwiteit. Dit dui daarop dat die totale DNS skade 'n bepalende faktor moet wees in stralings-sensitiwiteit. Die herstel van DNS skade deur die nie-homoloë eindpunt samevoeging (NHES) meganisme, geïdentifiseer deur inaktiveering deur Wortmannin, het 'n invloed op oorblywende DNS skade en sellulêre oorlewing. Beide die totale DNS skade en DNS herstel was beïnvloed deur die chromatien struktuur. Chromatien struktuur

was gemoduleer deur hoë sout konsentrasies en deur Nocodazole, en is geïdentifiseer as a belangrike parameter wat stralings-sensitiwiteit beïnvloed.

DEDICATION

FOR MY PARENTS

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LIST OF ABBREVIATIONS

α: linear coefficient of inactivation after ^{60}Co γ-irradiation.

AUC: area under the curve.

β: quadratic coefficient of inactivation after ^{60}Co γ-irradiation.

cdk: cyclin dependent kinases.

CFGE: constant field gel electrophoresis.

$\overline{D}$:   mean inactivation dose after ^{60}Co γ-irradiation.

DNA-PK: DNA-dependent protein kinase.

DNA-PK$_{cs}$:   DNA-dependent protein kinase catalytic subunit.

EDTA: ethylene diamine tetra-acid (disodium salt).

FADU: fluorometric analysis of DNA unwinding.

FBS: foetal bovine serum.

FIGE: field inversion gel electrophoresis.

HR: homologous recombination.

LMDS: local multiply damaged sites

NHEJ: non-homologous end joining.

P53:   tumour-suppressor protein 53.

PARP: poly-(ADP-ribose) polymerase.

PBS: phosphate-buffered saline.

PFGE: pulsed-field gel electrophoresis.

REF: radiotoxicity enhancement factor.

RIF: repair inhibition factor

SD/SEM: standard deviation/standard error margins.

SF2:   surviving fraction at 2 Gy of ^{60}Co γ-irradiation.

CHAPTER 1

Introduction

1.1. Radiosensitivity

Irradiation produces a variety of responses in tumours and in normal tissue. The degree to which normal tissue can be spared in radiotherapy is strongly influenced by the radiosensitivity of the malignant tissue. The radiosensitivity of tumour cells in culture varies widely, ranging from the radiosensitive lymphomas, seminomas and neuroblastomas to the very resistant melanomas and glioblastomas (Arlett *et al.* 1980, Fertil *et al.* 1984, 1985, Peacock *et al.* 1988 and Weichselbaum *et al.* 1989).

In vitro radiosensitivity has been known to depend on the susceptibility of a cell line or tissue to express apoptosis (Stephens *et al.* 1993, Tauchi and Sawada 1994, Olive *et al.* 1996, Hu and Hill 1996) and its ability to repair DNA damage (Fertil and Malaise 1985, Wlodek and Hittelman 1988b, Durante *et al.* 1998, Dolling *et al.* 1998). The proliferative state of the cell line in question would also influence its response to irradiation and its subsequent survival level (Fowler 1986, Begg *et al.* 1990, Budach *et al.* 1997). Another factor influencing radiosensitivity is the status of the tumour suppressor protein (p53) of the cells (Budach, 1997, Chiarugi *et al.* 1998, Dahm-Daphi, 2000).

It is accepted knowledge that unrepaired DNA double-strand breaks are lethal lesions (Frankenberg-Schwager 1989, Steel *et al.* 1989). DNA double-strand

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breaks can be induced by ionising radiation (Frankenberg *et al.* 1981, Kampf, 1988, Rudoltz *et al.* 1996) and by drugs (Snyder, 2000). Although DNA double-strand breaks are the most crucial lesions effecting cell survival, other forms of DNA damage should not be ignored. These lesions include single-strand breaks, nucleotide dimers and various cross links between DNA and between DNA and nuclear proteins (Arrand and Michael, 1992).

The relationship between radiosensitivity and DNA-damage is not clear. Both the initial DNA damage and the residual unrepaired DNA damage may be determinants in the processes leading to cell death. Furthermore, the molecular mechanisms which control variations in radiation-induced DNA damage between cell types and cell cycle phases is not understood and no reliable predictive assay for tumour radiation responsiveness exists (McMillan *et al.* 1994, Terry *et al.* 1993).

1.2. Cell cycle checkpoints

Irradiation of cells causes a transient division delay, which may include a G1 arrest, an S-phase delay or a G2 arrest (Dirks and Rutka 1997). Cell cycle delays are likely to play a role in cell survival following DNA damage and there is currently a great interest in the mechanisms controlling cellular responses to damage. The progression of cells through the cell cycle, and the transition of cell cycle checkpoints are controlled by cyclin proteins and enzymes called cyclin dependent kinases (cdk's) (Desai *et al.* 1992). Specific cyclins operate with specific cdk's at the G1/S and G2/M transition points as shown in figure 1.1. While the molecular events resulting in the formation of the G1 block are

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relatively well established and involve the p53 pathway (Kastan *et al.* 1991), very little is known about the signalling initiated by DNA damage which gives rise to a G2 delay. The G2 block can be induced as a result of many different types of DNA damage and is independent of the p53 status (Hwang and Muschel, 1998). Some evidence does exist for a role of p53 in the G2 block (Winters *et al.* 1998), but other p53 independent mechanisms must play a role.

In p53 wild-type cells, the irradiation-induced G1 arrest is associated with DNA repair (Lane, 1994). Whether the G2 block is also induced to facilitate DNA repair is an attractive possibility because p53 mutant cells cannot arrest in G1 (Russell et al. 1996, Hwang and Muschel, 1998) and hence would require time to prevent the propagation of a defective genome into mitosis.

Figure 1.1
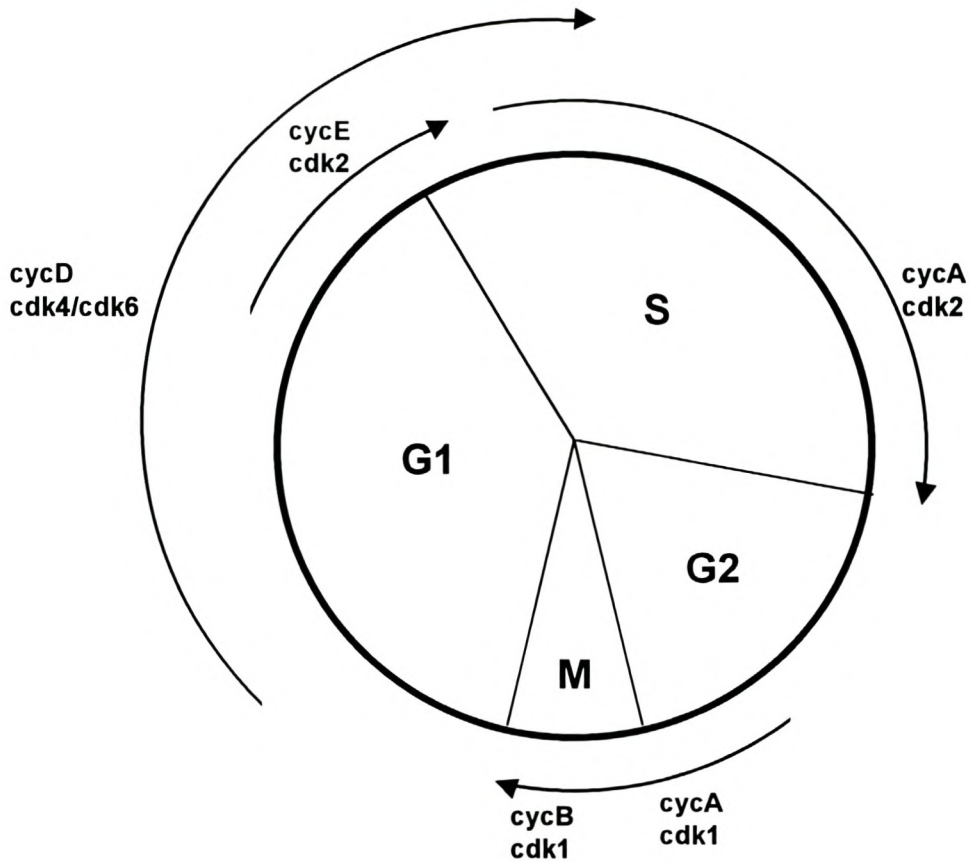
Schematic diagram of the cell cycle showing the regions of action of cyclins (cyc) and cyclin dependent kinases (cdk's). Cyclins are the regulatory subunits of cdk's. Cyclins associate with different cdk's at various stages of the cell cycle. A cdk can associate with more than one cyclin and a cyclin can interact with more than one cdk. Cyclins target cdk's to their substrates and determine the timing of cdk activity.

1.3. DNA-damage induction

Irradiation induces DNA damage by direct ionisation and displacement of bonding electrons (photoelectron effects) and indirectly via free radicals (figure 1.2). To a first approximation a cell can be considered as an aqueous system. The indirect effects of ionising irradiation on DNA in aqueous solutions in the presence of O_2 are almost exclusively mediated by $^•OH$ radicals as the result of radiolysis of H_2O molecules (Scholes, 1983). The direct effect of irradiation occurs as the result of the displacement of electrons giving rise to ionised molecules, which are highly reactive and chemically unstable. This excess of energy in higher orbitals can be dissipated either by emission of photons (fluorescence) on return to the initial state or by rupture of a covalent bond and scission of the molecule into two radicals. Bond scission is more probable after an ionisation than after excitation because the amount of energy received is greater in the first case (Halliwell and Gutteridge, 1991).

Figure 1.2
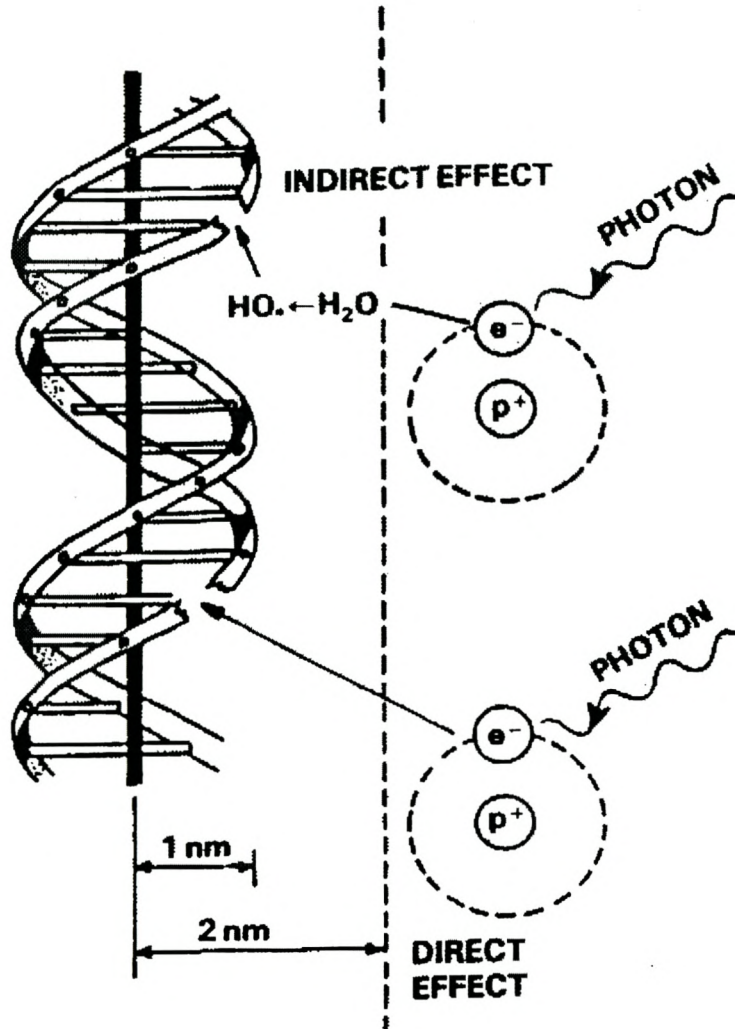
The direct and indirect effect of X- and γ-rays (Tubiana *et al*. 1990).

The concept of Local Multiply Damaged Sites (LMDS), which has emerged from considerations of the fine structure of radiation damage to DNA, was first discussed by Ward (1985). Calculations show that clusters of ionisations occur at the end of electron tracks, containing 10 or more ionisations within a diameter of perhaps 5 or more nanometers (Goodhead, 1989, Johnston and Bryant, 1994, Nikjoo *et al.* 1998, Prise *et al.* 1998). If such an event occurs in close proximity to DNA, which has a diameter of 2 nm, it would be expected to give rise to considerable local damage, perhaps containing a few double- and single-strand breaks plus base damage (Ward, 1990). A cell, which is capable of repairing isolated breaks, might well be overwhelmed by LMDS lesions. Since the incidence of single-strand breaks (~1000 per Gy) (Elkind, 1979) and double-strand breaks (~40 per Gy) (Blöcher, 1982) is very much greater than the frequency of lethal lesions (~1 per Gy), it is clear that cells have a remarkably high ability of repairing DNA damage induced by irradiation. DNA lesions, which cannot be repaired, will attain critical importance because they become lethal. The LMDS lesions are prominent candidates for lethal lesions.

1.4. DNA Repair

Ionising radiation induces various types of DNA damage, including double-strand breaks, single-strand breaks, base damage and DNA-protein crosslinks (Arrand and Michael, 1992). DNA double-strand break induction and repair, and the fidelity of DNA repair, seem to be critical factors which determine radiosensitivity in human cell lines (Nunez *et al.* 1996). Because of this, much effort has been expended in trying to understand the molecular mechanisms

leading to the repair of irradiation-induced DNA damage. Two independent double-strand break repair pathways have been identified to date (figure 1.3). Firstly the Non-Homologous End Joining (NHEJ) pathway and secondly the Homologous Recombination (HR) pathway. Operation of the NHEJ pathway requires the XRCC5 gene, which encodes the 80-kD subunit of Ku (Jeggo *et al.* 1999, Weaver, 1995). Along with another protein called p350, Ku80 and Ku70 forms the DNA-dependent protein kinase (DNA-PK) complex which is involved in DNA double-strand break repair (Anderson, 1993). The Ku proteins target the damaged DNA by attaching to the DNA termini, whereas p350 is the catalytic subunit containing kinase activity. The binding of Ku to the DNA termini fulfils the important function of protecting the DNA termini from exonuclease digestion (Polotnianka *et al.* 1998) and targeting the lesions for the DNA-PK catalytic site.

The HR pathway, which is mainly found in bacteria and yeast, is less rapid and in contrast to the error prone NHEJ pathway, the HR pathway is error free (Liefshitz *et al.* 1998). HR is initiated by the binding of Rad52 protein to free 3' DNA termini at the lesion (Parsons *et al.* 2000). The Rad52 binding to the DNA 3' termini also serves a protecting function against DNA digestion by exonucleases. Nucleolytic processing of the 5' terminus in the double-strand break generates a gap and 3' single strand DNA tail associates with Rad51 adjacent to Rad52 (figure 1.3). This complex loses Rad52 and invades base pairs of homologous DNA from the other chromosome and generates a 4 way heteroduplex junction (Holliday, 1964) which facilitates recombination between homologous DNA sequences (Resnick, 1976 and 1996). This is followed by

repair of the missing nucleotides by base pairing and nucleotide incorporation (figure 1.3).

Enzymes, which are activated by DNA strand breaks, e.g. poly(ADP-ribose) polymerase (PARP) and Mdm-2, have been implicated in the sensing of DNA damage (Buerkle *et al.* 1992, Momand and Zambetti, 1997). PARP is thought to indirectly activate p53, p21 and DNA-PK (Le Ruhn *et al.* 1998).

Proteins, which are involved in the detection and repair of irradiation-induced DNA damage, may have potential clinical applications. One could target Ku, p350, PARP, or other proteins yet to be discovered which act downstream, thus rendering cells more radiosensitive by preventing the recognition or repair of DNA damage. An example is Wortmannin, which inhibits the Ku subunit function of DNA-PK (Cheong *et al.* 1999, Rosenzweig *et al.* 1997). More drugs capable of inactivating these repair proteins and capable of inhibiting double-strand break repair are being sought. In this strategy, the preferential targeting of tumour cells over normal tissue remains an ever important problem.

The availability of Wortmannin, which inhibits DNA repair by non-homologous end joining (NHEJ) (Cheong *et al.* 1999, Rosenzweig *et al.* 1997), prompted an important range of experiments on the role of DNA repair in cell survival (see below).

Figure1.3
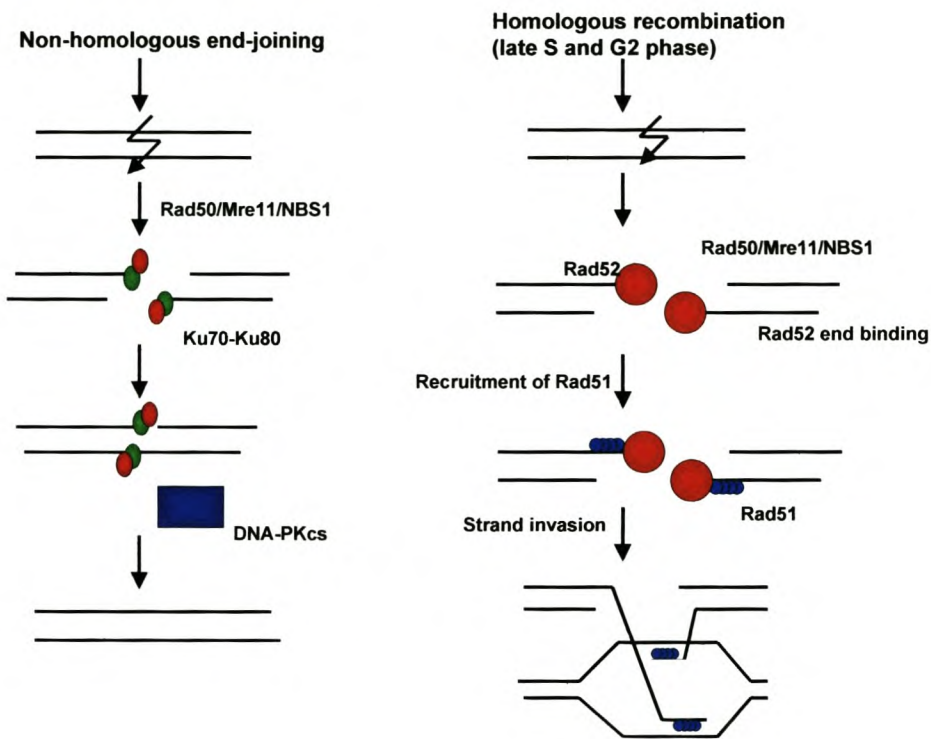
Schematic diagram of two important DNA repair pathways. Non-homologous end-joining (NHEJ) is initiated by the binding of Ku70 and Ku80 subunit, which protect the broken DNA ends. The Ku70/Ku80 proteins associate with the catalytic subunit of DNA-PK to form the active repair enzyme to close the gap by filling in new nucleotides in the absence of any homology. Homologous recombination (HR) is initiated by the binding of Rad52 protein to the DNA ends. Nucleolytic processing of the double-strand breaks generates a 3' single strand DNA tail and binds Rad51. This complex invades base pairs with homologous DNA from the other chromosome facilitating repair of the missing nucleotides.

1.5. Initial vs. residual DNA damage

Initial DNA double-strand breaks and residual double-strand breaks have been measured at high doses (10-100 Gy) of irradiation by the method of pulsed-field gel electrophoresis (PFGE). Such experiments have shown that radiosensitivity of human tumour cells correlates with the initial DNA double-strand breaks (Ruiz De Almodovar *et al.* 1994, Whitaker *et al.* 1995). Similar experiments using PFGE (Wurm *et al.* 1994), constant-field gel electrophoresis (CFGE) (Dikomey *et al.* 1998) and neutral filter elution (Schwartz *et al.* 1990, Zaffaroni *et al.* 1994) have suggested a correlation between radiosensitivity and residual unrepaired DNA double strand breaks. On the other hand field inversion gel electrophoresis (FIGE) (Smeets *et al.* 1993) and neutral filter elution (Olive *et al.* 1994) data showed no correlation between initial or residual DNA double strand breaks and radiosensitivity.

A common and important aspect of the above experiments is that they directly measure DNA double-strand breaks. Sensitivity considerations dictate the use of high doses of irradiation (10-100 Gy) which are beyond the clinical range. The 25-fold lower yield of DNA double-strand breaks compared to DNA single-strand breaks (Elkind, 1979) and generation of only 40 DNA double-strand breaks per Gy per cell (Blöcher, 1982) impose difficulties of measuring DNA double-strand breaks from low dose experiments. A considerable degree of discomfort therefore exists in relating *in vitro* radiosensitivity obtained at high dose with clinical tumour response at low dose. Knowledge of the extent of DNA

damage generated at low dose could help to predict tumour sensitivity from *in vitro* measurements.

Another cause for discomfort is the fact that the high dose experiments (Kohn *et al.* 1973, Wlodek and Hittelman, 1988a, Schwarts *et al.* 1990, Dahm-Daphi *et al.* 1994, Ruis De Almodovat *et al.* 1994, Wurm *et al.* 1994, Zaffaroni *et al.* 1994, Whitaker *et al.* 1995, Dikomey *et al.* 1998) do not assay the LMDS lesions. Since the LMDS consist of DNA double-strand breaks, DNA single-strand breaks and base damage, measurement of the total DNA damage (or the undamaged DNA) would give a better picture as to the influence of DNA damage on radiosensitivity.

1.6. Influence of chromatin structure on DNA damage

Differences in the initial DNA damage between cells ultimately may arise from higher order chromatin structure. Data on decondensed hyperacetylated chromatin in nuclei (Nackerdien *et al.* 1989), H1 depleted chromatin in suspension (Heussen *et al.* 1987) and histone depleted chromatin in nuclear and nucleoid monolayers (Ljungman, 1991) support this assumption.

Further support for a role of chromatin structure comes from the fact that radiosensitivity of cells varies through the cell cycle (Terasima and Tolmack, 1963). Mitotic cells exhibit similar and maximum radiosensitivities (Biade *et al.* 1997) while S phase cells are more radioresistant (Iliakis and Okayasu, 1990). The dependence of radiosensitivity on cell cycle phases has been attributed to changes in chromatin structure (for review see Leitch 2000). The LMDS

damage, which takes place within a 5 to 10 nanometer diameter, will cause more damage when these ionisations occur within a chromosome than in a single DNA strand.

I have examined the irradiation response of cells by modulating the chromatin structure in whole cells using Nocodazole to induce condensed G2/M phase chromatin and hypertonic medium to destabilise chromatin structure. Nocodazole inhibits the function of microtubules, both in interphase and in mitotic cells (Brabander *et al.* 1976) and promotes chromatin compaction. Hypertonic medium induces dehydration (Szekely *et al.* 1983), chromatin destabilisation (Iliakis *et al.* 1993), chromosomal swelling and chromatin dissociation (Szekely *et al.* 1983).

1.7. Thesis objective

The factors which determine radiosensitivity at clinical doses have not been identified. Candidates include the total amount of DNA damage and the potential of the cell to correct this damage. The mechanisms for controlling these processes in whole cells are also not clear.

This thesis addresses the following important questions:

1. Is it possible to measure radiation induced DNA damage at low doses and does the DNA damage at low dose differ between cell lines.

2. Is the DNA repair completed before or only after restoration of the G2/M delay?

3. Does the initial DNA damage at low doses (1-10 Gy) of ionising irradiation correlate with cell survival?

4. What is the influence of initial DNA damage at low doses (1-10 Gy) on radiosensitivity?

5. What is the influence of DNA repair on radiosensitivity?

6. Does chromatin structure influence DNA damage and can this be related to radiosensitivity?

It is expected that this information will advance the understanding of the mechanisms controlling radiosensitivity. Knowledge of the factors controlling radiosensitivity may provide a basis for the improvement of cancer therapy.

CHAPTER 2

Materials and Methods

2.1. Cells and cell culture

Normal human lung epithelial L132 cells, HT29 human colon adenocarcinoma cells and the radiosensitive ATs4 human ataxia telangiectasia cells were grown in Eagle MEM supplemented with 10 % fetal bovine serum (FBS). Normal CHO-K1 hamster cell line and the repair deficient mutants xrs1 and xrs5 were grown in MEM alpha medium with 5 % FBS. The repair deficient SCID mouse cell line was grown in Dulbecco's MEM with 10 % FBS. The human fibroblast cell lines 1BR.3 normal, AT1BR ataxia telangiectasia mutant, 180BR.B ligase IV mutant and 46BR.1 ligase I mutant were grown in Eagle MEM with 15 % FBS. All cultures were kept at $37^{o}C$ in a humidified atmosphere of 95 % air and 5 % CO_2. The cells were harvested with trypsin (0.05 %) for 2 min and neutralised with medium/10 % FBS.

2.2. Drug toxicity

The cytotoxicity of Wortmannin (Sigma, St. Louis) in the CHO-K1 and xrs1 cell lines over a dose range of 0-25 μM was measured using the colony assay. Wortmannin was added to cells seeded at a density of 1200 cells/flask (25 cm^2) for CHO-K1 and 8000 cells/flask for xrs1, depending on the plating efficiency of each cell line, and medium changed after 24 hours. After 7-10 days cultures were fixed in acetic acid: methanol: H_2O 1:1:8, stained in 0.01 % amido black

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and colonies containing 50-100 cells were counted. The scored colonies were plotted against drug dose to obtain the drug concentration at which the cell survival was reduced to 50 % (TD50).

2.3. Irradiation procedures

Exponentially growing cells were irradiated with 1-10 Gy of a ^{60}Co-γ-irradiation at a dose rate of 1.45 Gy/min. For colony formation, cultures were irradiated at room temperature. For the determination of initial DNA damage cell suspensions were irradiated on ice. The 12 hour repair samples were irradiated at room temperature in the cell culture flasks and then returned to the incubator at 37°C for 12 hours. Constant-field gel electrophoresis (CFGE) plugs were irradiated on ice over a dose range of 10-100 Gy at a dose rate of 2.78 Gy/min.

2.4. Flow cytometric determination of G2 block expression

Exponentially growing ATs4, HT29 and L123 cells in 25 cm^2 culture flasks were irradiated with a dose corresponding to a survival fraction of 0.16 and 0.02 ^{60}Co γ-irradiation. This was followed by harvesting cells at 2-hourly time intervals for up to 60 hours by trypsinisation, centrifugation and fixation in 70 % ethanol at -20°C. The DNA content of these cells was measured to determine the time of maximum G2 block expression. Briefly, cells were stained in PBS containing 10μg/ml propidium iodide solution (PI, Sigma, St. Louis) and 100μg/ml RNase (Boehringer Mannheim) at 37°C for 30 minutes. Analysis was done on a FACScan (Becton Dickinson, San Diego, USA) flow cytometer at 488nm. Red fluorescence (PI) was collected as a linear signal and recorded as a measure of

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the total DNA content. Cell doublets were gated out by processing red fluorescence into area and width. Estimates of cells in the different cell cycle stages, obtained by placing markers on DNA histograms, revealed the time of maximum G2 block expression as well as the time it took for the G2 block to disappear.

2.5. Cell survival

Colony assays were performed in monolayers (Wilson 1992), with cells growing in log phase. Cells were seeded in triplicate at appropriate numbers in 25 cm^2 flasks to yield approximately 100 surviving colonies. After 4 hours when cells were attached, cells were exposed to graded doses of ^{60}Co γ-irradiation. After 7-10 days, cultures were fixed in acetic acid: methanol: H$_2$O (v:v:v, 1:1:8), stained in 0.01 % amido black and colonies containing 50-100 cells were counted. Colony determination for the fibroblast cell lines was done using the method by Rodemann *et al.* (1991).

For survival in the presence of Nocodazole, cells were incubated at 37°C in medium containing 0.4μg/ml Nocodazole in DMSO for 24 hours before irradiation. Cells incubated in a corresponding amount of DMSO in medium for 24 hours at 37°C before irradiation served as a control. After irradiation Nocodazole and DMSO was removed by 3x washing with medium. To examine the influence of Wortmannin, cells were incubated at 37°C for 1 hour in 15μM Wortmannin added from a 10mM stock solution in DMSO before irradiation. DMSO controls were treated the same. Wortmannin and DMSO were not removed after irradiation. For the survival of cells in hypertonic medium, cells

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were incubated for 40 min in medium containing 500mM NaCl before irradiation. Hypertonic medium was removed after irradiation by 3x washing with normal medium.

Survival fractions were fitted to the linear-quadratic equation to obtain α and β values and $\overline{D}$ was derived as described (Kiltie *et al.* 1997).

2.6. Determination of mitotic index

The percentage of cells in mitosis was determined by fluorescence microscopy after staining with acridine orange. Exponentially growing cells were trypsinised into single-cell suspensions and plated (4×10^4 cells per plate) into 35 mm plastic petri dishes each containing a 22 mm glass coverslip to a final medium volume of 2ml. After cells were attached 0.4µg/ml Nocodazole was added to the samples and the cells were incubated for 24 hours at $37^{\circ}C$ in a humidified atmosphere of 95 % air and 5 % CO_2. Samples were fixed for 5 minutes at room temperature in a mixture of methanol and glacial acetic acid in the ratio of 3:1 (v/v) and air-dried.

Samples were stained by adding 2.9ml acridine orange (10µg/ml in PBS).

Coverslips were mounted on glass microscope slides for fluorescence microscopy. Cells containing chromosomes were scored as cells in mitosis. Mitotic index was expressed as the percentage of cells in mitosis.

2.7. DNA damage (FADU) assay

All experimental steps were done at 0^oC unless stated otherwise. The fluorometric analysis of DNA unwinding (FADU) method (Birnboim and Jevcak, 1981, Ogiu *et al.* 1992) was modified as follows: 200μl aliquots (solution B: 0.25M meso-inositol, 10mM sodium phosphate buffer (pH 7.2), 1mM $MgCl_2$) containing approximately 1 million cells were distributed between three sets of disposable glass tubes, designated T, P and B. Where T will be total fluorescence with no unwinding, P was partial unwinding and B was total unwinding. To each tube 200μl solution C (9M urea, 10mM NaOH, 2.5mM diaminocyclohexanediaminetetraacetate, 0.1 % sodium dodecyl sulphate) was added without mixing. The tubes were incubated at 0^oC for 10 minutes to allow for cell lysis and chromatin disruption.

Into tube P 200μl solution D (0.15M NaOH) was carefully added without mixing. This increases the pH and facilitates DNA unwinding. Tube P was then incubated at 15^oC for 2 hours in the dark. Alkaline unwinding was stopped by addition of 400μl solution F (0.125M glucose containing freshly added 14mM mercaptoethanol) and placing the samples on ice (0^oC).

Tube T was neutralised with 400μl solution F before the addition of 200μl solution D.

Into tube B 200μl solution D was added and mixed well. The chromatin suspension in tube B was then sonicated for 1 min at 10 % duty cycle with a Branson sonifier 450. This destroys all double-stranded DNA by allowing the

alkali to disassociate all double-stranded DNA into single stranded DNA. 400µl solution F was then added.

Solution G (6.7µg/ml ethidium bromide, 13.3mM NaOH) was added to each tube and mixed well by pipetting. The fluorescence was measured at room temperature with a RF-540 Shimadzu recording spectrofluorophotometer. Excitation was at 520nm and emission at 590nm.

Each dose point was measured in triplicate and the experiments were repeated three times.

The percentage residual double-stranded DNA after alkaline unwinding was calculated from the fluorescence values of the T, P and B samples.

$$\text{Residual double-stranded DNA (\%)} = \frac{(P-B)}{(T-B)} \times 100 \,,$$

where T is the total fluorescence, P is the fluorescence after partial unwinding, and B is the background fluorescence.

For DNA-damage in cells exposed to Nocodazole, cells were grown in medium containing 0.4µg/ml Nocodazole for 24 hours at $37^{o}C$ and irradiated in suspension containing the same concentration Nocodazole. The DMSO controls were treated the same way. For DNA-damage in hypertonic treated cells, cells in suspension were exposed to 500mM NaCl for 40 min at $37^{o}C$ and then irradiated on ice in cold hypertonic medium.

To facilitate comparison of data sets between cell lines, the fluorescence of zero Gy (control) was used to represent 100 % undamaged double-stranded DNA. Statistical analysis was by GraphPad Prism software.

2.8. Isolation of nuclei

Cells from semi-confluent cultures were washed and incubated for 30 min on ice in the presence of 10mM Tris-HCl; 10mM NaCl; 5mM $MgCl_2$; pH 7.4. The swollen cells were lysed by dropwise addition of 10 % (v/v) NP-40 (Sigma, St. Louis) dissolved in lysis buffer to a final concentration of 0.5 % (v/v). The suspension was gently mixed by vortexing. The released nuclei were sedimented at 300g for 5 min in a swinging bucket rotor and resuspended gently by stepwise addition of small volumes of a modified Hewish and Burgoyne buffer containing 15mM Tris-HCl; 15mM 2-mercaptoethanol; 0.2mM spermine; 1mM spermidine; pH 7.4 as detailed in Gelderblom *et al.* (1984). If necessary, the resulting nuclear pellet can be freed of most cellular debris and detergent by repeated washings in the modified Hewish and Burgoyne buffer.

For the isolation of nuclei from hypertonic treated cells the whole cells were first subjected to medium containing 0.5M NaCl for either 40 min or 120 min before nuclei were isolated.

2.9. Histone extraction from nuclei

Nuclei were centrifuged and the pellet was suspended in 2N H_2SO_4. The suspension was centrifuged and 7 volumes of ice cold acetone was added to the supernatant containing the histones to precipitate the proteins overnight.

After centrifugation most of the acetone was siphoned off and the pellet was washed in PBS before the acetone washing step was repeated to remove any traces of acid.

2.10. SDS-PAGE of histones

A 15 % SDS-PAGE according to the method of Laemmli (1970) was used to monitor histone isolation. Shortly, the 15 % separating gel was made by mixing appropriate volumes of the following solutions: solution 1: 30ml of 30 % w/v acrylamide; 2.7 % w/v NN'-methylene-bisacrylamide, 15ml of solution 2: 1.5M Tris; pH 8.8, 0.6ml of solution 3: 10 % SDS, 14.1ml water and 0.3ml of 10 % ammonium persulphate. 20µl of TEMED was added to initiate the reaction after degassing the mixture. After pouring the gel, a running gel overlay (0.375M Tris; pH 8.8, 0.1 % SDS) was added.

The stacking gel was prepared as follows: 2.66 ml of Solution 1 was mixed with 5 ml of 0.5 M Tris, pH 6.8, 0.2 ml of 10 % SDS, 12.2 ml water and 100 µl 10 % Ammonium persulphate. After degassing, 10 µl TEMED was added.

The tank was prepared as follows: 12 g Tris (hydroxymethyl) aminomethane, 57.6 g Glycine, 40 ml of 10 % SDS was dissolved in water to give 0.025 M, 0.192 M and 0.1 % SDS respectively. The final pH was 8.3 and the buffer was filtered prior to use.

Histone samples were mixed 1:1 with 0.125 M Tris pH 6.8; 4 % SDS; 20 % glycerol and 10 % 2-mercaptoethanol.

2.11. DNA repair (CFGE) assay

The amount of DNA double-strand break damage was determined by constant-field gel electrophoresis (CFGE) as described previously (Wlodek *et al.* 1991). Confluent cultures were used to avoid S-phase variations between cell lines (Dikomey *et al.* 1998). Irradiation of cells encapsulated in agarose was optimised according to Kysela *et al.* (1993) to minimise non-specific DNA damage. Cells harvested by trypsinisation were resuspended in a 0.5 % low melting point agarose solution and aliquots of 30 µl, containing ~0.5 x 10^5 cells, were placed into each well of a disposable plug mold (BioRad), and allowed to solidify at 4°C for 45 min. Plugs were irradiated in ice-cold MEM containing 2 % HEPES, over a dose range of 0-100 Gy ^{60}Co γ-irradiation on ice. Samples for the determination of initial damage were immediately submitted to lysing and washing steps. The residual damage was determined by incubating plugs at 37°C in growth medium for periods of 2 hours and 20 hours.

For repair in the presence of Wortmannin the cells were irradiated with ^{60}Co γ-irradiation in ice-cold MEM containing 2 % HEPES and 15 µM Wortmannin over a dose range of 0-100 Gy on ice. The DMSO controls were treated the same. After irradiation the plugs were transferred to preheated (37°C) MEM alpha medium containing 15µM Wortmannin or an appropriate amount of DMSO and incubated for 2 and 20 hours to allow for repair.

For both protocols (initial and residual damage), plugs were submersed in an ice-cold lysing solution containing 50mM EDTA, 1 % N-lauryl-sarcosine and 1 mg/ml Proteinase K. Incubation of 1 hour at 4°C was followed by lysing at 37°C

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for 20 hours. Agarose plugs were then washed five times (50mM EDTA) and stored in 2ml of 50mM EDTA solution.

Agarose plugs were loaded into a 20 x 20 cm 0.6 % agarose gel and run in 0.5 x TBE buffer for 30 hours at a constant field strength of 1.2 V/cm. Gels were stained with ethidium bromide (0.5µg/ml in 0.5 x TBE) and subjected to fluorometric analysis with a GeneSnap (VacuTec) image analysis system. The fraction of DNA released from the plug was obtained from the following equation:

$$F_{rel} = \frac{fl_{rel}}{(fl_{plug} + fl_{rel})},$$

where fl_{rel} and fl_{plug} correspond to fluorescence measured in the lane (DNA released), and in the plug respectively. Untreated control samples were used for each sample subset to subtract background fluorescence caused by non-specific DNA degradation.

Dose response curves were obtained by plotting dose (Gy) vs. the fraction of DNA released (F_{rel}) as calculated above, representing initial damage (0 hours), residual damage (2 hours) and residual damage (20 hours). Since data could not be fitted by linear regression, data points were connected and the area under the curve (AUC) was calculated for each curve in the GraphPad Prism (GraphPad software, San Diago, USA) computer program.

2.12. Data evaluation

All experiments were repeated at least three times, and the data are given as a mean ± standard deviation (SD) or standard error margin (SEM) for the independent experiments. Statistical analysis and data fitting were performed by means of the GraphPad Prism (GraphPad Software, San Diego, USA) computer program. A two-sided t test was used to compare the means between sample groups.

CHAPTER 3

Results

3.1. Clonogenic survival

Cell survival from doses of 0-10 Gy ^{60}Co γ-irradiation is shown in figure 3.1. SF2, α and β values were obtained from the mean survival data fitted the linear-quadratic model, $-\ln S=\alpha D+\beta D^2$. $\overline{D}$ values were obtained by calculation of the area under the curve in linear plots. The cell inactivation parameters are summarised in table 3.1. The $\overline{D}$ values for the most radioresistant and the most radiosensitive cell lines were found to be 2.755 and 0.6464 Gy, respectively. The cell lines CHO-K1, L132 and HT29 are deemed radioresistant because the $\overline{D}$ values exceeded 2.400 Gy. SCID, xrs1, xrs5, 180BR.B, AT1BR, ATs4 and 46BR.1 cells display a $\overline{D}$ smaller than 1.500 Gy and are deemed radiosensitive. The $\overline{D}$ values for 1BR.3 cells was found to be 1.735 Gy and fall into an intermediate category of radiosensitivity.

Figure 3.1
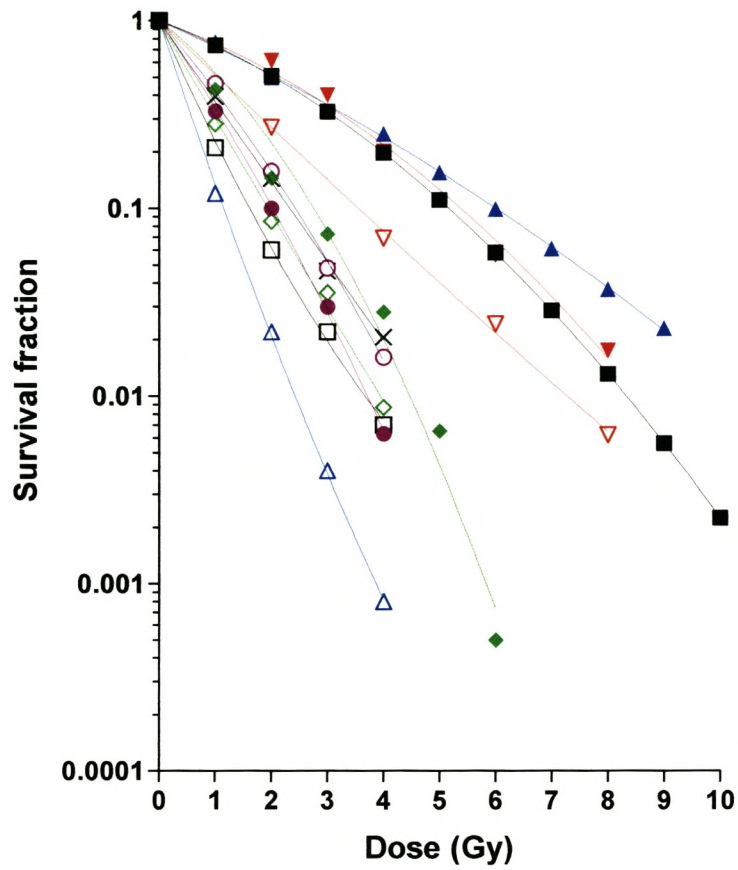
Survival curves for eleven cell lines in response to Cobalt irradiation: CHO-K1 (■), L132 (▲), HT29 (▼), ATs4 (♦), SCID (●), xrs1 (□), xrs5 (Δ), 1BR.3 (▽), 180BR.B (◇), AT1BR (O) and 46BR.1 (x).

Table 3.1

Cell line	α (Gy^{-1})	β (Gy^{-2})	SF2	$\overline{D}$ (Gy)
CHO-K1	$0.2700 \pm 3.291 \times 10^{-8}$	$0.0340 \pm 4.057 \times 10^{-9}$	0.51	2.489
L132	0.3063 ± 0.008837	0.01290 ± 0.001189	0.52	2.755
HT29	0.2436 ± 0.04512	0.03426 ± 0.006762	0.54	2.752
ATs4	0.5225 ± 0.1542	0.1136 ± 0.03084	0.22	1.182
SCID	1.010 ± 0.04452	0.06188 ± 0.01296	0.10	0.9632
xrs1	1.562 ± 0.06707	-0.08366 ± 0.01952	0.06	0.7955
xrs5	2.091 ± 0.05645	-0.07892 ± 0.01643	0.02	0.6464
1BR.3	0.6621 ± 0.03192	-0.003983 ± 0.004645	0.27	1.735
180BR.B	1.205 ± 0.1036	-0.009659 ± 0.03016	0.09	0.9078
AT1BR	0.8023 ± 0.05686	0.06011 ± 0.01655	0.16	1.176
46BR.1	0.9940 ± 0.06338	-0.002666 ± 0.01845	0.14	1.095

Radiosensitivity parameters α and β were determined with the linear quadratic equation. SF2 is the surviving fraction at 2 Gy and $\overline{D}$ is the mean inactivation dose.

3.2. G2 cell cycle block induction and recovery

Survival data for ATs4, HT29 and L132 served to identify isodoses leading to the same cellular inactivation (table 3.2). The three cell lines were then irradiated to the same survival level and the cell cycle distribution was monitored by flow cytometry.

The G2 block maximum in response to 2 Gy, 4.5 Gy and 5 Gy for ATs4, HT29 and L132, respectively, was reached at 12 hours post-irradiation. Figure 3.2 shows the typical G1, S and G2 cell cycle distribution in ATs4, HT29 and L132. The times of recovery from the G2 block in irradiated cells were similar in ATs4, HT29 and L132 when irradiated to the same survival fraction. The recovery data are given in table 3.3.

Table 3.2

ATs4		HT29		L132	
Survival fraction	Dose (Gy)	Survival fraction	Dose (Gy)	Survival fraction	Dose (Gy)
0.1649	2	0.1531	4.5	0.1568	5
0.0202	4	0.0270	6.5	0.0260	8.5
0.0063	5	0.0051	8	0.0056	11

Doses corresponding to a specific survival fraction for the cell lines ATs4, HT29 and L132.

Figure 3.2
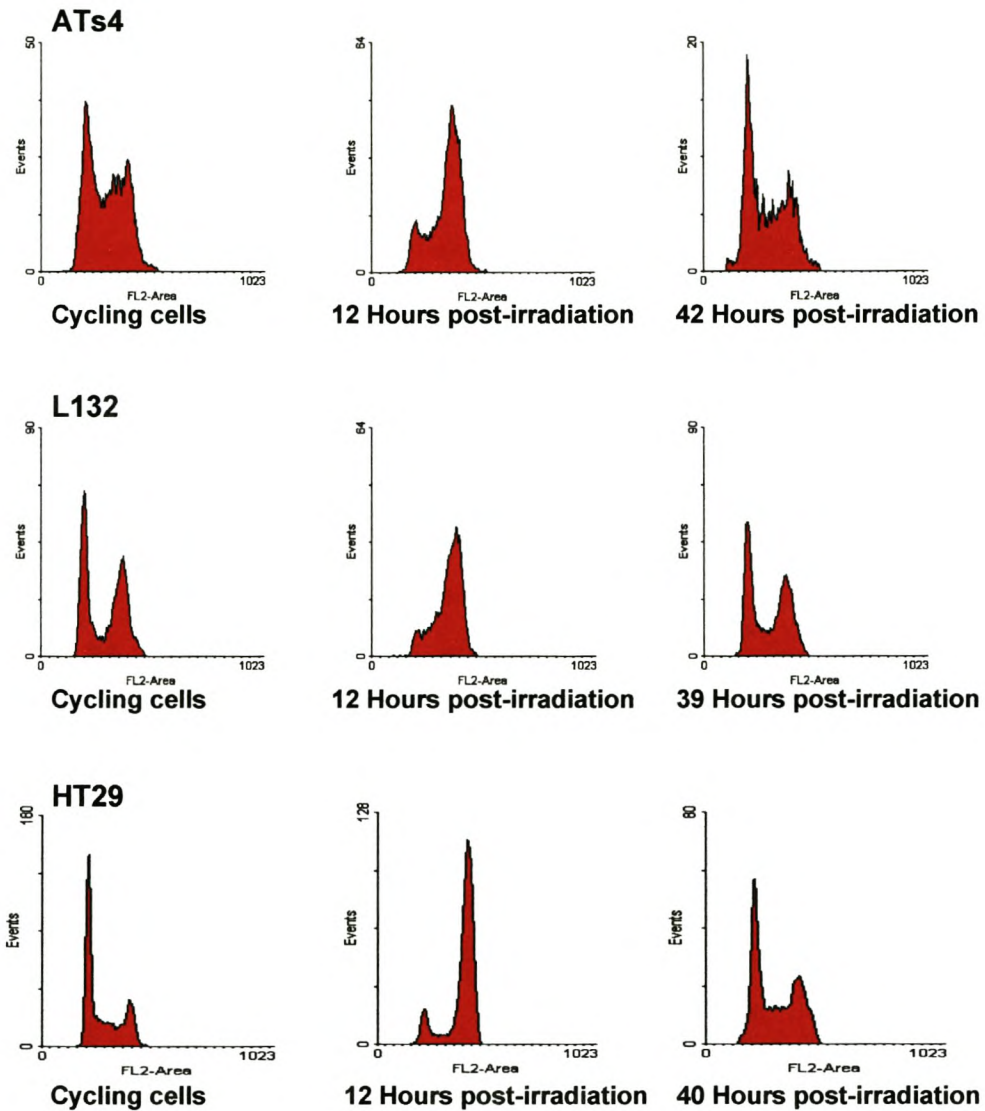
DNA histograms showing the influence of 2, 5 and 4.5 Gy of irradiation for ATs4, L132 and HT29, respectively, on the distribution of cells in G1 and G2 cell cycle phases at various post-irradiation times. G2 maximum was reached 12 hours after irradiation. The cell cycle returned to normal 42, 39 and 40 hours after irradiation for ATs4, L132 and HT29, respectively.

Table 3.3

Cell line	Surviving fraction	Time (hours)	Surviving fraction	Time (hours)
ATs4	0.1649	42	0.0202	-
HT29	0.1531	40	0.0270	53
L132	0.1568	39	0.0262	50

Time it takes the G2/M block to disappear after irradiation to the same surviving fraction for the ATs4, HT29 and L132 cell lines. Cells were irradiated with isodoses leading to the same survival fraction and then the time for G2/M recovery was determined by flow cytometry.

3.3. Irradiation-induced DNA damage and DNA repair

The dose-response curves for DNA damage induction, expressed as per cent remaining double-stranded DNA, for 0 hours repair time (initial damage) and for 12 hours repair time (residual damage) are given in figures 3.3 and 3.4, respectively. It is apparent that increase of the irradiation dose gives rise to a decrease of undamaged DNA. This implies that increases of the irradiation dose increases DNA strand breaks and hence DNA damage. The DNA unwinding slopes, calculated from figure 3.3 and 3.4, and the per cent double-stranded DNA remaining after exposure to 5 Gy of irradiation for initial damage and residual damage are given in table 3.4.

It is noted that in the FADU assay, five out of six double-strand break repair deficient cell lines (SCID, xrs1, 180BR.B, AT1BR and 46BR.1) show low to very low amount of repair after 12 hours (figure 3.4). This emphasises the dominant role of double-strand breaks in the unwinding process.

At 0 hours repair time, the slopes representing the unwinding rate of the DNA for the sensitive xrs5 cell line and the resistant L132 cell line were found to be –8.81 and –2.73, respectively, indicating that the initial DNA damage differs between cell lines differs by a factor of up to 3.

Figure 3.3
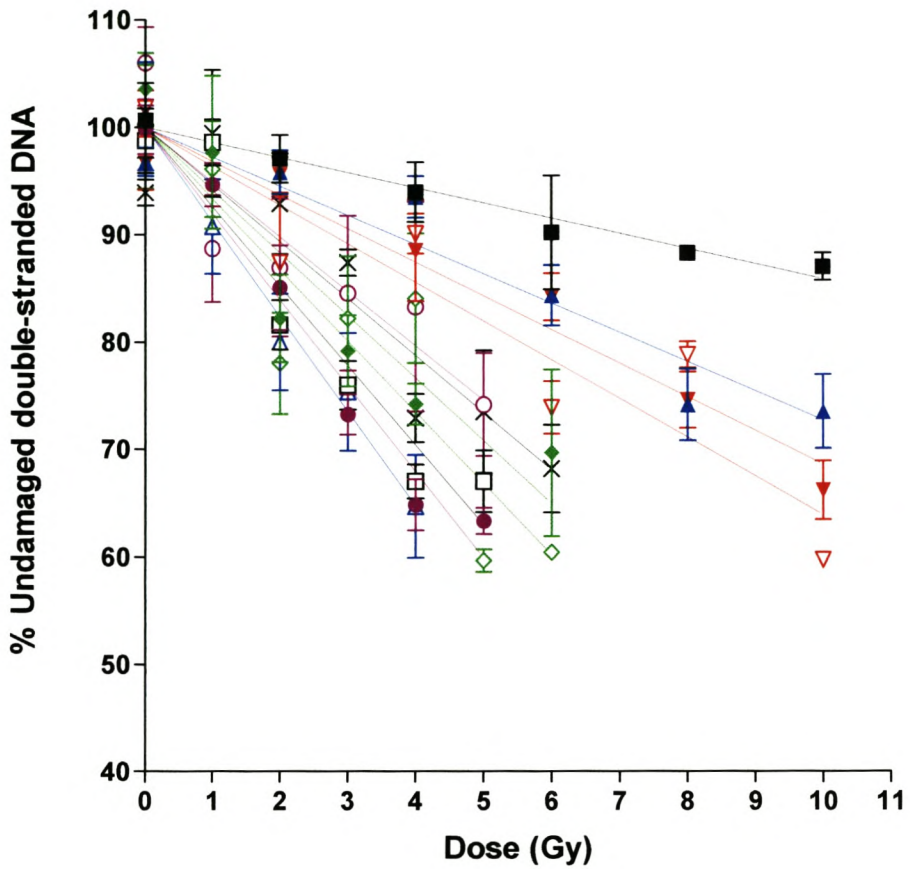
Total amount of DNA damage (initial damage) as measured by the fluorometric analysis of DNA unwinding: CHO-K1 (■), L132 (▲), HT29 (▼), ATs4 (♦), SCID (●), xrs1 (□), xrs5 (Δ), 1BR.3 (▽), 180BR.B (◇), AT1BR (O) and 46BR.1 (x).

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Figure 3.4
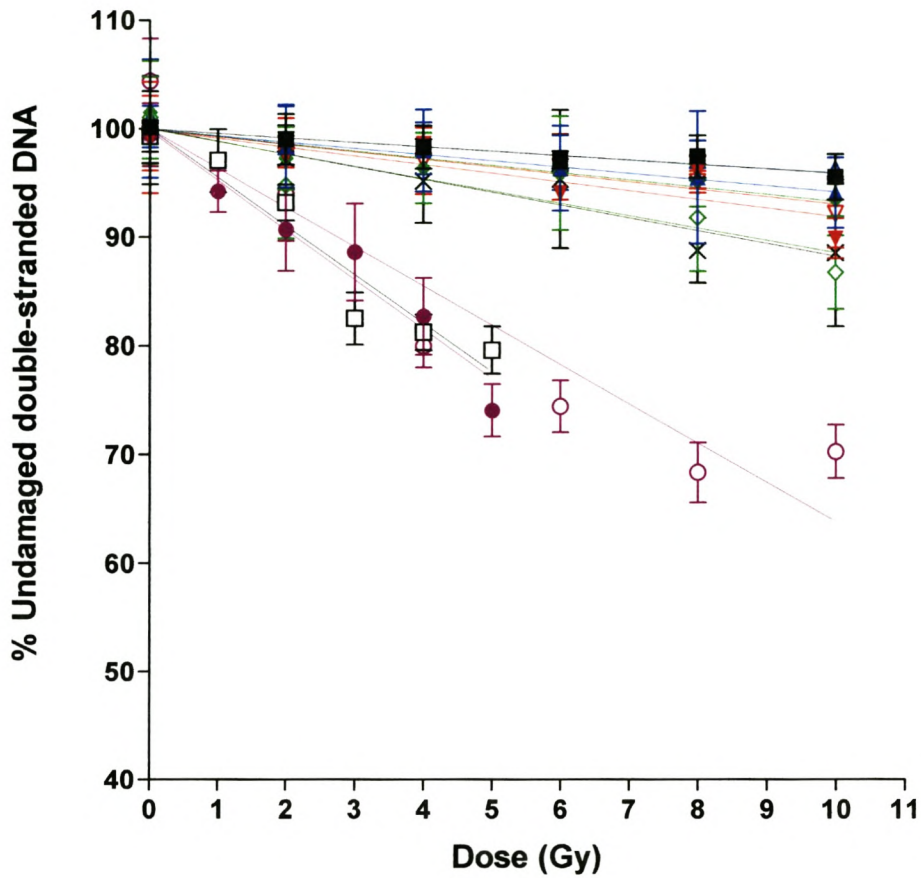
DNA damage remaining after 12 hours repair as measured by the fluorometric analysis of DNA unwinding method: CHO-K1 (■), L132 (▲), HT29 (▼), ATs4 (◆), SCID (●), xrs1 (□), xrs5 (Δ), 1BR.3 (▽), 180BR.B (◇), AT1BR (O) and 46BR.1 (x).

Table 3.4

	Slope		Double-stranded DNA at 5 Gy (%)	
Cell line	Initial damage	Residual damage	Initial damage	Residual damage
CHO-K1	-1.408 ± 0.1160	-0.4155 ± 0.05622	93	98
L132	-2.723 ± 0.4178	-0.5450 ± 0.03911	86	97
HT29	-3.140 ± 0.3320	-0.7966 ± 0.2409	84	96
ATs4	-5.840 ± 1.044	-0.6726 ± 0.1374	71	97
SCID	-8.104 ± 0.7251	-4.676 ± 0.4962	60	77
xrs1	-7.403 ± 1.079	-4.476 ± 0.6373	63	78
xrs5	-8.811 ± 0.5783	-0.4104 ± 0.09039	56	98
1BR.3	-3.609 ± 0.7297	-0.6966 ± 0.08249	82	97
180BR.B	-6.637 ± 1.342	-1.144 ± 0.3221	67	94
AT1BR	-5.083 ± 1.171	-3.620 ± 0.6416	75	82
46BR.1	-5.320 ± 0.9146	-1.170 ± 0.1826	73	94

Data for fluorometric analysis of DNA unwinding (FADU). Slopes were determined by linear regression. Per cent double-stranded DNA was read from the linear fit at 5 Gy.

3.4 Correlation between radiation-induced DNA damage and radiosensitivity

The relationship between radiosensitivity and DNA strand breaks was examined by plotting the mean inactivation dose ($\overline{D}$) versus per cent residual undamaged double-stranded DNA for the 5 Gy dose point. The initial DNA damage (0 hours repair time) and residual undamaged double-stranded DNA after 12 hours repair is given in figure 3.5. A correlation was found to exist between initial DNA damage and radiosensitivity. The correlation coefficient r^2 was found to be 0.81 (P-value=0.0002). For residual DNA damage remaining after 12 hours repair the correlation coefficient was found to be 0.22 (P-value=0.1508).

Using other accepted radiosensitivity parameters the corresponding correlation coefficients and P-values for initial and residual damage were found to be r^2=0.79 (P=0.0003) and r^2=0.06 (P=0.4665) for the α-coefficient and r^2=0.79 (P=0.0002) and r^2=0.21 (P=0.1569) for SF2-values, respectively (figures not shown).

Figure 3.5
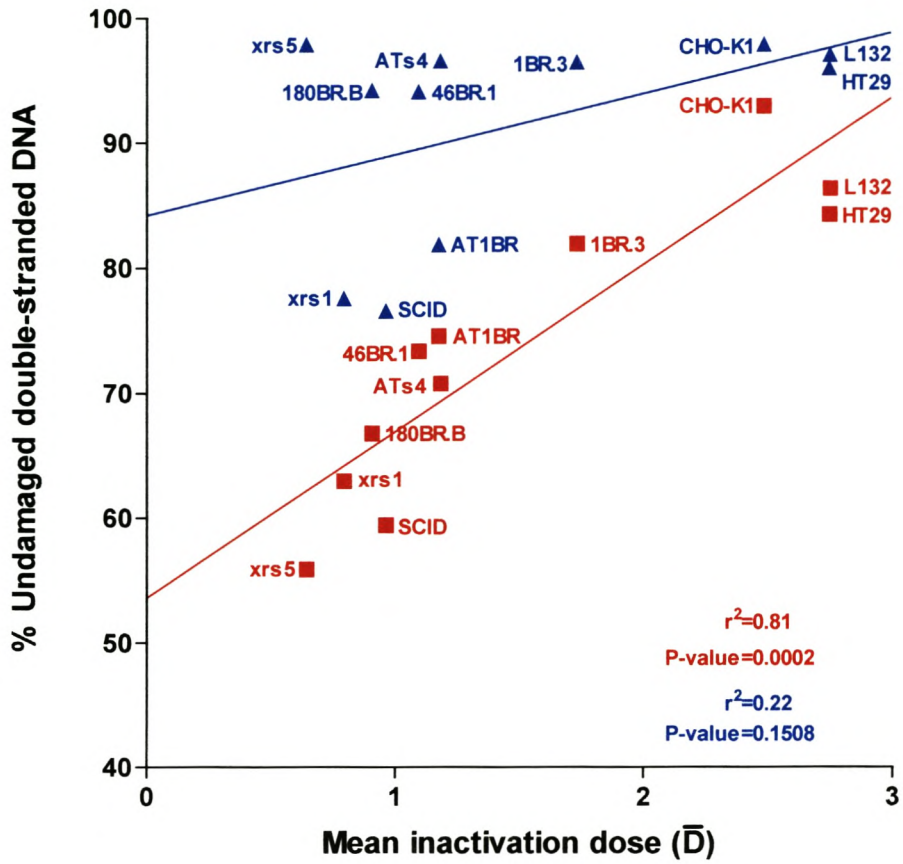
Relationship between remaining undamaged double-stranded DNA and radiosensitivity after 5 Gy irradiation Initial DNA damage (red) and residual DNA damage (blue).

3.5. Cells in mitosis

The percentage of cells in mitosis was determined for normal cycling cells and cells exposed to Nocodazole for 24 hours. CHO-K1 and xrs1 cells showed similar amounts of cells in mitosis, 8 % and 3 % respectively for untreated cells and 24 % and 35 % respectively for Nocodazole treated cells (figure 3.6).

3.6. Clonogenic survival after chemical modifications

Cell survival from ^{60}Co γ-irradiation was measured for the dose range 0-10 Gy for irradiation alone, and for irradiation in the presence of Nocodazole, Wortmannin, DMSO and hypertonic medium. Figures 3.7 A and B show survival curves obtained from colony assay data in CHO-K1 and xrs1 cells, respectively. Survival curves were fitted to the linear-quadratic equation. CHO-K1 cells show the highest radioresistance with a surviving fraction at 2 Gy (SF2) of 0.71, while xrs1 cells showed radiosensitivity with a SF2 of 0.17. Table 3.5 lists the radiosensitivity parameters. Small differences in relation to the earlier survival data (figure 3.1) can be explained by the fact that cells with later passage numbers were used for these experiments.

The shape of the slope of the survival curves of xrs1 corresponds to published data (Dahm-Daphi et al. 1994, Dikomey et al. 1998).

Irradiation of CHO-K1 cells under different conditions effects radiosensitivity as follows: DMSO decrease radiosensitivity and shows a radioprotection effect by increasing the SF2 from 0.71 to 0.93. Nocodazole decreases the radioresistance of CHO-K1 cells from a $\overline{D}$ of 4.446 to 4.376 Gy. Hypertonic

treatment decreases the radioresistance even more from a SF2 of 0.71 to 0.60 and Wortmannin decreases the radioresistance of CHO-K1 from a SF2 of 0.93 in DMSO controls to 0.52.

Treatment of xrs1 cells under these conditions altered the radiosensitivity as follows: In xrs1 cells DMSO decreases the radiosensitivity from a SF2 of 0.17 to 0.28. Nocodazole radiosensitises xrs1 from a SF2 of 0.17 to 0.06. Hypertonic treatment increases the radioresistance from a SF2 of 0.17 to 0.31 and addition of Wortmannin increases radioresistance from a SF2 of 0.28 in DMSO controls to 0.31.

3.7. Modulation of DNA damage

Initial DNA damage induced by doses of 0-10 Gy ^{60}Co γ-irradiation was measured for irradiation alone, and for irradiation in the presence of Nocodazole, DMSO and hypertonic medium. Figures 3.8 A and B show dose-response curves for DNA damage induction, expressed as per cent remaining double-stranded DNA for 0 hours repair time in CHO-K1 and xrs1 cells, respectively. The DNA unwinding slopes and the per cent double-stranded DNA remaining after unwinding at 5 Gy irradiation are given in table 3.6.

After exposure to 5 Gy, CHO-K1 cells show the least amount of DNA damage with 93 % of undamaged double-stranded DNA, while xrs1 cells show more DNA damage with only 57 % undamaged double-stranded DNA remaining (figure 3.8 A and B). Treatment of CHO-K1 under different conditions altered the remaining undamaged double-stranded DNA after 5 Gy irradiation as

follows: Nocodazole decreased undamaged DNA from 93 % to 87 %, hypertonic treatment decreased undamaged DNA further from 93 % to 85 % and DMSO samples decreased the undamaged DNA from 93 % to 83 % (figure 3.8A). Treatment of xrs1 under these conditions effected the per cent undamaged double-stranded DNA remaining after 5 Gy irradiation as follows: Nocodazole decreased undamaged DNA from 57 % to 43 %, hypertonic treatment increased undamaged DNA at 5 Gy from 57 % to 64 % and DMSO increased undamaged DNA from 57 % to 66 %.

Figure 3.6
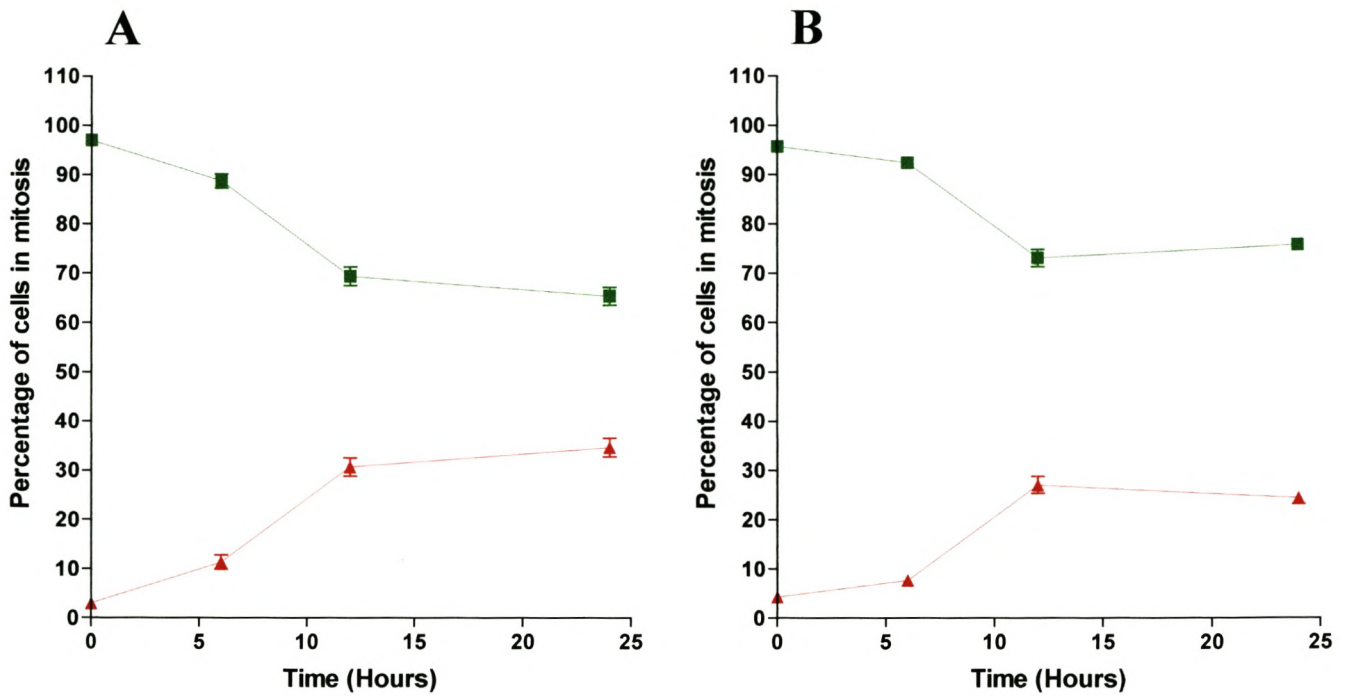
Cells in mitosis for CHO-K1 (A) and xrs1 (B). Cells were grown in medium containing 0.4mg/ml Nocodazole for different times at 37°C. Cells showing chromosomes were scored as cells in mitosis. Cells in mitosis (red), and cells in interphase (green).

Figure 3.7
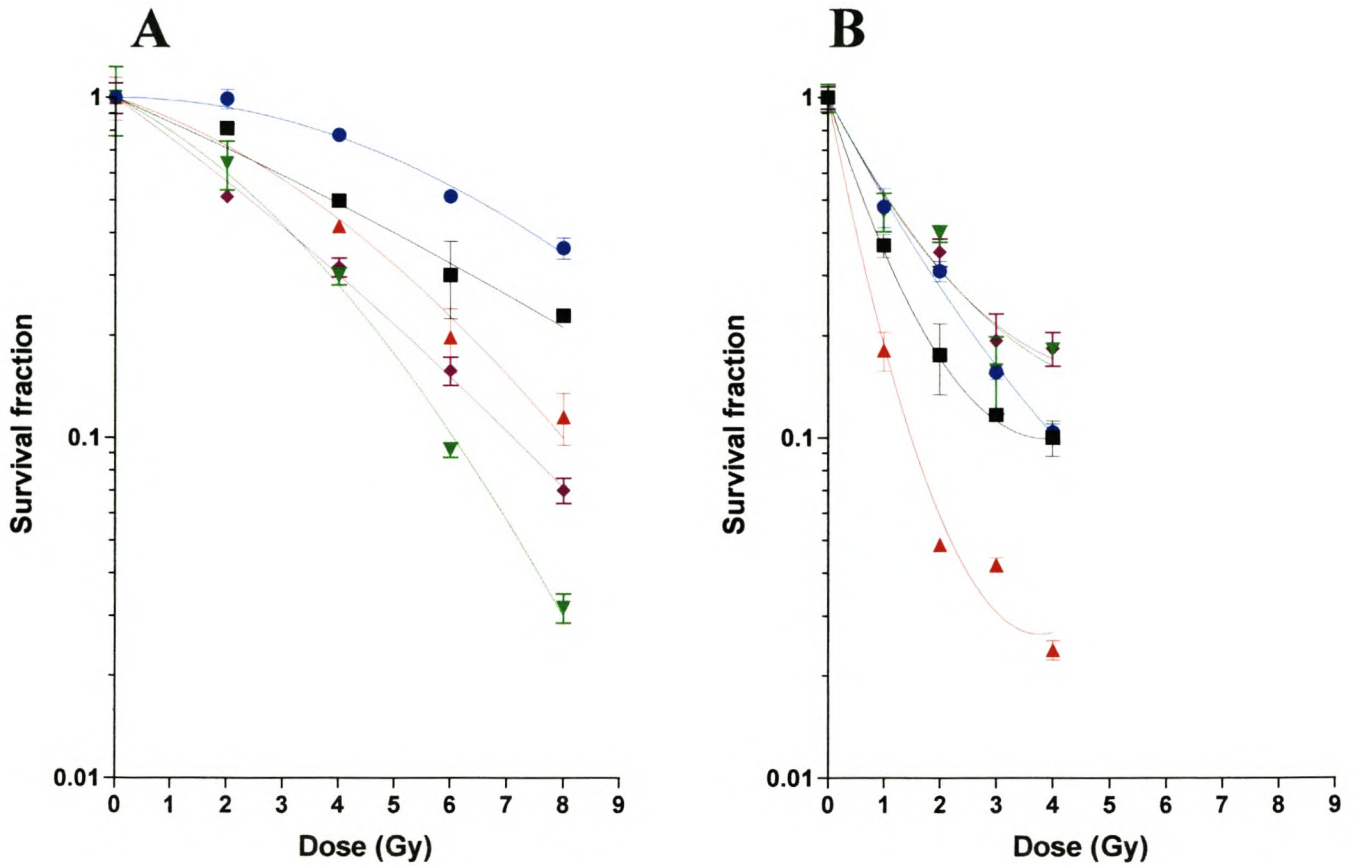
Survival curves for CHO-K1 (A) and xrs1 (B) cells following irradiation (■). Cells were grown in medium containing 0.4mg/ml Nocodazole for 24 hours at 37^oC before irradiation (▲), hypertonic medium (500mM NaCl) was added to the cells 40 min before irradiation (▼), Wortmannin (15mM) was added to the cells 1 hour prior to irradiation (◆) and DMSO controls were treated similarly as drug treated samples (●). Cell survival was measured by colony assay, and data were fitted to the linear-quadratic equation.

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Table 3.5

	α(Gy^{-1})	β(Gy^{-2})	SF2	$\overline{D}$ (Gy)
CHO-K1	0.165 ± 0.053	0.004 ± 0.008	0.71	4.446
CHO-K1 + DMSO	0.002 ± 0.017	0.016 ± 0.003	0.93	5.914
CHO-K1 + Wortmannin	0.271 ± 0.030	0.007 ± 0.004	0.52	3.043
CHO-K1 + Nocodazole	0.124 ± 0.078	0.020 ± 0.011	0.72	4.376
CHO-K1 + hypertonic treatment	0.197 ± 0.055	0.030 ± 0.008	0.60	3.092
xrs1	1.186 ± 0.098	-0.152 ± 0.028	0.17	1.209
xrs1 + DMSO	0.709 ± 0.062	-0.035 ± 0.018	0.28	1.493
xrs1 + Wortmannin	0.724 ± 0.116	-0.071 ± 0.033	0.31	1.811
xrs1 + Nocodazole	1.928 ± 0.107	-0.256 ± 0.031	0.05	0.7836
xrs1 + hypertonic treatment	0.705 ± 0.127	-0.063 ± 0.038	0.31	1.609

Radiosensitivity parameters α and β were obtained by fitting the survival data to the linear quadratic equation. SF2 is the surviving fraction at 2 Gy and $\overline{D}$ is the mean inactivation dose. Cells were grown in medium containing 0.4mg/ml Nocodazole for 24 hours at 37°C before irradiation, hypertonic medium (500mM NaCl) was added to the cells 40 min before irradiation, Wortmannin (15mM) were added to the cells 1 hour prior to irradiation and DMSO controls were treated similarly as drug treated samples.

Figure 3.8
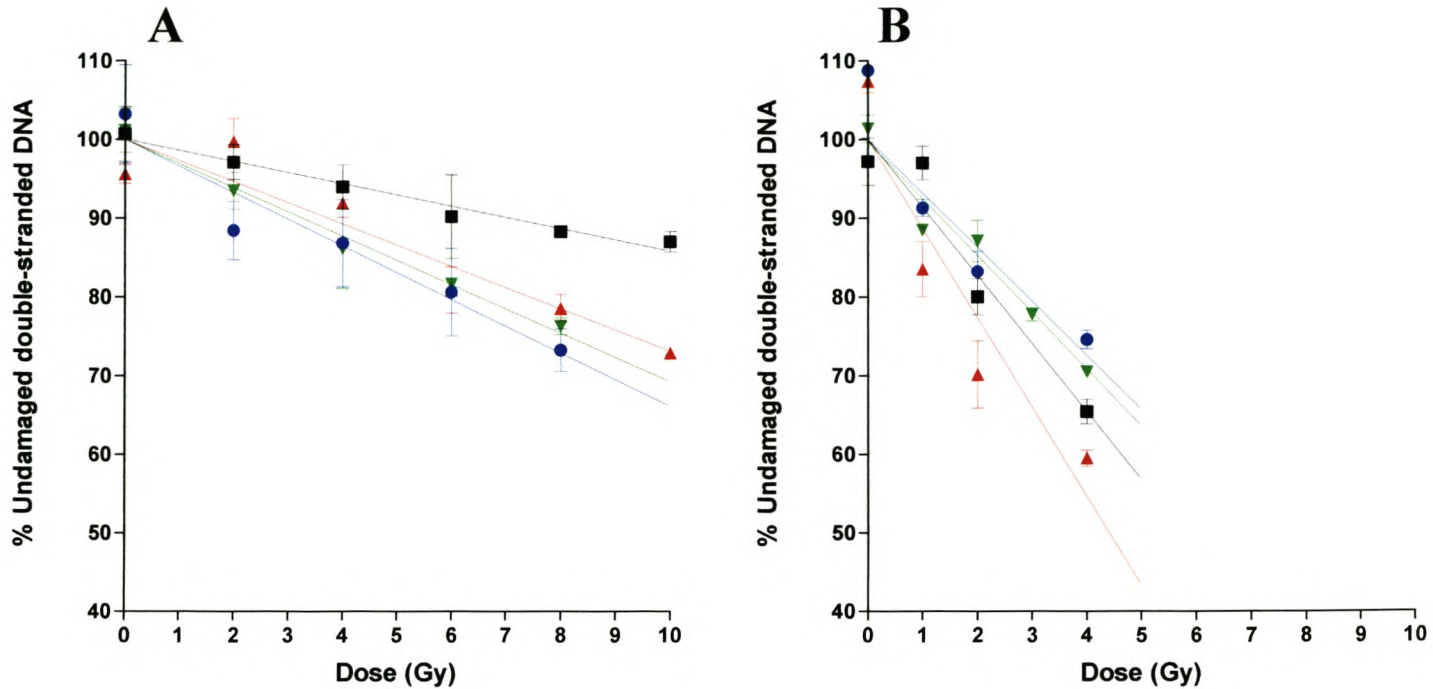
Total amount of DNA damage (initial damage) for CHO-K1 (A) and xrs1 (B) cells following irradiation (■). Cells were grown in medium containing 0.4mg/ml Nocodazole for 24 hours at 37°C before irradiation (▲), hypertonic medium (500mM NaCl) was added to the cells 40 min before irradiation (▼) and DMSO controls were treated similarly as drug treated samples (●). Initial damage was measured by fluorometric analysis of DNA unwinding, and data were fitted to the linear equation.

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Table 3.6:

	Slope	Double-stranded DNA at 5 Gy (%)
CHO-K1	-1.419 ± 0.329	93
CHO-K1 + DMSO	-3.386 ± 0.729	83
CHO-K1 + Nocodazole	-2.684 ± 0.350	87
CHO-K1 + hypertonic treatment	-3.072 ± 0.372	85
xrs1	-8.640 ± 1.014	57
xrs1 + DMSO	-6.857 ± 1.101	66
xrs1 + Nocodazole	-11.34 ± 1.601	43
xrs1 + hypertonic treatment	-7.203 ± 0.884	64

Data for fluorometric analysis of DNA unwinding (FADU). Cells were grown in medium containing 0.4mg/ml Nocodazole for 24 hours at $37^{o}C$ before irradiation, hypertonic medium (500mM NaCl) was added to the cells 40 min before irradiation, and DMSO controls were treated similarly as drug treated samples. Slopes were determined by linear regression. Per cent double-stranded DNA was read from the linear fit at 5 Gy.

3.8. Effect of hypertonic treatment on histone H1 levels in whole cells

Cells were exposed to 40 and 120 min of 0.5M NaCl hypertonic medium before isolation of nuclei. The histones were then extracted and separated on a 15 % SDS gel (figure 3.9). Lane 1 shows whole chicken histone standards. Lanes 2, 3 and 4 contained CHO-K1 histones after treatment for 0, 40 and 120 min, respectively. Lanes 5, 6 and 7 contained xrs1 histones after treatment for 0, 40 and 120 min, respectively.

The SDS-PAGE gel was stained and subjected to densitromic analysis with a GeneSnap image analysis system. The percentage histone H1 compared to total histones was calculated and plotted (figure 3.10). In CHO-K1 cells, the percentage histone H1 in untreated cells was 8.8 %. After 40 and 120 min treatment, histone H1 levels were 11.7 % and 9.3 %, respectively. In xrs1 cells the percentage histone H1 in untreated cells was 12.5 %. For 40 and 120 min treatment, histone H1 levels were 11.1 % and 12.2 %, respectively.

3.9. Fluorescent microscopy analysis after hypertonic treatment

Cells were exposed to 40 min and 120 min of 0.5M NaCl hypertonic medium before staining with acridine orange. Photographs are shown for CHO-K1 cells (figure 3.11) and xrs1 cells (figure 3.12). Increase of time in hypertonic medium increased cellular dehydration observed in the cytoplasm and in the nucleus. In the nucleus the fluorescence increased with exposure time. In mitotic cells hypertonic treatment induced a loss of chromosome definition (figure 3.13 and figure 3.14).

3.10. Influence of hypertonic treatment on initial radiation damage to DNA

Cells were subjected to 40 min and 120 min of 0.5M NaCl hypertonic medium, irradiated with 0-10 Gy and then subjected to analysis of DNA damage by the FADU assay (figure 3.15). In CHO-K1 cells, 40 min treatment decreases the undamaged DNA from 93 % to 85 %. After 120 min treatment in 0.5M NaCl the irradiation-induced DNA damage was higher as shown by the undamaged DNA decreasing from 93 % to 82 %.

In xrs1 cells exposure to 0-10 Gy after 40 and 120 min incubation in 0.5M NaCl led to a increase of the undamaged DNA from 57 % (control) to 64 % (40 min) and 80 % (120 min) (figure 3.15).

Figure 3.9
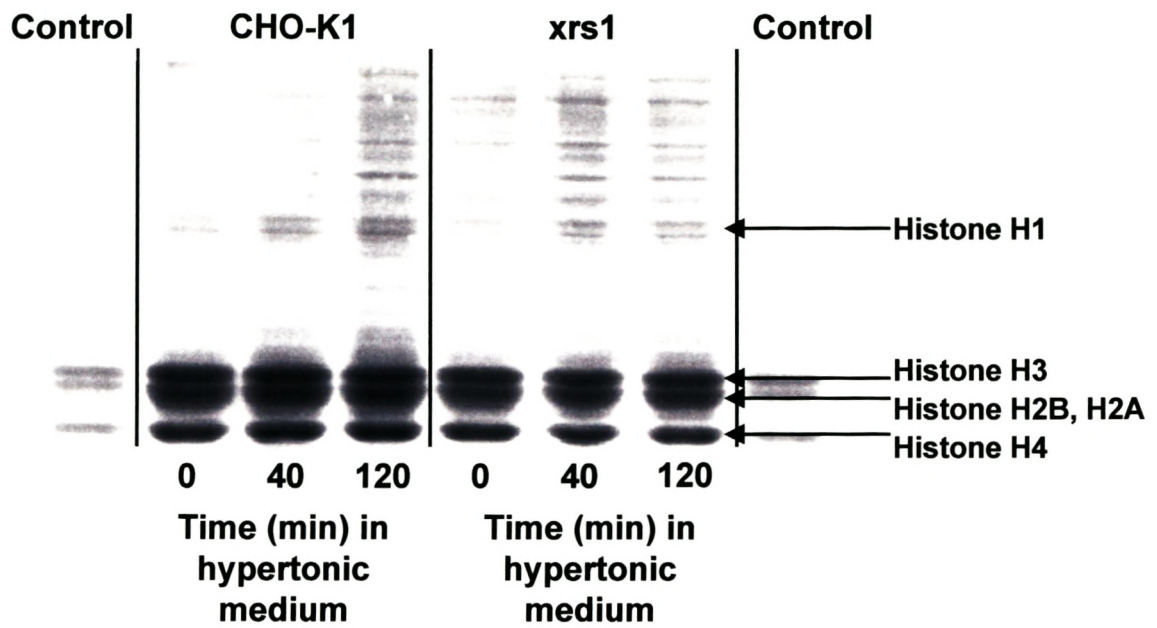
SDS-PAGE for the determination of histone H1 in whole cells after treatment with hypertonic medium. A 15 % gel was electrophoresed for 2 hours at 18mA.

Figure 3.10
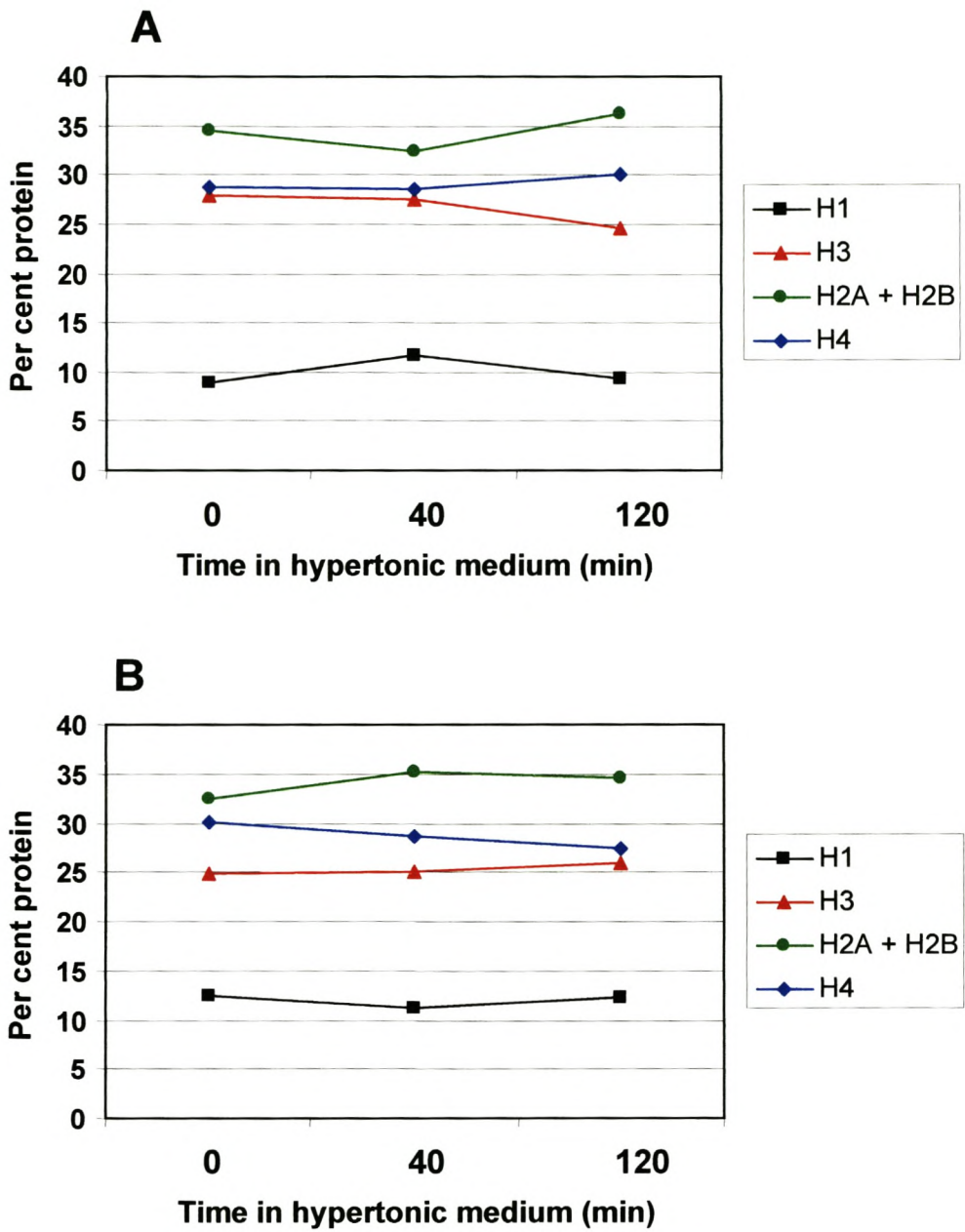
Histone levels in CHO-K1 (A) and xrs1 (B) after treatment with hypertonic medium.

Figure 3.11
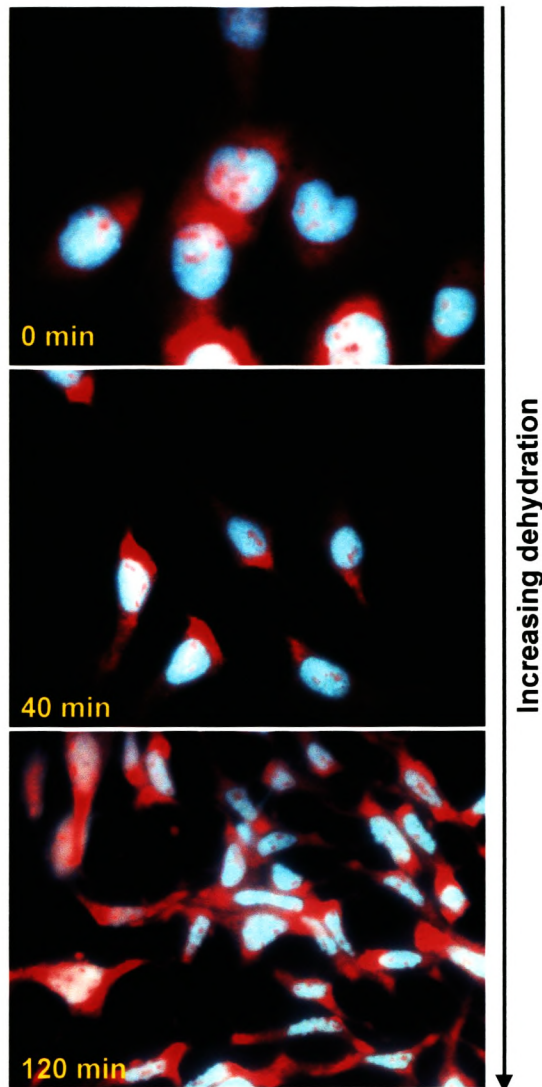
CHO-K1 cells stained with acridine orange after treatment with hypertonic medium.

Figure 3.12
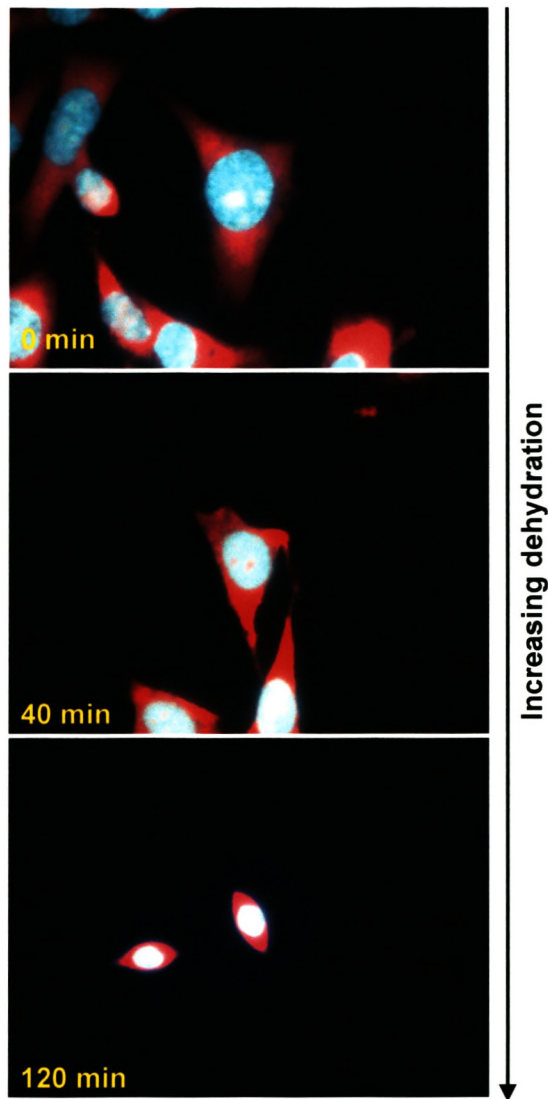
Xrs1 cells stained with acridine orange after treatment with hypertonic medium.

Figure 3.13
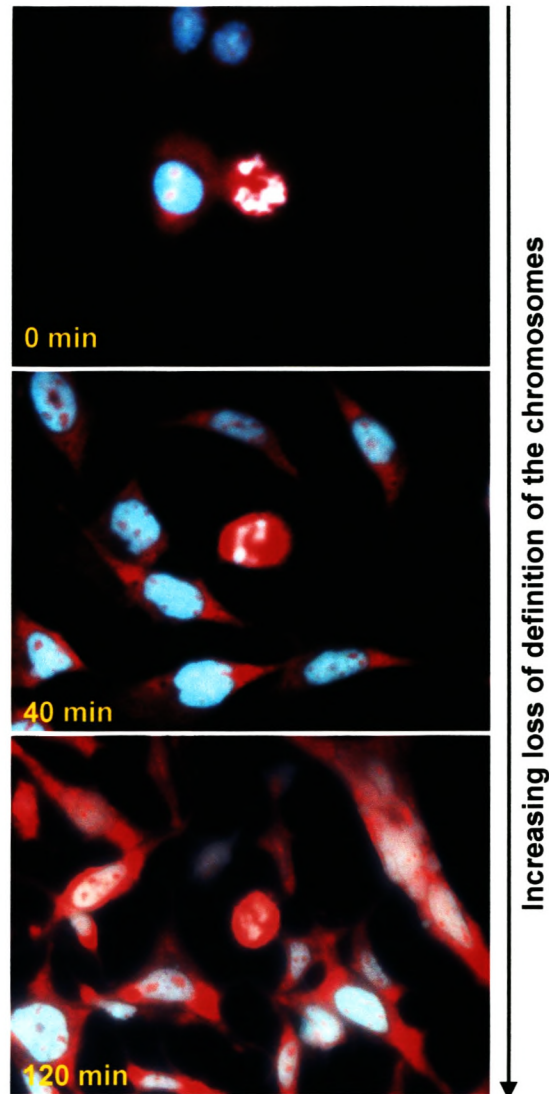
CHO-K1 cells stained with acridine orange after treatment with hypertonic medium showing cells in mitosis.

Figure 3.14
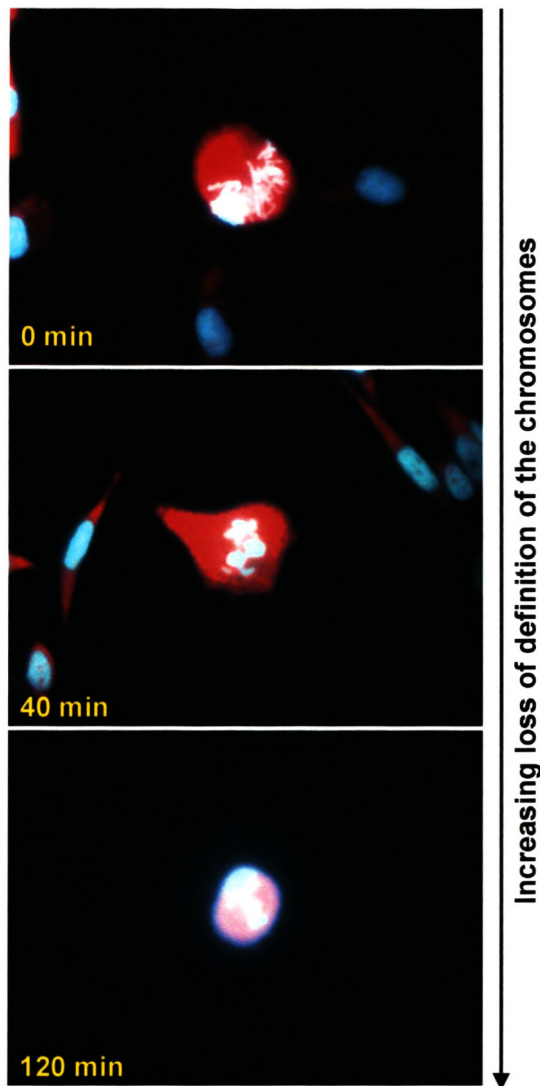
Xrs1 cells stained with acridine orange after treatment with hypertonic medium showing cells in mitosis.

Figure 3.15
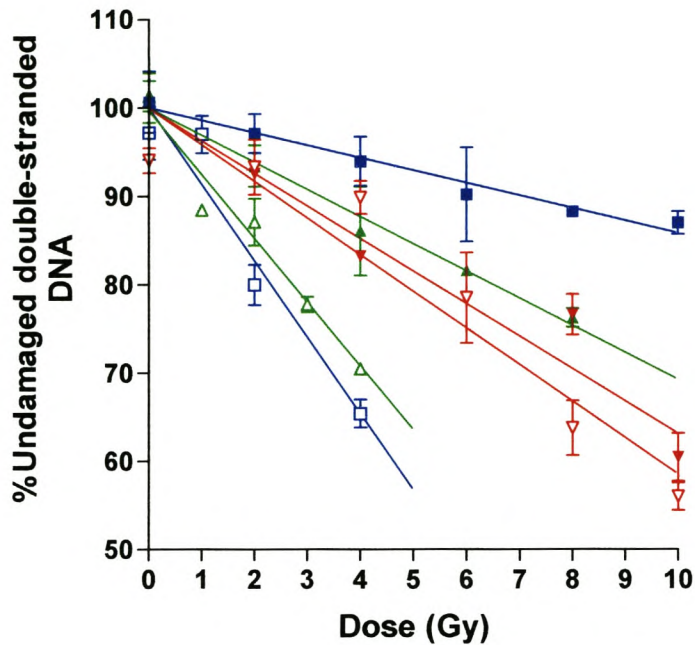
Total amount of DNA damage (initial damage) for CHO-K1 cells (■) and xrs1 cells (□) following irradiation. Cells were grown in medium containing 0.5M NaCl for 40 min (△ for xrs1 and ▲ for CHO-K1) and 120 min (▽ for xrs1 and ▼ for CHO-K1) before irradiation. Initial damage was measured by flourometric analysis of DNA unwinding, data were analysed by linear equation.

3.11. DNA repair

DNA repair was assayed by CFGE method measuring the concentration of mobile (low molecular weight) DNA arising from DNA double-strand breaks after high doses of 10-100 Gy. A typical gel is shown in figure 3.16. Lanes 1 to 3 shows DNA after 0 Gy irradiation. The effect of irradiation dose on DNA mobility after 10, 20, 40, 80 and 100 Gy is shown in lanes 4 to 6, lanes 7 to 9, lanes 10 to 12, lanes 13 to 15 and lanes 16 to 18, respectively. These results were used to plot the dose-response curves.

Dose-response curves representing initial double-strand breaks and residual double-strand breaks after 2 and 20 hours repair time for the dose range 0-100 Gy are given in figure 3.17 A and B. Fractions of DNA released (Frel) were plotted against dose, and the area under each curve (AUC) was calculated to compare repair in the presence or absence of Wortmannin. These results are summarised in table 3.7. The differences between residual damage after 2 and 20 hours in the presence of Wortmannin were significant in CHO-K1 (2 hours: $p<0.0001$, 20 hours: $p=0.0095$) compared to the DMSO controls. The xrs1 cells did not show a significant difference after 2 or 20 hours in the presence of Wortmannin (2 hours: $p=0.6082$, 20 hours: $p=0.6069$) compared to the DMSO controls.

To assess the rate of double-strand repair, ratios of the AUC values representing initial damage versus residual damage after 2 hours and after 20 hours were calculated. These ratios are summarized in table 3.8. After 2 hours of repair, the CHO-K1 and xrs1 cells showed repair ratios of 3.60 and 1.61,

respectively. In the presence of Wortmannin, the ratio for CHO-K1 and xrs1 were 1.64 and 1.65, respectively, and for the DMSO controls they were 2.91 and 1.73, respectively. The 20 hours repair ratios were found to be 16.67 and 2.05 for CHO-K1 and xrs1, respectively. In the presence of Wortmannin, the ratios were 6.03 and 3.07 for CHO-K1 and xrs1 respectively and for the DMSO controls they were 14.61 and 2.44 respectively. These repair ratios (table 3.8) were used to calculate repair inhibition factors (RIF) for the two repair incubation periods (table 3.9). After 2 hours of repair in the presence of Wortmannin, RIFs were found to be 2.20 and 0.98 in CHO-K1 and xrs1, respectively. In the DMSO controls, the RIFs were 1.24 and 0.93for CHO-K1 and xrs1, respectively. The 20 hours repair data showed RIFs of 2.76 and 0.67 in the presence of Wortmannin and 1.14 and 0.84 for the DMSO controls in CHO-K1 and xrs1, respectively (table 3.9).

Figure 3.16
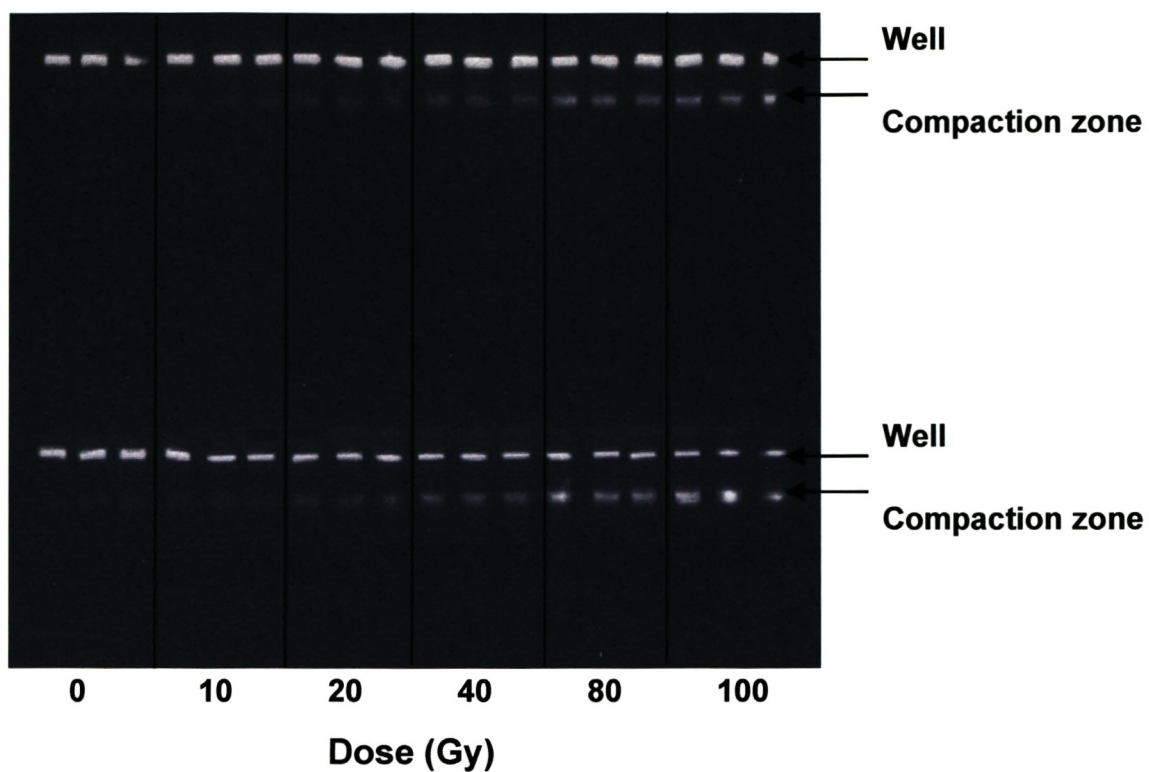
Constant field gel electrophoresis (CFGE) gel showing DNA released for the dose range of 0-100 Gy in CHO-K1.

Figure 3.17
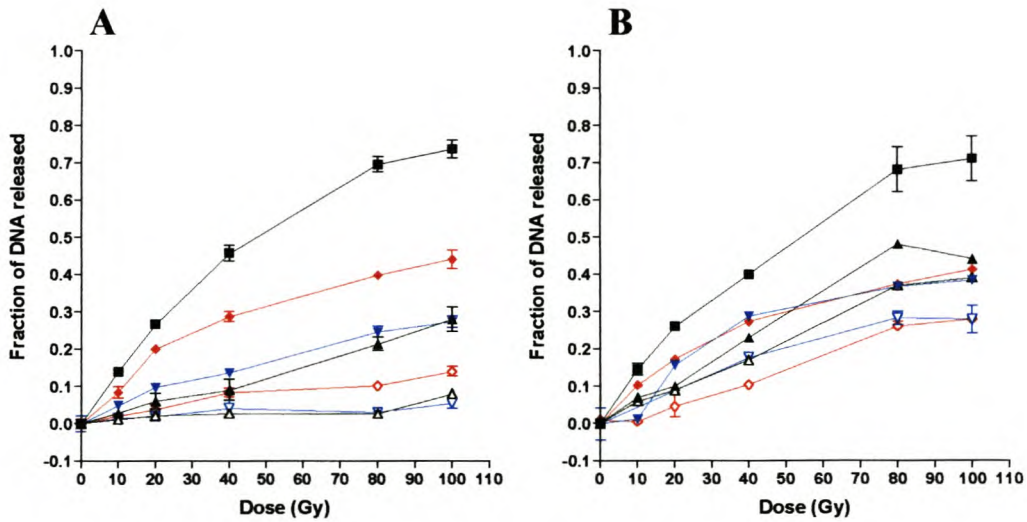
Constant field gel electrophoresis (CFGE) data showing fractions of DNA released for the dose range of 0-100 Gy in CHO-K1 (A) and xrs1 (B) cells, as determined by fluorescent densitometry of EtBr stained gels. Area under the curve (AUC) was calculated for each dose-response curve and is given in table 3.7. Initial DNA damage measured immediately after irradiation (■); residual DNA damage measured after 2 hours repair incubation at 37°C (▲); residual DNA damage measured after 2 hours repair incubation at 37°C in the presence of DMSO (▼); residual DNA damage measured after 2 hours repair incubation at 37°C in the presence of Wortmannin (◆); residual DNA damage measured after 20 hours repair incubation at 37°C (△); residual DNA damage measured after 20 hours repair incubation at 37°C in the presence of DMSO (▽); residual DNA damage measured after 20 hours repair incubation at 37°C in the presence of Wortmannin (◇).

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Table 3.7

	CHO-K1	xrs1
Initial DNA damage	47.33	44.85
2 hours repair	13.13	27.90
2 hours repair + DMSO	16.26	26.00
2 hours repair + Wortmannin	28.94	27.20
20 hours repair	2.84	22.03
20 hours repair + DMSO	3.24	18.38
20 hours repair + Wortmannin	7.85	14.59

Area under the dose-response curves (AUC) calculated from fractions of DNA released against irradiation dose (figure 3.17) for 0, 2 and 20 hours after irradiation over a dose range of 0-100 Gy.

Table 3.8

	CHO-K1	xrs1
2 hours repair	3.60	1.61
2 hours repair + DMSO	2.91	1.73
2 hours repair + Wortmannin	1.64	1.65
20 hours repair	16.67	2.05
20 hours repair + DMSO	14.61	2.44
20 hours repair + Wortmannin	6.03	3.07

Ratios of initial versus residual DNA damage derived from AUC data (table 3.7) for various repair times in the absence or presence of Wortmannin.

Table 3.9

	CHO-K1	xrs1
RIF 2 hours DMSO control	1.24	0.93
RIF 2 hours Wortmannin	2.20	0.98
RIF 20 hours DMSO control	1.14	0.84
RIF 20 hours Wortmannin	2.76	0.67

Repair inhibition factors (RIF) as calculated from repair ratios (table 3.8) in the absence and presence of Wortmannin.

CHAPTER 4

Discussion

4.1. Influence of cellular and DNA damage on G2/M block duration

The dependence of the G2/M block duration on dose was measured by irradiating HT29, L132 and ATs4 cells to the same survival level (table 3.2). In all three cell lines the G2/M maximum was reached 12 hours after irradiation (figure 3.2). The duration of the G2/M block was approximately 40 hours. The dose dependence of the G2/M block duration corresponded well with published data (Smeeds *et al.* 1994) The amount of undamaged DNA at 4.5, 5 and 2 Gy was found to be 86 %, 86 % and 88 % (calculated from table 3.4) for HT29, L132 and ATs4 respectively, showing that G2/M block is dependent on the amount of DNA damage induced by irradiation. After 12 hours repair, when the G2/M reaches its maximum (figure 3.2), the residual undamaged DNA showed levels of 96 %, 97 % and 99 % for HT29, L132 and ATs4 respectively (calculated from table 3.4) leaving approximately 3 % of DNA damage to be repaired during the G2/M block. That the remaining 28 hours of the G2/M block would be needed exclusively for the repair of the remaining 3 % DNA damage seems very questionable.

4.2. Influence of initial DNA damage DNA repair on radiosensitivity

The initial DNA damage and the unrepaired DNA damage induced by low doses of irradiation in cell lines of widely differing radiosensitivity was examined. DNA

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damage was inferred from the remaining double-stranded DNA after unwinding in alkali. The intact undamaged double stranded DNA was measured by a fluorescence signal of intercalated ethidium bromide. The method (Birnboim and Jevcak, 1981, Ogiu *et al.* 1992) is closely related to the comet assay (Olive *et al.* 1994, Olive and Banath, 1995) and relies on the fact that strand breaks and DNA modifications enhance the unwinding rate and produce lower amount of intact double-stranded DNA after irradiation damage in a dose dependent manner.

The FADU assay does not exclusively measure DNA double-strand breaks. The assay is based on the principle that any modification to the double helical DNA structure, which influences the association of strands, would influence the rate of unwinding and hence be detected by the ethidium bromide fluorescence signal emanating from residual double-stranded DNA. The method is based on the observation that short bacteriophage DNA unwinds faster (Davison, 1966) than long mammalian DNA (Ahnstrom and Erixon, 1973). The FADU assay is more sensitive to DNA double-strand breaks, but the 25-fold higher abundance of single-strand breaks (Elkind, 1979) will also contribute to DNA unwinding.

The experiments with repair proficient and repair deficient cell lines show that the initial amount of undamaged DNA remaining after doses of 1-10 Gy declines linearly with dose (figure 3.3). The ranking of slopes closely correlates with cell survival curves (figure 3.1). For a given dose, radiosensitive cell lines show a low level of residual intact DNA and radioresistant cell lines show a high level of residual intact DNA (figure 3.3). For the same dose radiosensitive cell lines

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show less intact DNA than radioresistant cell lines (figure 3.3). These observations indicate that cell survival and hence radiosensitivity is a reflection of the initial DNA damage.

The FADU data presented show that large differences exist in the induction of initial DNA damage (strand breaks). These differences were not apparent in the methods of alkaline elution (Kohn *et al*. 1976) and alkaline unwinding (Ahnstrom and Erixon, 1973). The differences can be attributed to sample preparation. In the latter 2 methods the chromatin structure is not completely destroyed. It appears now that DNA constraints probably play a role in the unwinding process (Olive *et al*. 1986, Jorgensen *et al*. 1990). Such constraints could arise from the attachment of DNA to the nuclear protein matrix, which would limit the extent of DNA unwinding. In the FADU assay the chromatin structure is disrupted by the addition of urea, to a final concentration of 4.5M, which effectively removes DNA from the matrix attachment sites.

Another factor, which may affect unwinding, is increased radioresistance due to differences in the shape of cells conferred by cell to cell contact as compared to single cells (Olive et al. 1986). My experiments were done on single cells harvested from cultures in logarithmic growth phase. The influence of "cell shape" is therefore minimal, as there is no contact inhibition.

In an analysis of the relative contributions of single event inactivation (α-term) and double event inactivation (β-term) it has previously been shown by Steel and Peacock (1989) that radiosensitivity as defined by cell survival at 2 Gy (SF2) is mainly given by the α-term and that the β-term contributes very little to

cell inactivation in the low dose range. In eleven cell lines differing widely in radiosensitivity use of the α-coefficient can explain 80 %-90 % of the survival seen at 2 Gy. At this dose, survival due to the β-coefficient alone accounts for 10 %-20 % of the actual survival. This means that low dose cell survival is mainly determined by the single event killing.

Factors, which could influence and regulate the initial DNA damage, are chromatin loop size (Heng *et al.* 1996) and chromatin compaction (Chapman *et al.* 1999). Indeed recent analysis of chromatin compaction by transmission electron microscopy in CHO-K1 cells and xrs5 cells has come to the conclusion that cells with the highest level of compacted chromatin in interphase show the highest radiosensitivity (Chapman *et al.* 1999). Repair fidelity could clearly also be a determinant for radiosensitivity, however comparison of the ranking of cell lines according to SF2 determined by colony assays (figure 3.1) with the initial DNA damage determined at 0°C or the residual double-stranded DNA after unwinding (figure 3.3) clearly demonstrates that cells showing a high initial DNA damage are poor survivors whereas those showing a low initial DNA damage rank as radioresistant in survival curves. This closely agrees with the conclusions of Steel and Peacock (1989) as outlined above.

The whole envelope of the initial DNA lesions can be considered to consist of repairable and non-repairable strand breaks which differ between cell lines in terms of proportions and magnitude. The repair rate data demonstrate that the vast differences of initial DNA damage at 5 Gy which range from 56 % to 93 % undamaged DNA have disappeared after 12 hours. Also, all cell lines except

AT1BR, xrs1 and SCID show a similar level of undamaged DNA in the range of 94 % to 98 %. After 12 hours repair the repair deficient lines xrs1 and SCID and the ataxia telangiectasia cell line AT1BR show only a marginal increase of undamaged DNA from approximately 66 % to approximately 79 % at the 5 Gy dose point (figure 3.4).

That AT1BR, xrs1 and SCID cells should fall into a different category and fail to recover to the same level of intact DNA as the other cell lines is not unexpected for two reasons. These cell lines undergo a markedly higher initial damage, perhaps as a result of higher levels of compacted chromatin in G1 as shown for xrs5 cells (Chapman *et al*. 1999), and they lack vital repair components. SCID cells lack the DNA-dependent protein kinase catalytic subunit (DNA-PK$_{CS}$) (Araki *et al*. 1997) and xrs1 cells lack the DNA-PK Ku80 subunit (Singleton *et al*. 1997). AT1BR has been shown to be repair deficient (Foray *et al*. 1997).

An unexpected result of the repair experiments (figure 3.4) is the excellent recovery of xrs5 cells. The xrs5 cells are only mutated in one repair component, namely the Ku80 subunit of DNA-PK (Stevens *et al*. 1999). DNA double-strand breaks are repaired by either non-homologous end joining (NHEJ) or by homologous recombination (HR) (Haber, 1999). The Ku proteins are required for non-homologous end joining but the radiation-induced double-strand breaks can still be repaired by Rad52-dependent homologous recombination (Van Dyck *et al*. 1999). In fact it has been shown that xrs5 cells display the same repair half time for double-strand breaks in G2 as CHO-K1 cells (Mateos *et al*.

1994). Another reason could be that the xrs5 cells have reverted back to the wildtype Ku80 status.

Both the ligase I mutant, 46BR.1 (Henderson *et al*. 1985), and the ligase IV mutant, 180BR.B (Riballo *et al*. 1999), repair to a level of 94 %. Although these mutations do not cause such an extreme repair deficiency they still cause the lowest repair capacity of the first group (figure 3.4).

A repair period of 12 hours separates the relationship between undamaged DNA and dose into two different sets of curves (figure 3.4), beyond which time the individual cell lines can no longer be distinguished. This shows that differences in detectable DNA damage, by unwinding, no longer persist after 12 hours of repair (except between the two groups). It therefore appears that repair competent cell lines do not differ vastly in terms of the non-repairable lesions as both types of cells essentially restore 97 % of their double-stranded DNA after 12 hours.

The vast differences of initial DNA damage between cell lines and the small differences of remaining DNA damage after 12 hours of repair are also reflected in the relationship between the mean inactivation dose ($\overline{D}$) and undamaged double stranded DNA. This plot shows an excellent correlation at 0 hours of repair and (as expected) a poor correlation after 12 hours of repair (figure 3.5).

Some recent experiments using high dose and measuring the induced double-strand breaks by PFGE and CFGE methods also have come to the conclusion that radiosensitivity of tumour cell lines correlates with the initial DNA double-strand breaks (Ruis De Almodovar *et al*. 1994, Whitaker *et al*. 1995). Other

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studies using similar techniques and high doses demonstrate that radiosensitivity does not correlate with initial induced DNA double-strand breaks (Dikomey *et al*. 1998, Schwartz *et al*. 1990, Wurm *et al*. 1994, Zaffaroni *et al*. 1994) but does correlate with the residual unrepaired DNA double-strand breaks. The strength of these data (Dikomey *et al*. 1998, Ruis De Almodovar *et al*. 1994, Smeets *et al*. 1993, Whitaker *et al*. 1995, Wurm *et al*. 1994) is that they rest upon measurements of DNA double-strand breaks. A weakness of this approach is that it requires high doses, which are beyond the clinical range. A weakness of the unwinding data by FADU is that they cannot measure DNA double-strand breaks but only residual intact double-stranded DNA. At the low dose of 2 Gy the remaining unrepaired DNA double-strand breaks would be in the region of 2-20 double-strand breaks (Blöcher, 1982, Dikomey *et al*. 1998) and below the detection threshold of PFGE methods. It is also not possible to obtain the unrepaired DNA double-strand breaks from the undamaged DNA determined by the FADU method as shown (figures 3.3 and 3.4). A strength of the FADU assay is that it takes into account the influence of LMDS as all lesions will have an effect on the unwinding rate.

Since the initial DNA damage can be measured reliably at low doses and since cell lines show enormous variation in initial DNA damage, which correlates with radiosensitivity, it follows that the initial DNA damage must be considered as a criterion which can distinguish cellular radiosensitivity.

4.3. Modulation of DNA damage, DNA repair and radiosensitivity

Survival curves show that xrs1 cells are more radiosensitive than CHO-K1 cells (figure 3.6). Published data on the xrs5 repair deficient cells and parental CHO-K1 cells also show a higher radiosensitivity in xrs5 cells than in CHO-K1 cells (Chapman *et al*. 1999). Using synchronised cells, the authors showed the concentration of condensed patches of chromatin in G1 phase to be higher in xrs5 cells than in CHO-K1, and concluded that condensed chromatin contributes to the higher radiosensitivity. In my experiments, low dose irradiation also induced more DNA damage in repair deficient xrs1 cells than in the parental CHO-K1 cells (figure 3.8). This is in agreement with other published data on xrs5 and CHO cells showing chromosome damage per unit absorbed radiation dose measured by premature chromosome condensation to be higher in xrs5 cells than in CHO cells (Iliakis and Pantelias, 1990). The authors attributed the higher chromosome damage in xrs5 cells to changes in chromatin structure, which increases the probability of a DNA double-strand break developing into a chromosome break.

It has previously been shown that differences in chromatin structure affect the yield of DNA double-strand breaks per unit dose. By alkaline sucrose density sedimentation it was found that H1-depleted chromatin in solution undergoes more DNA damage per unit dose than H1 containing chromatin condensed by Mg^{2+} (Heussen *et al*. 1987). Using the same damage assay, it was shown that hyperacetylated decondensed chromatin in nuclei received higher levels of DNA damage than controls (Nackerdien *et al*. 1989). In nuclear and nucleoid monolayers, and in chromatin depleted of histones by addition of high salt, DNA

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damage per unit dose was also higher than in control samples (Ljungman, 1991).

A different picture emerges when the influence of chromatin structure on radiosensitivity is tested in intact cells. In CHO-K1 and xrs1 cells grown in Nocodazole for 24 hours, mitotic levels were found to be 35 % and 24 % respectively (figure 3.6). It was observed that an increase in mitotic levels gives rise to an increase in radiosensitivity (figure 3.7). In CHO-K1 cells, addition of Nocodazole increased the radiosensitivity as shown by a decrease in the mean inactivation dose ($\overline{D}$) from 4.446 to 4.376 Gy. In xrs1 cells, Nocodazole also increased the radiosensitivity as shown by a decrease in $\overline{D}$ value from 1.209 to 0.7836 Gy. Prolongation of the G2/M-phase by pre-incubation with Nocodazole has been found to enhance radiation-induced cell killing (Ning and Knox, 1999), conceivably resulting from an inability of mitotic cells to repair DNA damage (Giulotto et al. 1978).

When the total amount of DNA damage was measured in the low dose range by the FADU assay, presence of Nocodazole was found to increase the mitotic index and to increase DNA damage (figure 3.8). In CHO-K1 cells exposed to 5 Gy, addition of Nocodazole increased the DNA damage from 7 % (control) to 13 %, while in xrs1 cells the DNA damage increased from 43 % to 57 % (figure 3.8 and table 3.6). This is consistent with the correlation between DNA damage and cell survival (Roos et al. 2000).

To further test the influence of chromatin structure on radiosensitivity, cells were subjected to a hypertonic medium of 0.5 M. Presence of a hypertonic medium

for 40 min preceding irradiation leads to an increase in radiosensitivity in normal cycling CHO-K1 cells as shown by a decrease of $\overline{D}$ from 4.446 to 3.092 Gy, while normal cycling xrs1 cells showed a decrease in radiosensitivity from a $\overline{D}$ of 1.209 Gy to a $\overline{D}$ of 1.609 Gy (table 3.5). Hypertonic treatment of cells causes dehydration (Szekely *et al.* 1983), chromatin destabilisation (Iliakis *et al.* 1993), dissociation of chromatin, chromosomal clumping and chromosomal swelling (Szekely et al. 1983).

It is well known that subjection of chromatin to high concentrations of NaCl will remove the histone H1 from DNA (Hoffmann and Chalkey, 1978). Therefore the effect of hypertonic treatment on H1 levels in intact cells was examined. After hypertonic treatment of CHO-K1 and xrs1 cells for 40 and 120 min, the histone H1 levels were compared with control levels (figure 3.9). Some fluctuation of histone H1 levels was observed but it was within 2 % of the norm and can be attributed to experimental error (figure 3.10).

The effect of hypertonic treatment on CHO-K1 and xrs1 cells was further examined by fluorescence microscopy after staining with acridine orange (figure 3.11 and 3.12). An increase in dehydration of the cytoplasm and the nucleus was observed with increasing treatment time. The increase in fluorescence in the nucleus (figure 3.11 and 3.12) is attributed to the fact that as a result dehydration the DNA is crammed into a smaller volume and the DNA concentration per nucleus increases. This "tighter" packing of DNA into the nucleus can be expected to invoke a profound effect on chromatin conformation. The change in chromatin structure is most clearly seen in the

chromosomes during mitosis (figure 3.13 and 3.14). As the time of hypertonic treatment increases the chromosomes lose definition due to swelling and clumping.

The influence of hypertonic treatment on initial DNA damage was also examined (figure 3.15). In CHO-K1 cells, an increase in DNA damage versus treatment time was observed and in xrs1 cells a decrease in DNA damage versus time was observed. The slopes for the two cell lines converge as the treatment time increased (figure 3.15). This implies that the differences in initial DNA damage and radiosensitivity between CHO-K1 and xrs1 cells disappear. Exposure of cells to hypertonic medium also abolishes the existing differences in native chromatin structure between cell lines as shown by fluorescence microscopy (figure 3.11, 3.12, 3.13 and 3.14).

In CHO-K1 cells, hypertonic treatment before 5 Gy irradiation increased DNA damage from 7 % to 15 %, while in xrs1 cells DNA damage decreased from 43 % to 36 % (table 3.6). The observation that the same modulator can induce opposite effects in terms of DNA damage in different cell lines is attributed to changes of chromatin structure.

When the per cent undamaged double-strand DNA remaining after 5 Gy irradiation (table 3.6) in control cells and cells treated with Nocodazole and hypertonic medium are examined, it emerges that the change of DNA damage follows the same trend as the change of radiosensitivity measured by colony assay (table 3.5).

The modulation of DNA repair was accomplished by the repair inhibitor Wortmannin. This inhibitor inactivates non-homologous end joining (NHEJ) repair (Rosenzweig *et al.* 1997, Sarkaria *et al.* 1998, Cheong *et al.* 1999, Chernikova *et al.* 1999) by inhibiting the catalytic subunit of DNA-PK. DNA double-strand break repair was monitored by the CFGE method because it assays specifically for DNA double-strand breaks.

Inspection of residual DNA double-strand breaks after 20 hours of repair in CHO-K1 and xrs1 cells, shows AUC values of 2.84 and 22.03, respectively (table 3.7). The 7 times higher AUC values in xrs1 cells demonstrates a vastly higher number of unrepaired DNA double-strand breaks in xrs1 than in CHO-K1 cells. This is in agreement with the proposed correlation between radiosensitivity and unrepaired DNA double-strand breaks (Schwartz *et al.* 1990, Whitaker *et al.* 1995, Dikomey *et al.* 1998). The reasons that this interrelationship is disputed by other groups (Smeets *et al.* 1993, Olive *et al.* 1994) are not known at present, but may lie in repair quality. The presence or absence of unrepaired DNA strand breaks measured by CFGE assay do not give any information about the quality of the repaired DNA. Mutations and misrepair are not detected by the CFGE method and will feature as repaired DNA although loss of clonogenicity may ensue later. The observed correlation between unrepaired DNA double-strand breaks and radiosensitivity (Dikomey *et al.* 1998) does not deal with these uncertainties. Since the abundance of unrepaired DNA double-strand breaks and misrepaired DNA double-strand breaks must be much lower at 2 Gy (calculations show 0.8 to 8 double-strand breaks per cell for 2 Gy using Frankenberg-Schwager (1989) and Dikomey *et*

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al. (1998) data), it is clear that a low dose parameter i.e. DNA damage which can be accurately determined, may be more meaningful for radiosensitivity considerations.

The inhibition of DNA double-strand break repair, by Wortmannin, in the CHO-K1 cell line greatly increased the radiosensitivity from a $\overline{D}$ of 5.914 Gy in the DMSO controls to a $\overline{D}$ of 3.043 Gy (table 3.5). In xrs1 cells no significant increase in radiosensitivity was seen with a $\overline{D}$ of 1.493 Gy for the DMSO control to $\overline{D}$ of 1.811 Gy after Wortmannin inhibition. Xrs1 cells are mutated in the Ku80 subunit of DNA-PK and Wortmannin should not effect radiosensitivity. In CHO-K1, the radiosensitising effect correlates with the inhibition of DNA double-strand break repair as seen from the 20 hours repair data in relation to controls (p=0.0095) (figure 3.17). No significant inhibition of DNA double-strand break repair was seen in xrs1 cells after 20 hours repair (p=0.6069). The Wortmannin data for CHO-K1 and xrs1 cells clearly demonstrate the importance of NHEJ DNA double-strand break repair in radiosensitivity.

Data on CHO-K1 and xrs1 cells confirm the previous findings that the initial DNA damage is a determinant of radiosensitivity (Roos *et al.* 2000). It is shown here that condensed chromatin in intact cells gives rise to more DNA damage and that processes which abolish chromatin structure reduce differences of radiosensitivity between cells. The observation that Nocodazole-induced chromatin condensation in intact cells gives rise to more DNA damage (figure 3.8) and less cell survival (figure 3.6) is not in contradiction to results on chromatin in solution, where a higher DNA damage was observed in

decondensed (open) chromatin and not in compact condensed chromatin (Heussen *et al.* 1987, Nackerdien *et al.* 1989, Ljungman, 1991). Intact cells and intact nuclei differ from chromatin in solution, and especially from salt disaggregated chromatin in repair competency. The high radiosensitivity of G2/M phase cells is commonly attributed to reduced repair competency and reduced access to damaged DNA sites (Johnston and Bryant, 1994). The present data indicate that repair plays an important role in radiosensitivity as measured by clonogenic assays. This is evident from the fact that the repair inhibitor Wortmannin (Rosenzweig et al. 1997, Cheong et al. 1999) increases the radiosensitivity in repair competent CHO-K1 cells but not in repair deficient xrs1 cells. The operation of repair in CHO-K1 and absence of repair in xrs1 cells is also indicated by the CFGE data which demonstrate that restoration of DNA damage in repair competent CHO-K1 cells is profoundly inhibited by Wortmannin but not in repair deficient xrs1 cells. It can therefore be deduced that the initial DNA damage and NHEJ DNA double-strand break repair competency are major factors which influence radiosensitivity and cell survival. Furthermore it is shown that both processes are profoundly influenced by chromatin structure.

CONCLUSIONS

1. DNA damage at low doses (1-10 Gy) was measured by the fluorometric analysis of DNA unwinding (FADU) method. In all eleven cell lines a linear induction of DNA damage was observed showing FADU to be a sensitive assay capable of measuring DNA damage at low doses of irradiation.

2. When looking at the effect of irradiation on G2/M delay in HT29, L132 and ATs4 cell lines, the G2/M maximum was reached 12 hours after irradiation with isodoses and lasted for approximately 40 hours. The isodoses induced similar amounts of DNA damage and after 12 hours repair only 3 % DNA damage remained. The remaining 28 hours of the G2/M block therefore seems to have a different function than DNA repair.

3. In all eleven cell lines the linear slopes of the DNA damage induction curves differed from each other. This showed that initial DNA damage induction by irradiation per unit dose differed between cell lines.

4. When comparing the $\overline{D}$ values of eleven cell lines that differ greatly in radiosensitivity with the initial DNA damage slopes the values followed the same order. A correlation between the two sets of values produced the following results: r^2=0.81 and P-value=0.0002. This shows that initial DNA damage is a determining factor of radiosensitivity.

5. When comparing the $\overline{D}$ values of eleven cell lines that differ greatly in radiosensitivity with the residual DNA damage remaining after 12 hours

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repair the following results were found: r^2=0.22 and P-value=0.1508. When inhibiting the NHEJ DNA double-strand break repair in CHO-K1, the radiosensitivity was greatly increased. This shows that although no correlation exist between the rate of DNA repair and radiosensitivity, NHEJ does play a part in radiosensitivity.

6. The modulation of chromatin structure by Nocodazole and hypertonic treatment had a profound effect on radiosensitivity and initial DNA damage. This shows that chromatin structure determines the amount of cell death induced by irradiation by influencing the initial DNA damage levels.

PUBLICATIONS FROM THIS THESIS

Roos, W. P., Binder, A. and Böhm, L. 2000, Determination of the initial DNA damage and residual DNA damage remaining after 12 of repair in eleven cell lines at low doses of irradiation. *International Journal of Radiation Biology* **76(11)**, 1493-1500.

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