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Cellular Responses to Ionizing Radiation and Cisplatin

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"Of all the vegetable kingdom, the vine alone makes intelligible the true savor of the earth." - Colette

Abstract

This thesis tested the hypothesis that the synergistic effect of cisplatin in combination with radiation was due to cisplatin inhibition of repair of radiation-induced DNA damage. Two model cell systems were used. DNA repair inhibition caused by cisplatin and the fundamental mechanism of DNA double strand break repair were studied in human fibroblast cells. The effect of cisplatin on stress response and the induction of radiation resistance was studied in yeast.

It was shown that normal human fibroblasts cells (AG1522), irradiated in the presence or absence of cisplatin adducts have biphasic DNA repair curves with a fast and slow component. When cells were treated with cisplatin immediately before irradiation, fast repair was not affected but slow repair was inhibited. The rate of the slow repair in the presence of cisplatin adducts was ten fold less than the rate in the absence of cisplatin adducts. Cisplatin treatment 24 hours prior to radiation resulted in inhibition of both fast and slow repair.

Yeast experiments tested whether defects in DNA repair mechanisms would amplify the radiosensitizing effect of cisplatin. It was shown that cisplatin sensitized cells with a competent recombinational repair mechanism, but did not sensitize cells defective in recombinational repair. It was also shown that under certain circumstances cisplatin did not radiosensitize normal, repair competent cells but induced stress response and cellular radiation resistance. Repair proficient wild type yeast cells became thermal tolerant and radiation resistant two hours after a sublethal cisplatin treatment. Cisplatin pretreatment also suppressed mutations caused by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treatment, a response previously shown in wild type cells with radiation pretreatment. Like radiation, cisplatin-induced stress response did not confer radiation resistance or suppress MNNG mutations in a recombinational repair deficient mutant (rad52). These results support the idea that cisplatin crosslinks in DNA can induce a stress response which subsequently confers radiation

resistance, thermal tolerance, and mutation resistance in yeast. The mechanism of this cisplatin-induced resistance was determined to be dependent on an error free recombinational repair pathway.

The results obtained in this thesis indicated that error free recombinational repair plays a major role in the interaction between cisplatin and radiation. To further examine error-free recombinational repair in mammalian cells, confocal microscopy in conjunction with fluorescence *in situ* hybridization was used to show that the distance between homologous chromosome domains was reduced after radiation. The mechanism for homologous chromosome rearrangement following exposure to radiation is not known, however, it is plausible that a recombinational repair process is responsible.

Overall, this work has shown that the radiosensitizing effect of cisplatin was due to inhibition of DNA repair processes involving recombinational repair. However, it was also shown that, under specific conditions, cisplatin treatment induced a stress response that conferred cellular radiation resistance. Therefore, the effect of cisplatin in combination with radiation was variable depending upon the cellular processes influenced by cisplatin.

Résumé

Le travail présenté dans cette thèse visait à vérifier l'hypothèse que l'effet synergistique du cisplatine combiné à la radiation était dû à l'inhibition, par le cisplatine, de la réparation du dommage à l'ADN provoqué par la radiation. En utilisant des cellules fibroblastes humaines, nous avons etudié l'inhibition de la réparation de l'ADN causée par le cisplatine et le mécanisme fondamental de la réparation de l'ADN avec les deux brins brisés. Dans des expériences connexes, les cellules de la levure ont été utilisées pour étudier l'effet du cisplatine sur la réponse au stress et l'induction de la résistance à la radiation.

Il a été démontré que les cellules fibroblastes humaines normales (AG1522), irradiées en présence ou en absence des produits du cisplatine, démontrent des courbes de réparation d'ADN à deux phases, l'une rapide et l'autre lente. Quand les cellules ont été traitées avec le cisplatine immédiatement avant l'irradiation, la réparation rapide n'a pas été affectée mais la réparation lente a été inhibée. Le taux de la réparation lente en présence des adduits du cisplatine était dix fois plus faible qu'en l'absence de ces mêmes adduits. Un traitement au cisplatine 24 heures avant l'irradiation a causé l'inhibition des deux réparations, rapide et lente.

Les expériences avec les cellules de la levure ont permis de vérifier si les défauts dans les mécanismes de réparation de l'ADN amplifient l'effet de la sensibilisation à la radiation causée par le cisplatine. Il a été démontré que le cisplatine a sensibilisé seulement les cellules avec un mécanisme de réparation de la recombinaison compétente. Dans d'autres conditions expérimentales, le cisplatine n'a pas rendu les cellules plus sensibles à la radiation mais a plutôt provoqué une réponse de stress et une résistance cellulaire à la radiation. Les cellules de la levure du type compétentes sauvages ont développé une tolérance thermique et une résistance à la radiation deux heures après un traitement au cisplatine subléthal. Des prétraitements au cisplatine ont aussi

réduit les mutations causées par le N-méthyl-N-nitro-N-nitrosoguanidine (MNNG), une réponse observée précédemment dans les cellules du type sauvage avec un prétraitement radiatif. Tout comme pour la radiation, la réponse de stress provoquée par le cisplatine n'a pas conféré de résistance à la radiation ou n'a pas supprimé les mutations causées par le MNNG dans un mutant défectueux (rad52) au niveau de la réparation de recombinaison. Ces résultats confirment l'idée que les liens croisés du cisplatine dans l'ADN peuvent induire une réponse de stress qui confère par la suite une résistance à la radiation, une tolérance thermique, et une résistance aux mutations dans la levure. Le mécanisme de cette résistance induite par le cisplatine implique selon nos résultats la réparation de la recombinaison sans erreur.

Les résultats présentés dans cette thèse indiquent que la réparation de la recombinaison sans erreur joue un rôle majeur dans l'interaction entre le cisplatine et la radiation. Pour mieux comprendre la réparation de la recombinaison dans les cellules de mammifères, la microscopie confocale a été utilisée conjointement avec l'hybridation fluorescente in situ. Ces experiences ont révélé que l'irradiation a causé une réduction de la distance entre les domaines des chromosomes homologues. Le mécanisme pour ce réarrangement des chromosomes homologues demeure obscur, cependant il est plausible qu'un processus de réparation de recombinaison soit impliqué.

Globalement, ce travail a montré que l'effet de la sensibilisation à la radiation causée par le cisplatine était dû à l'inhibition des processus de réparation, en particulier la réparation de la recombinaison. De plus, des traitements avec le cisplatine sous des conditions spécifiques ont permis d'induire une réponse de stress qui a conféré une résistance cellulaire envers la radiation. Par conséquent, la synergie entre le cisplatine et la radiation découle directement de l'influence de cette drogue sur les processus cellulaires.

Table of Contents

List of Figures

List of Tables

Cellular Responses to Ionizing Radiation and Cisplatin

General Introduction

This thesis investigates cellular reponses to ionizing radiation and cisplatin. Alone or in combination, these two agents are used to treat cancer. A better understanding of the DNA repair mechanisms induced by these agents should assist in enhancing their therapeutic effectiveness in cancer treatment.

Ionizing Radiation

Ionizing radiation exerts its biological effect by breaking chemical bonds in molecules. It is generally agreed that the critical cellular molecule (critical target) damaged by ionizing radiation is DNA (Hall 1988). Exposure to ionizing radiation causes a variety of DNA lesions including base alterations, DNA-protein crosslinks, single and double strand breaks (Ward 1988, Frankenberg-Schwager 1989). Approximate yields of the types of DNA damage produced by ionizing radiation (per Gray[1]) have been determined and are listed in Table 1.

Table 1. Number of radiation-induced DNA lesions per cell per Gray (Ward 1988)[a]

Lesion	Number
Single strand break	1000
Double strand break	40
DNA-protein crosslink	150
Base damage	950

(a) Based on 6 pg DNA per cell

Cellular DNA repair mechanisms can restore the integrity of the DNA molecule. If a base is damaged or a break occurs in one strand of the DNA molecule, they can be repaired rapidly ($t_{1/2}$=2 to 10 minutes) by an excision repair process in which the complementary DNA strand is used as a template (Frankenberg-Schwager 1989, Sancar 1994). If there is a break in both DNA strands, at the same or a nearby position, a double strand break results. Even

[1]Gray (Gy) is a unit of absorbed dose and is equivalent to 1 joule/kg

though the yield of double strand breaks is low compared to other types of damage, double strand breaks are considered to be the most biologically relevant lesion leading to mutations, transformation and cell death (Ward 1988, Frankenberg-Schwager 1989, Frankenberg-Schwager and Frankenberg 1990a). The complete repair of double strand breaks requires several hours to occur ($t_{1/2}$=40 minutes to 4 hours) (Frankenberg-Schwager 1989). To repair a double strand break, genetic information must be copied from a duplicate source (homologous chromosome or sister chromatid) through a process of recombinational repair (Resnick 1976, Szostak et al. 1983, Sancar and Sancar 1988).

The process of recombinational repair has been well documented in yeast. It has been shown that recombinational repair capacity can be enhanced by exposure to a variety of sublethal stresses including radiation (Mitchel and Morrison 1982, Mitchel and Morrison 1987, Boreham et al. 1990). Increased repair capacity results from increased levels of recombinational repair gene transcripts and proteins (Boreham 1990, Game 1993) which complex to form a "recombinosome" (Hays et al. 1995) and consequently generates a transient radioresistant phenotype. Yeast cells induced for radioresistance are protected from the lethal and mutagenic effects of a subsequent radiation exposure (Mitchel and Morrison 1982, 1987) . The understanding of inducible repair processes is limited in mammalian cells, although it has been demonstrated that, as in yeast, the induction of repair processes by a sublethal dose of radiation is a protective mechanism that confers resistance to the damaging effects of subsequent radiation exposures (Wolff 1992, Wojcik and Streffer 1994). The induction of this protective mechanism is known as the adaptive response. Mammalian cells exposed to a low dose of radiation become less susceptible to radiation-induced micronucleus and chromosome aberration formation (Shadely and Dai 1992, Azzam et al. 1994a, Wojcik and Streffer 1994), mutagenesis (Kelsey et al. 1991), and transformation to malignancy (Azzam et al. 1994, Azzam et al. 1996).

Cisplatin

Cis-diamminedichloroplatinum II (cisplatin) is a chemotherapeutic drug which exerts a cytotoxic effect through damaging DNA. Cisplatin in its native form is a small, neutral platinum based molecule (Bruhn et al. 1990, Douple 1990). In the cell, where chloride ion concentration is relatively low (4 mM), cisplatin molecules undergo hydrolysis and become reactive species (Lippard 1982). In the hydrolyzed state, cisplatin can then bind to DNA through a two step process (Figure 1).

Initially, monofunctional adducts are formed primarily at the N7 positions of guanine and adenine. These monofunctional platinum-DNA adducts react further to form bifunctional adducts (inter- or intrastrand DNA crosslinks) again with adenine or guanine. The relative amount of each cisplatin-DNA adduct has been determined in cell culture systems and with isolated DNA and the reported proportions are listed in Table 2.

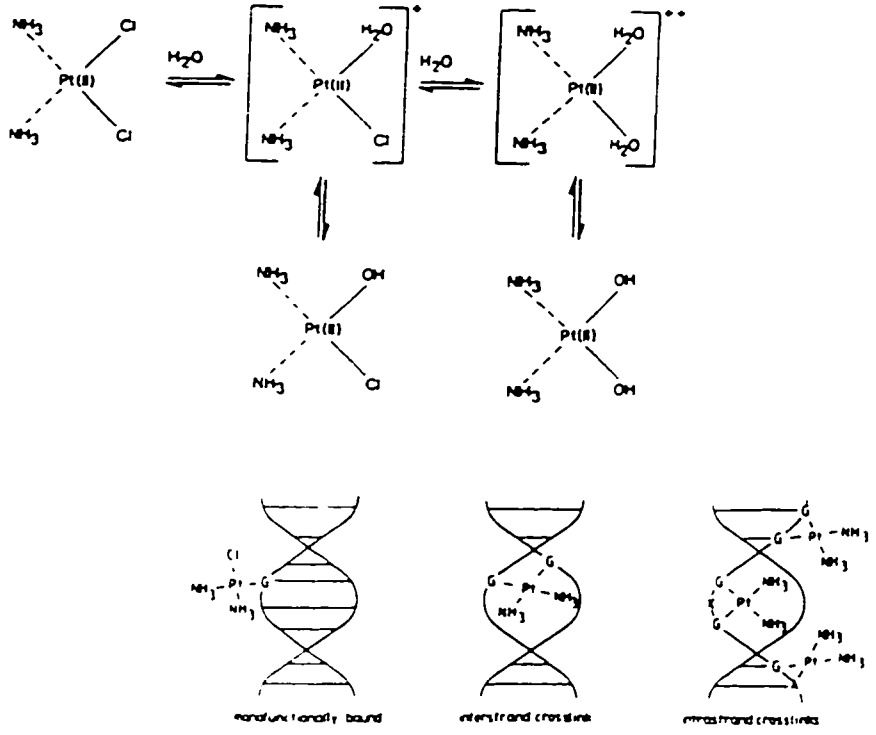


Figure 1. Formation of reactive cisplatin species and cisplatin-DNA adducts (modified from Dewitt 1987).

Table 2. Relative proportions of cisplatin-DNA adducts (from (A) Fichtinger-Schepman et al. 1985, (B) Fichtinger-Schepman et al. 1987, (C) Dijt et al. 1988).

Cisplatin Adduct		Proportion		
		A	B	C
Time between cisplatin treatment and adduct analysis (hr)		5	3	3
Intrastrand crosslinks between adjacent bases	cis-[$Pt(NH_3)_2${d(GpG)}]	47-50%	65%	62-75%
	cis-[$Pt(NH_3)_2${d(ApG)}]	23-28%	22%	9-19%
Intrastrand crosslinks between nonadjacent guanines and interstrand crosslinks	cis-[$Pt(NH_3)_2${$d(GMP)_2$}]	8-10%	13%	9-15%
Monofunctional adducts	cis-[$Pt(NH_3)_2${dGMP)}]	2-3%	0.7%	2-14%

A number of studies have investigated the formation and removal of cisplatin-DNA adducts. After short incubations with cisplatin (15 minutes), a prevalent lesion is the monofunctional adduct (Eastman 1986). With time during or after exposure to cisplatin, the number of monofunctional adducts decreases and the number of bifunctional adducts increases. In the absence of repair processes to remove cisplatin adducts, the number of bifunctional adducts increases because the second chloride ion of the cisplatin molecule becomes hydrolyzed which then reacts with DNA in a second reaction (see Figure 1). In several cell lines studied, the number of bifunctional adducts continued to increase for many hours (12-24h) after cisplatin treatment (Zwelling et al. 1978, Plooy et al. 1985). Accumulation of crosslinks eventually stops and repair processes begin to reduce the number of crosslinks. However, for cells from humans with Fanconi's anaemia which are crosslink repair deficient [2], the removal of lesions does not occur and the accumulation of interstrand crosslinks continues for days (Plooy et al. 1985).

[2]Fanconi's anaemia is a human genetic disorder characterized by increased susceptibility to malignant disease, and the cells from these patients are defective in repair of DNA crosslinks.

Accumulation of crosslinks is not, however, a universal finding and may be dependent upon the repair capabilities of individual cell lines (Plooy et al 1985, Hill et al. 1994) and the concomittant conversion of monofuntional adducts to inter- and intrastrand crosslinks over time (Jones et al. 1991).

Removal or repair of cisplatin adducts is thought to be mainly by a nucleotide excision repair mechanism (Sancar and Sancar 1988, Sibghat-Ullah et al. 1989, Calsou and Salles 1993), although there is evidence that repair of interstrand crosslinks involves a combination of excision and recombinational repair pathways (Cole 1973, Hannan et al. 1984, Sancar and Sancar 1988, Calsou and Salles 1993). Compared to repair of radiation-induced lesions, restoring cisplatinated DNA to its original form is a slow process. The time required to repair DNA crosslinks varies depending upon cell type and growth state and ranges from one to four days (Fraval and Roberts 1979, Dijt et al. 1988, Jones et al. 1991, Larminat et al. 1993, Hill et al 1994) demonstrating the persistence of cisplatin-DNA adducts.

DNA damage produced by cisplatin may signal the induction of elevated repair capacity and confer cellular radiation resistance (induction of the adaptive response). Nuclear extracts from mammalian cells treated with cisplatin, catalyzed homologous recombination in an *in vitro* assay, demonstrating that cisplatin-induced DNA damage signalled the induction of a process likely involved in repair of DNA double strand breaks (Lehnert and Chow 1996). Further, treatment of mammalian cells with cisplatin, four to six hours prior to a radiation exposure, led to an increased survival in the initial portion of radiation survival curves indicating enhanced repair capacity for radiation damage (Skov et al 1996), and research using mice demonstrated that when mice are pretreated with cisplatin 24 hours prior to irradiation, they develop resistance to the lethal effects of radiation (Zak and Drobnik 1971). The evidence indicates therefore that DNA damage caused by cisplatin seems to induce an adaptive response *in vitro* and *in vivo*.

Combination Cisplatin and Radiation

In contrast to the above mentioned results, cisplatin used in combination with radiation may not lead to enhanced repair capacity but may produce greater cell kill than would be expected if the effects were additive. This synergistic effect has been reported in both prokaryotic (Richmond and Powers 1976, Richmond et al. 1977) and eukaryotic systems (Wodinsky et al. 1974, Zak and Drobnik 1971, Overgaard and Khan 1981, Alvarez et al. 1978, Douple and Richmond 1978, Korbelik and Skov 1989, Skov and MacPhail 1991). The synergistic effect is evident, for cell killing, when cells are irradiated under hypoxic conditions and is less pronounced or absent when cells are irradiated in air (Richmond and Powers 1976, Richmond et al. 1977, Douple and Richmond 1978, Dewitt 1987, Korbelik and Skov 1989, Skov and MacPhail 1991). The reasons for this observation are not known, although oxygen modification of radiation-induced lesions may affect the interaction between cisplatin- and radiation-induced damage (Korbelik and Skov 1989). Comparisons of radiosensitization by cisplatin at low (0-4 Gy) versus high (5-30 Gy) radiation doses under hypoxic conditions have revealed increased enhancement in the low dose range. It has been suggested that this increased enhancement is due to the interaction between a specific type(s) of radiation damage (induced predominantly at low doses) and cisplatin DNA adducts at the level of repair of DNA damage (Korbelik and Skov 1989, Skov and MacPhail 1991).

There is evidence to indicate that cisplatin-induced radiosensitization is in part due to inhibited repair of radiation-induced DNA damage (Douple 1990). This has been demonstrated as a reduction in the shoulder region of radiation survival curves (Alvarez et al. 1978, Begg 1987, Douple 1990), a reduction in potentially lethal damage recovery (Carde and Laval 1981, Wilkins et al. 1993), a decreased sparing effect of split dose radiation exposures (Schwachofer et al. 1991) and low dose rate irradiation (Wilkins et al. 1996). However, the mechanism of inhibition of radiation repair processes by cisplatin has not been established.

Chadwick et al. (1976) analyzed data from experiments using a cisplatin related complex in combination with radiation on Chinese hamster ovary cells and postulated that the synergism was due to the interaction of a radiation-induced single strand break opposite a platinum intrastrand DNA crosslink. The authors proposed that a radiation-induced strand break in the opposite DNA strand and close to a platinum crosslink would not be repaired and that subsequent replication of the single strand break would give rise to a double strand break, which if unrepaired would lead to cell death (Figure 2A). The potential for generation of a double strand break also exists when a platinum adduct, located in close proximity to a radiation-induced single strand break on the complementary strand, is excised during enzymatic repair (Figure 2B). The resulting nick or gap across from a single strand break produces a potentially lethal double strand break. It has been reported that carboplatin, an analogue of cisplatin, enhances the production and persistence of radiation-induced single strand breaks (Yang et al. 1995). Yang et al. (1995) also found that carboplatin increased the yield of double-strand breaks in both wild type and excision repair deficient Chinese hamster ovary cell lines. The authors suggested that in repair proficient cells, more DNA double strand breaks may have resulted from excision of a platinum adduct across from a radiation-induced single strand break (Figure 2B), where as in excision repair deficient cells a carboplatin adduct nearby a DNA double strand break may have inhibited the repair of the double strand break (Figure 2C).

Alternatively, synergistic cell killing effects of combined cisplatin and radiation exposure may be due in part to radiation potentiation of cisplatin cytotoxicity. It has been shown that large doses of X rays (10 - 50 Gy) increased cellular uptake of carboplatin, presumably due to radiation damage to membranes and consequent change in permeability (Yang et al. 1995). The same study found no increase in cellular uptake for a second platinum complex and concluded that the change in membrane permeability is dependent upon the

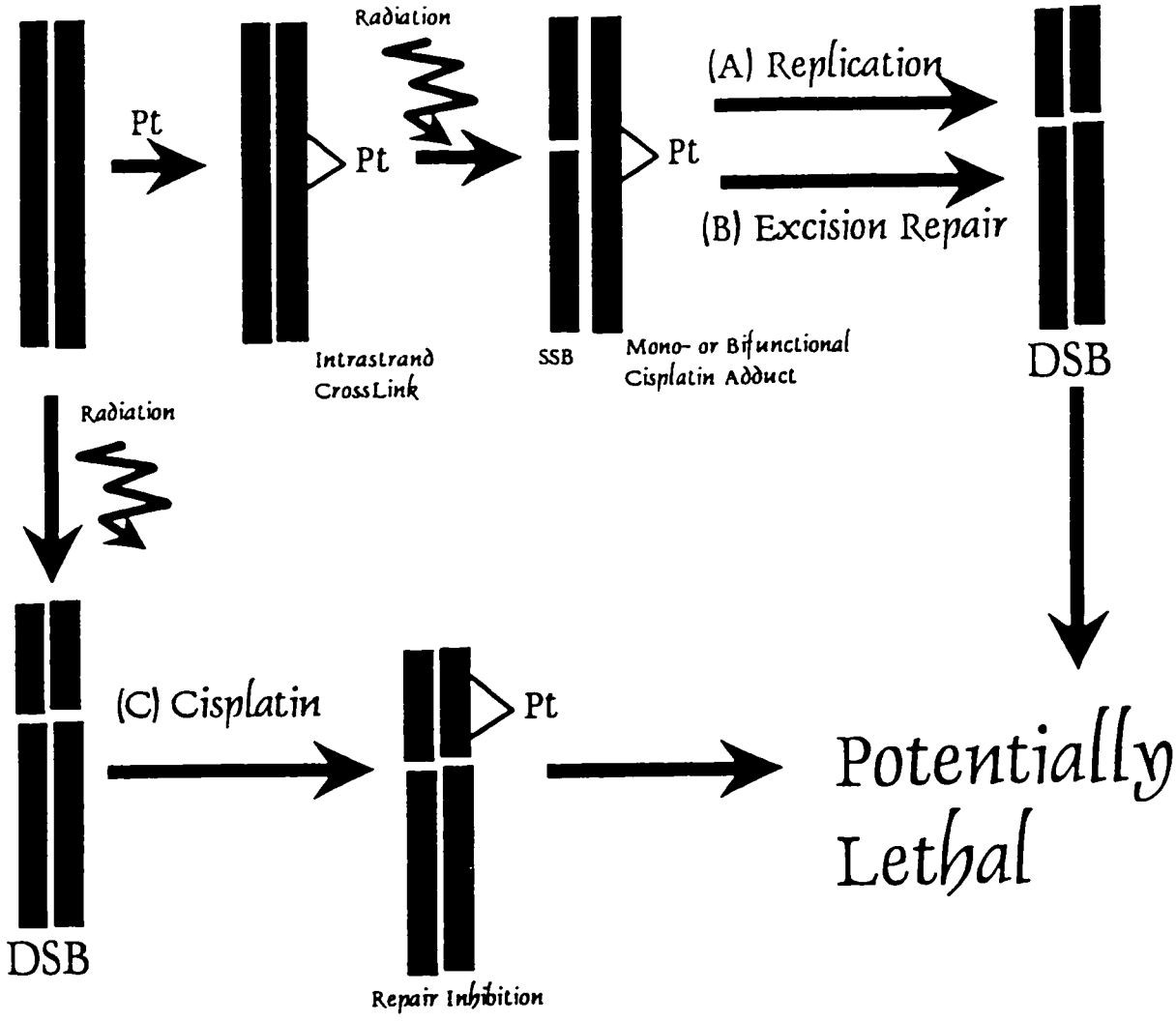


Figure 2. Production of Potentially Lethal Double Strand Breaks by Interaction of Cisplatin DNA Adducts and Radiation-Induced DNA Damage. A potentially lethal DNA double strand break may arise from (A) cisplatin crosslink inhibition of single strand break repair and subsequent replication of the unrepaired single strand break, (B) excision repair of a cisplatin crosslink across from a radiation-induced single strand break or (C) cisplatin crosslink inhibition of radiation-induced double strand breaks.

properties of the platinum compound used. In addition, it has been suggested that cisplatin can scavenge hydrated electrons and prevent the interaction of the hydrated electrons with hydroxyl radicals (e^-_{aq} + OH. → OH-), thereby increasing the number of hydroxyl radicals that can interact with DNA (Richmond and Powers, 1976).

The interference of a cisplatin adduct with the repair of radiation-induced DNA damage, or the formation of a potentially lethal double strand break during the repair of a cisplatin adduct implies that both types of damage (strand breaks caused by radiation and cisplatin-DNA adducts) must occur at the same time or that the lesions are persistent allowing for interaction to occur over long periods of time. Maximal radiosensitization by cisplatin has been found when cells are treated with cisplatin either shortly before or immediately after exposure to radiation (Dewitt 1987, Douple 1990). However, in a few studies , the largest effect was found when the time interval between cisplatin and subsequent radiation treatment was increased to 24 hours (Douple and Richmond 1978, Dewitt 1987). In contrast, at least one study (Zak and Drobnick 1971) found that separating the radiation exposure from the cisplatin treatment by 24 hours did not result in enhanced killing by radiation but led to enhanced survival instead. To understand these variable results, further elucidation of the interactions between cisplatin-DNA adducts, radiation-induced DNA damage and DNA repair processes is required.

Research on the combined effects of cisplatin-DNA adducts and radiation-induced DNA damage is presented in the following thesis. An investigation was conducted of repair mechanisms in mammalian cells exposed to a combination of cisplatin and radiation. The influence of cisplatin-DNA adducts on the repair of strand breaks caused by radiation when cisplatin was delivered immediately before, or 24 hours before radiation exposure is presented. In addition, the effect of a combined cisplatin and radiation treatment on the formation

of micronuclei, which are thought to arise from unrepaired DNA double strand breaks, was also examined.

Yeast have been used to investigate numerous aspects of DNA repair and have proven to a powerful model system for elucidation of various mechanisms (Kiefer and Muller 1990). To better understand the results obtained using mammalian cells in this work, yeast were utilized. The radiosensitizing effect of cisplatin on recombinational deficient (rad52) and excision repair deficient (rad3) yeast mutants was investigated to determine whether cisplatin-DNA adducts generated immediately before radiation interfered with specific repair processes. In subsequent experiments, the time between cisplatin and radiation exposures was extended to several hours to determine whether cisplatin-DNA adducts, which are known to distort the DNA helix (Bruhn et al. 1990), are effective signals for the induction of the adaptive response. Further, by employing an N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-generated mutation assay, the question of whether cisplatin-DNA adducts were signalling the induction of an error prone or error free recombinational repair system was investigated (Mitchel and Morrison 1987).

The experiments presented in chapters one, two and three of this thesis, were designed to determine whether cisplatin-DNA adducts influence, positively or negatively, the error free recombinational repair processes of cells. The error free repair process has been well documented in yeast, but evidence for its existence in mammalian cells is limited. In contrast to yeast, it is thought that in mammalian cells the predominant process for repair of complex DNA damage such as DNA double strand breaks is via an illegitimate process involving a repair complex DNA-PK (Wood 1996). In this process, proteins (Ku70, Ku80) bind to the broken ends and mediate ligation of the break. This process is considered illegitimate because the missing genetic information is not replaced. Such a process may be tolerated in non-gene coding regions within the genome, but repair of gene coding DNA presumably must be carried out by a recombinational repair process involving chromatids or homologous chromosomes in order to limit detrimental genetic

effects to the cell. Chapter four presents experiments that test for the existence of this latter repair system in mammalian cells after the cells have been exposed to radiation.

In summary, this thesis examines mechanisms of DNA repair in lower and higher eukaryotic cells. The effect of DNA crosslinks was used as a tool to elucidate the signalling, inhibition and induction of DNA repair. The results obtained within this thesis provide a better understanding of the adaptive response and DNA repair mechanisms.

References

Alvarez M.V., G. Cobreros, A. Heras and M.C. Lopez Zumel. Studies on cis-dichlorodiammineplatinum(II) as a radiosensitizer. Br. J. Cancer 37:68-72 (1978).

Azzam, E.I., S.M. deToledo, G.P. Raaphorst and R.E.J. Mitchel. Low dose ionizing radiation decreases the frequency of neoplastic formation to a level below the spontaneous rate in C3H 10T1/2 cells. Radiat. Res. 146: 369-373 (1996).

Azzam, E.I., G.P. Raaphorst and R.E.J. Mitchel. Radiation-induced adaptive response for protection against micronucleus formation and neoplastic transformation in C3H 10T1/2 mouse embryo cells. Radiat. Res. 138: S28-S31 (1994).

Begg, A.C. Additivity versus repair inhibition in fractionated treatments combining drugs and X-rays: A theoretical approach. Int. J. Radiation Oncology Biol. Phys. 13: 921-927 (1987).

Boreham, D.R. Ph.D. Thesis, The mechanism of induced radiation resistance (University of Ottawa, Ottawa, Canada) (1990).

Boreham, D.R., A. Trivedi and R.E.J. Mitchel. Radiation and stress response in *Saccharomyces cerevisiae*. In Yeast Molecular Biology and Biotechnology (R. Prasad, Ed.) pp294-314 Omega Scientific, New Delhi (1990).

Bruhn, S.L., J.H. Toney and S.J. Lippard. Biological processing of DNA modified by platinum compounds. Progress in Inorganic Chemistry: Bioinorganic Chemistry 38:477-517 (1990).

Calsou, P. and B. Salles. Role of DNA repair in the mechanisms of cell resistance to alkylating agents and cisplatin. Cancer Chemother. Pharmacol. 32:85-89 (1993).

Carde,P. and F. Laval. Effects of cis-dichlorodiammine platinum II and X-rays on mammalian cell survival. Int. J. Radiation Oncology Biol. Phys. 7:929-933 (1981).

Chadwick, K.H., H.P. Leenhouts, I. Szumiel and A.H.W. Nias. An analysis of the interaction of a platinum complex and radiation with CHO cells using the molecular theory of cell survival. Int. J. Radiat. Biol. 30: 511-525 (1976).

Cole, R.S. Repair of DNA containing interstrand crosslinks in *Escherichia coli*: Sequential excision and recombination. Proc. Nat. Acad. Sci. 70: 1064-1068 (1973).

Dewitt, L. Combined treatment of radiation and cisdiamminedichloroplatinum (II): A review of experimental and clinical data. Int. J. Radiation Oncology Biol. Phys. 13: 403-426 (1987).

Dijt, F.J., A.M. Fichtinger-Schepman. F. Berends and J. Reedijk. Formation and repair of cisplatin-induced adducts to DNA in cultured normal and repair-deficient human fibroblasts. Cancer Res. 48: 6058-6062 (1988).

Douple, E.B. Interactions between platinum coordination complexes and radiation. In Antitumor Drug-Radiation Interactions (B.T. Hill and A.S. Bellamy, Eds.) pp171-190 CRC Press, Boca Raton, Florida (1990).

Douple, E.B. and R.C. Richmond. Platinum complexes as radiosensitizers of hypoxic mammalian cells. Br. J. Cancer 37:98-102 (1978).

Eastman, A. Reevaluation of interaction of cis-dichloro(ethylenediammine)-platinum(II) with DNA. Biochem. 25: 3912-3915 (1986).

Fichtinger-Schepman, A.M., A.T. van Oosterom, P.H.M. Lohman and F. Berends. Cis-diamminedichloroplatinum(II)-induced DNA adducts in peripheral leukocytes from seven cancer patients: Quantitative immunochemical detection of the adduct induction and removal after a single dose of cis-diamminedichloroplatinum(II). Cancer Res. 47: 3000-3004 (1987).

Fichtinger-Schepman, A.M., J.L. van der Veer, J.H.J. den Hartog, P.H.M. Lohman and J. Reedijk. Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: Formation, identification, and quantitation. Biochem. 24: 707-713 (1985).

Frankenberg-Schwager, M. Review of repair kinetics for DNA damage induced in eukaryotic cells in vitro by ionizing radiation. Radiotherapy and Oncology 14: 307-320 (1989).

Frankenberg-Schwager, M. and D. Frankenberg. DNA double-strand breaks: Their repair and relationship to cell killing in yeast. Int. J. Radiat. Biol. 58: 569-575 (1990a).

Fraval, H.N.A. and J.J. Roberts. Excision repair of cis-diamminedichloroplatinum(II)-induced damage to DNA of Chinese hamster cells. Cancer Res. 39:1793-1797 (1979).

Game, J.C. DNA double strand breaks and the RAD50-57 genes in Saccharomyces. Seminars in Cancer Biol. 4: 73-83 (1993).

Hall, E.J. Radiobiology for the Radiobiologist, Third Edition. J.B. Lippincott Company, Philadelphia (1988).

Hannan, M.A. S.G. Zimmer and J. Hazle. Mechanisms of cisplatin (cis-diamminedichloroplatinum II)-induced cytotoxicity and genotoxicity in yeast. Mutat. Res. 127: 23-30 (1984).

Hassard, T.H. Understanding Biostatistics. Mosby-Year Book Inc. St. Louis, Missoure (1991).

Hays, S.L., A.A. Firmenlich and P. Berg. Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55 and Rad57 proteins. Proc. Natl. Acad. Sci. 92: 6925-6929 (1995).

Hill, B.T. S.A. Shellard, A.M.J. Fichtinger-Schepman, H.J. Schmoll and A. Harstrick. Differential formation and enhanced removal of specific cisplatin-DNA adducts in two cisplatin-selected resistance human testicular teratoma sublines. Anti-Cancer Drugs 5:321-328 (1994).

Jones, J.C., W. Zhen, E. Reed, R.J. Parker, A. Sancar and V.A. Bohr. Gene-specific formation and repair of cisplatin intrastrand adducts and interstrand cross-links in Chinese hamster ovary cells. J. Biol. Chem. 266:7101-7107 (1991).

Kelsey, K.T., A. Menusoglu, D. Frenkel and H.L. Liber. Human lymphocytes exposed to low doses of X-rays are less susceptible to radiation-induced mutagenesis. Mutat. Res. 263: 197-201 (1991).

Kiefer, J. and J. Muller. Yeast as a model system to study DNA repair. In Yeast Molecular Biology and Biotechnology (R. Prasad Ed.) pp 172-187 (1990).

Korbelik, M. and K.A. Skov. Inactivation of hypoxic cells by cisplatin and radiation at clinically relevant doses. Radiat. Res. 119: 145-156 (1989).

Larminat, F., W. Zhen and V.A. Bohr. Gene-specific DNA repair of interstrand cross-links induced by chemotherapeutic agents can be preferential. J. Biol. Chem. 268: 2649-2654 (1993).

Lehnert, S. and T.Y.K. Chow. Low doses of ionizing radiation induce nuclear activity catalyzing double strand homologous DNA recombination. Abstract P14-262, 44th Annual Meeting of the Radiation Research Society, Chicago, Illinois (April 1996).

Lippard, S.J. New chemistry of an old molecule: cis-$[Pt(NH_3)_2Cl_2]$. Science 218: 1075-1082 (1982).

Mitchel, R.E.J. and D.P. Morrison. Heat shock induction of ionizing radiation resistance in *Saccharomyces cerevisiae*. Transient changes in growth cycle distribution and recombinational ability. Radiat. Res. 92: 182-187 (1982).

Mitchel, R.E.J. and D.P. Morrison. Inducible DNA repair systems in yeast. Competition for lesions. Mutat. Res. 183:149-159 (1987).

Overgaard, J. and A.R. Khan. Selective enhancement of radiation response in a C3H mammary carcinoma by cisplatin. Cancer Treat. Rep. 65: 501-503 (1981).

Plooy, A.C.M., M. van Dijk, F. Berends and P.H.M. Lohman. Formation and repair of DNA interstrand cross-links in relation to cytotoxicity and unscheduled DNA synthesis induced in control and mutant human cells treated with cis-diamminedichloroplatinum(II). Cancer Res. 45: 4178-4184 (1985).

Resnick, M.A. The repair of double-strand breaks in DNA: A model involving recombination. J.Theor. Biol. 59: 97-106 (1976).

Richmond, R.C. and E.L. Powers. Radiation sensitization of bacterial spores by cis-dichlorodiammineplatinum(II). Radiat. Res. 68:251-257 (1976).

Richmond, R.C., J.D. Zimbrick and D.L. Hykes. Radiation-induced DNA damage and lethality in *E. Coli* as modified by the antitumor agent cis-dichlorodiammineplatinum(II). Radiat. Res. 71: 447-460 (1977).

Sancar, A. Mechanisms of DNA excision repair. Science 266:1954-1956 (1994).

Sancar, A. and G.B. Sancar. DNA repair enzymes. Ann. Rev. Biochem. 57: 29-67 (1988).

Schwachofer, J.H.M., R.P.M.A. Crooijmans, J. Hoohenhout, H.B. Kal and A.G.M. Theeuwes. Effectiveness in inhibition of recovery of cell survival by cisplatin and carboplatin: Influence of treatment sequence. Int. J. Radiation Oncology Biol. Phys. 20:1235-1241 (1991).

Shadely, J.D. and G. Dai. Low dose X-ray-induced cytogenetic adaptive response in human lymphocytes. In Low Dose Irradiation and Biological Defense Mechanisms (T. Sugahara, L.A. Sagan and T. Aoyama, Eds.) pp283-286 Elsevier Science Publishers (1992).

Sibghat-Ullah, H. Intisar, W. Carlton and A. Sancar. Human nucleotide excision repair in vitro: Repair of pyrimidine dimers, psoralen and cisplatin adducts by HeLa cell-free extract. Nuc. Acids Res. 17:4471-4484 (1989).

Skov, K. and S. MacPhail. Interaction of platinum drugs with clinically relevant X-ray doses in mammalian cells: A comparison of cisplatin, carboplatin, iproplatin, and teraplatin. Int. J. Radiation Oncology Biol. Phys. 20: 221-225 (1991).

Skov, K., Mathews J. and H. Zhou. Further studies at low doses: factors relevant to hypersensitivity and increased resistance. Abstract P14-265, 44th Annual Meeting of the Radiation Research Society, Chicago, Illinois (April 1996).

Szostak, J.W., T.L. Orr-Weaver, R.J. Rothstein and F.W. Stahl. The double-strand break model for recombination. Cell 33: 25-35 (1983).

Ward, J.F. DNA damage produced by ionizing radiation in mammalian cells: Identities, mechanisms of formation, and reparability. Progress in Nucleic Acid Research and Molecular Biology 35: 95-125 (1988).

Wilkins, D.E., C.E. Ng and G.P. Raaphorst. Cisplatin and low dose rate irradiation in cisplatin resistant and sensitive human glioma cells. Int. J. Radiat. Oncol. Biol. Phys. 36: 105-111 (1996).

Wilkins, D.E., D.P. Heller and G.P. Raaphorst. Inhibition of potentially lethal damage recovery by cisplatin in a brain tumor cell line. Anticancer Res. 13: 2137-2142 (1993).

Wodinsky, I., J. Swiniarski, C.J. Kensler and J.M. Venditti. Combination radiotherapy and chemotherapy for P388 lymphocytic leukemia in vivo. Cancer Chemotherapy Rep. 4: 73-97 (1974).

Wojick A. and C. Streffer. Adaptive reponse to ionizing radiation in mammalian cells: a review. Biol. Zent. bl. 113: 417-434 (1994).

Wolff, S. Low-dose exposures and the induction of adaption. In Low Dose Irradiation and Biological Defense Mechansims (T. Sugahara, L.S. Sagan and T. Aoyama, Eds.) pp21-28 Elsevier Science Publishers (1992).

Wood, R. D. DNA repair in eukaryotes. Annu. Rev. Biochem. 65: 135-167 (1996).

Yang, L., E.B. Douple, J.A. O'Hara and H-J. Wang. Production of DNA double-strand breaks by interactions between carboplatin and radiation: a potential mechanism for radiopotentiation. Radiat. Res. 143: 309-315 (1995).

Yang. L., E.B. Douple and H-J. Wang. Irradiation enhances cellular uptake of carboplatin. Int. J. Radiat. Oncol. Biol. Phys. 33: 641-646 (1995).

Yang, L., E.B. Douple, J.A. O'Hara and H-J. Wang. Carboplatin enhances the production and persistence of radiation-induced DNA single-strand breaks. Radiat. Res. 143: 302-308 (1995).

Zak, M. and J. Drobnik. Effect of cis-dichlorodiammine platinum (II) on the post-irradiation lethality in mice after irradiation with X-rays. Strahlentherapie 142: 112-115 (1971).

Zwelling, L. A., K. W. Khon, W.E. Ross, R. A. G. Ewig and T. Anderson. Kinetics of formation and disappearance of a DNA cross-linking effect in mouse leukemic L1210 cells treated with cis and trans-diamminedichloroplatinum (II). Cancer Res. 38: 1762-1768 (1978).

Chapter 1. Modulation of Radiation-Induced Strand Break Repair by Cisplatin in Mammalian Cells

Introduction

Cisplatin, a chemotherapeutic drug, is used in combination with ionizing radiation to treat cancer and has been studied in both the laboratory and clinical setting. In vitro studies using mammalian cells have shown that cisplatin can sensitize cells to the killing effect of radiation. This has been demonstrated as a decrease in the shoulder portion of radiation survival curves (Alvarez et al. 1978, Begg 1987, Douple 1990) and/or an increase in the slope of the exponential portion (Alvarez et al. 1978, Carde and Laval 1981). Cisplatin has also been shown to reduce the sparing effect of split dose radiation exposures (Dritschilo et al. 1979, Schwachofer et al. 1991) and low dose rate irradiation (Wilkins et al. 1996, Raaphorst et al. 1996) and inhibit repair of potentially lethal damage (Carde and Laval 1981, Wilkins et al. 1993).

The mechanism(s) by which cisplatin potentiates the lethal effects of radiation has not been established. It has been demonstrated that carboplatinum, a second-generation cisplatin analogue, used in combination with radiation resulted in an increased yield of DNA double strand breaks four hours after treatment compared to irradiated control cells (Yang et al. 1995). A DNA double strand break resulting from excision repair of a platinum-DNA adduct in the vicinity of a radiation-induced single strand break on the opposite strand of DNA was suggested as a potential mechanism to account for the enhanced production of double strand breaks (Chadwick et al.1976, Yang et al. 1995). Another possibility was that platinum-DNA lesions may have interfered with the repair of radiation-induced single or double strand breaks (Chadwick et al. 1976, Yang et al. 1995). The latter has been observed for other drugs such as 1-ß-D-arabinofuranosylcytosine or diamide which are known to interfere with repair processes, and treatment of cells with these chemicals resulted in enhanced

radiosensitivity (Ward et al. 1984) Carboplatinum was also found to increase the number of radiation-induced single strand breaks caused by 10, 20 and 40 Gy radiation exposures, and retard the repair of single strand breaks after a 40 Gy exposure when present during the repair period (Yang et al. 1995). It is unclear however, that after 40 Gy, when virtually all cells are reproductively dead, whether the yield of single strand breaks is indicative of inhibited or inactivated repair processes that would otherwise be active in viable cells.

It is also not known whether the inhibitory effect of platinum-lesions on repair of radiation-induced strand breaks persists over time. It has been shown that maximal radiosensitization by cisplatin occurs when cells are treated with cisplatin either shortly before or immediately after exposure to radiation although these observations are not universally supported in the literature (reviewed in Dewitt 1987, Douple 1990). In contrast, separating the radiation exposure from cisplatin treatment by four to six hours was shown to result in increased survival in the initial portion of radiation survival curves for mammalian cells (Skov et al. 1996), and a 24 hour time interval in between the treatments led to enhanced survival of mice to radiation when compared to mice which only received a radiation exposure (Zak and Drobnick 1971).

This chapter investigates the repair of radiation-induced DNA lesions in viable cells in the presence of cisplatin-DNA adducts and studies the persistence of DNA repair inhibition by cisplatin.

Materials and Methods

Cell line and culture conditions. Normal human diploid skin fibroblasts (AG1522, passage 9) were obtained from NIH Aging Cell Repository. Cells were grown attached in flasks containing D-MEM/F-12 media (GIBCO) supplemented with 15% fetal calf serum, 2 mM L-glutamine and 25 µg/ml gentamicin (GIBCO) and incubated at 37° C in a humidified atmosphere of 98% air, 2.0% CO_2.

Prior to irradiations, cells were grown to confluence. Cells were seeded into T-25 tissue culture flasks at 2 X 10^5 cells per flask. The media was changed five and eight days after seeding, and experiments were performed on day 10. For experiments in which cisplatin treatment occurred 24 hours prior to irradiation, irradiations were carried out on day 11. At the time of irradiation, the cell cycle distribution was 88% ± 2% G_0/G_1, 6% ± 1% S, 7% ± 1% G_2/M as determined by flow cytometric analysis.

Irradiation. Confluent phase cell cultures were ^{60}Co gamma-irradiated (Atomic Energy of Canada Limited Gamma Cell 200) as monolayers at a dose rate of 0.02 Gy/s. Irradiations were carried out at 0°C by filling the tissue culture flasks with 0°C phosphate buffered saline (PBS - 0.137M NaCl, 0.00268M KCl, 0.0064M $Na_2HPO_4.7H_2O$, 0.00147M KH_2PO_4, pH 7.4) and submerging flasks in an ice water bath before and immediately after the exposure. Unirradiated control flasks were also filled with 0°C PBS and held in ice water.

Cisplatin treatment. Cisplatin (Bristol Myers Squibb) was diluted in complete media. Cells were treated with 4.0 ml of media containing cisplatin and the flasks were submerged in a 37°C covered, circulating water bath for 30 minutes. At the end of exposure the media was removed and the cells washed three times in 37°C PBS. For experiments in which cells were incubated at 37°C after drug treatment, cisplatin-free media was added to culture flasks. Untreated control flasks were handled in a similar fashion.

Detection of DNA strand breaks. After irradiation, the 0°C PBS in the tissue culture flasks was replaced with 4.0 ml of complete media at 37°C and the flasks were submerged in a 37°C water bath for repair times ranging from two to 60 minutes. For longer repair times of 90 and 135 minutes, flasks were initially warmed to 37°C for 10 minutes in a water bath and then transferred to a 37°C humidified incubator (98% air, 2% CO_2). At the end of incubation at 37°C, flasks were filled with 0°C PBS and placed in an ice water bath. The cells were then rinsed twice in 0°C PBS, incubated for two minutes at 37°C with 1.0 ml of 1X

trypsin-EDTA solution (Sigma), suspended in 10 ml of complete media at 0°C, returned to an ice water bath and prepared for analysis of strand breaks. Strand breaks were detected using the Fluorometric Analysis by DNA Unwinding (FADU) assay (Birnboim and Jevcak 1981). Briefly, cell samples were centrifuged at 0°C (200 Xg, 10 minutes) and resuspended in 3.0 mL of 0°C inositol (0.25 M mesoinositol, 10 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.2). Aliquots of cell suspension (200 µL) was placed into three sets of four 16 x 100 mm cold glass test tubes designated P (partial fluorescence), B (blank) and T (total fluorescence) samples. Cells in all samples were lysed at 0°C by addition of 200 µL of urea solution (9 M urea, 10 mM NH_4OH, 2.5 mM cyclohexanediaminetetraacetate, 0.1% sodium dodecyl sulfate). P samples were then subjected to an unwinding solution (100 µl of 55% urea solution in 0.2N NaOH, 100 µl of 45% urea solution in 0.2N NaOH, pH 12.8) for 30 minutes at 0°C followed by 60 minutes at 15°C. During this alkali treatment, the DNA unwinds at a rate which is proportional to the number of strand breaks. The unwinding of P samples was stopped by the addition of a neutralizing solution (1 M glucose, 14 mM mercaptoethanol) and samples were sonicated (Branson Sonifier) for 15 seconds. B samples were treated with unwinding solution, sonicated and left to totally unwind for 30 minutes at room temperature before addition of the neutralizing solution. DNA in T samples is prevented from unwinding by simultaneous addition of the neutralizing and unwinding solutions prior to sonication. Ethidium bromide (1.5 ml of 100 µg/ml) was then added to all tubes and the fluorescence was measured using a spectrofluorimeter (520 nm excitation, 590 nm emission). The fluorescence from treated and untreated samples was compared and the relative strand breakage was expressed in Q_d units, where $Q_d = -100 \log (D/D_c)$. D and D_c are the percentages of double stranded DNA (as determined fluorometrically) remaining in treated and untreated control samples respectively.

Micronucleus assay. The micronucleus assay was first used by Evans et al (1959) to quantitate chromosomal damage caused by ionizing radiation. A modified micronucleus (MN) assay was developed (Fenech and Morley 1985) to provide a more sensitive and accurate measure of MN induction. The modification utilized cytochalasin B, an inhibitor of actin polymerization, to inhibit cytokinesis but not karyokinesis. Cytochalasin B by itself does not induce micronuclei (Fenech and Morley 1985, Prosser et al. 1988). This cytochalasin B micronucleus assay was employed in the present study as described below. Immediately after irradiation (4 Gy) at 0°C, cells were trypsinized and cell suspensions were counted using an electronic cell counter and 4 X 10^4 cells in 5 mL of complete media (at 37°C) were pipetted into chambered slides (GIBCO). Cytochalasin B (1 μg/ml final concentration) (Sigma) was added immediately and cultures were incubated for 48 hours. After 48 hours, cells were washed twice with 37°C PBS. The cells were then incubated for five minutes with 2 ml of 1% sodium citrate solution (w/v) at 37°C. After the hypotonic treatment, an equal volume of fix (3:1 methanol: glacial acetic acid) at room temperature was added and left for five minutes at room temperature. This solution was replaced with 5 mL of fix for another five minutes. The fix was removed and slides were air dried. A mixture of propidium iodide/ antifade (0.2 μg/mL) and 4,6-diamino-2phenylindole (DAPI)/ antifade (0.4 μg/mL) (Vysis) was applied and the slides were coverslipped. Under UV light (546 nm excitation, 590 nm emission) DNA within main nuclei and micronuclei appeared blue and the cytoplasm appeared pale red. Criteria used for identifying and scoring micronuclei were those outlined by Fenech (1993).

Statistical analysis. All results are data combined from at least three experiments. Student's T-test and one-way analysis of variance were used to determine whether results obtained after the various treatments were different from one another and p-values are stated when data were significantly different.

Results

Effect of cisplatin on initial level of radiation-induced strand breaks. The level of initial DNA damage, as measured by the FADU assay was 28.8 ± 1.7 and 27.5 ± 3.0 Qd units for cells treated with cisplatin immediately (10 µg/ml, 30 min., 37°C) before irradiation and cells exposed to 4 Gy radiation alone, respectively. These values are not significantly different. For cells treated with cisplatin (10 µg/ml, 30 min., 37°C) 24 hours prior to irradiation, the level of DNA strand breakage was 31.1 ± 2.5 Qd units. This was not significantly different from their control cells (31.9 ± 1.8 Qd units) which were exposed to 4.0 Gy radiation alone.

Effect of cisplatin on repair of DNA strand breaks. The repair of DNA strand breaks caused by exposure to a 4 Gy dose of ^{60}Co gamma radiation is shown in Figure 1.1. The rejoining of strand breaks was biphasic with an initial fast rejoining component (up to 15 minutes of repair time) followed by a slower component after 15 minutes of repair to completion of rejoining. Cisplatin treatment (10 µg/ml, 30 minutes: 10.2%±2.1% survival) before irradiation had no effect on the fast rejoining component but inhibited the slow component ($p<0.01$). To a lesser extent, exposure of cells to cisplatin during the course of repair also resulted in an inhibition of the slow repair component ($p<0.01$).

The inhibitory effect of cisplatin on the repair of radiation-induced DNA strand breaks after 60 minutes of repair increased as the drug concentration was increased from 0.1 µg/ml to 1.0 µg/ml (Figure 1.2). The effect leveled off at about 1.0 µg/ml with no significant further inhibition at 10 µg/ml. At the highest concentration of cisplatin (10 µg/ml, 30 minutes) the inhibitory effect persisted and was evident at 24 hours after cisplatin exposure (Figure 1.3). This cisplatin treatment 24 hours prior to irradiation inhibited both the slow- and fast-rejoining of DNA strand breaks (Figure 1.3).

Exposure of cells to a mild cisplatin treatment (1 µg/ml, 30 min.: 95.3%±3.4% survival) 24 hours prior to irradiation did not retard the repair of

DNA strand breaks (Figure 1.4). In contrast, the amount of residual unrepaired DNA damage was less in cells treated with cisplatin 24 hours prior to radiation as compared to control cells. There was significantly less damage after 5, 15 and 45 minutes of repair ($p<0.05$).

Effect of cisplatin on radiation-induced micronucleus formation. The frequency of radiation-induced micronuclei following exposure of AG1522 cells 10 days after seeding was not significantly different from that observed when cells were irradiated 11 days after seeding (Figure 1.5A). Cisplatin treatment alone (0.1 or 1.0 µg/ml, 30 min.) did not induce formation of micronuclei above the spontaneous level of 0.042 ± .005 per binucleate cell. Cisplatin treatment (1 µg/ml, 30 minutes) immediately before exposure to radiation on day 10, resulted in fewer binucleate cells without micronuclei and more binucleate cells containing three or more micronuclei ($p<0.05$) (Figure 1.5B). In contrast, the same cisplatin treatment (1 µg/ml, 30 minutes) 24 hours prior to radiation produced the opposite result, a decreased number of radiation-induced micronuclei (Figure 1.5C). Under these conditions, there were significantly more binucleate cells without micronuclei when compared to cells which were not pretreated with cisplatin ($p<0.01$) and significantly less binucleate cells with two micronuclei ($p<0.05$). A similar result was obtained for cells treated with 0.1 µg/ml (100% survival) 24 hours before exposure to radiation (Figure 1.5D).

Effect of cisplatin on binucleate cell formation after exposure to radiation. Forty-eight hours after addition of cytochalasin B, 24.9% ± 2% of unirradiated control cells were binucleate (Figure 1.6). Cell cultures exposed to 4 Gy radiation or 1 µg/ml (but not 0.1 µg/ml) cisplatin showed significantly less binucleate cell formation ($p<0.01$). Combined treatments of radiation and cisplatin (1.0 or 0.1 µg/ml) did not result in a significant change in the binucleate cell frequency compared to radiation alone (Figure 1.6).

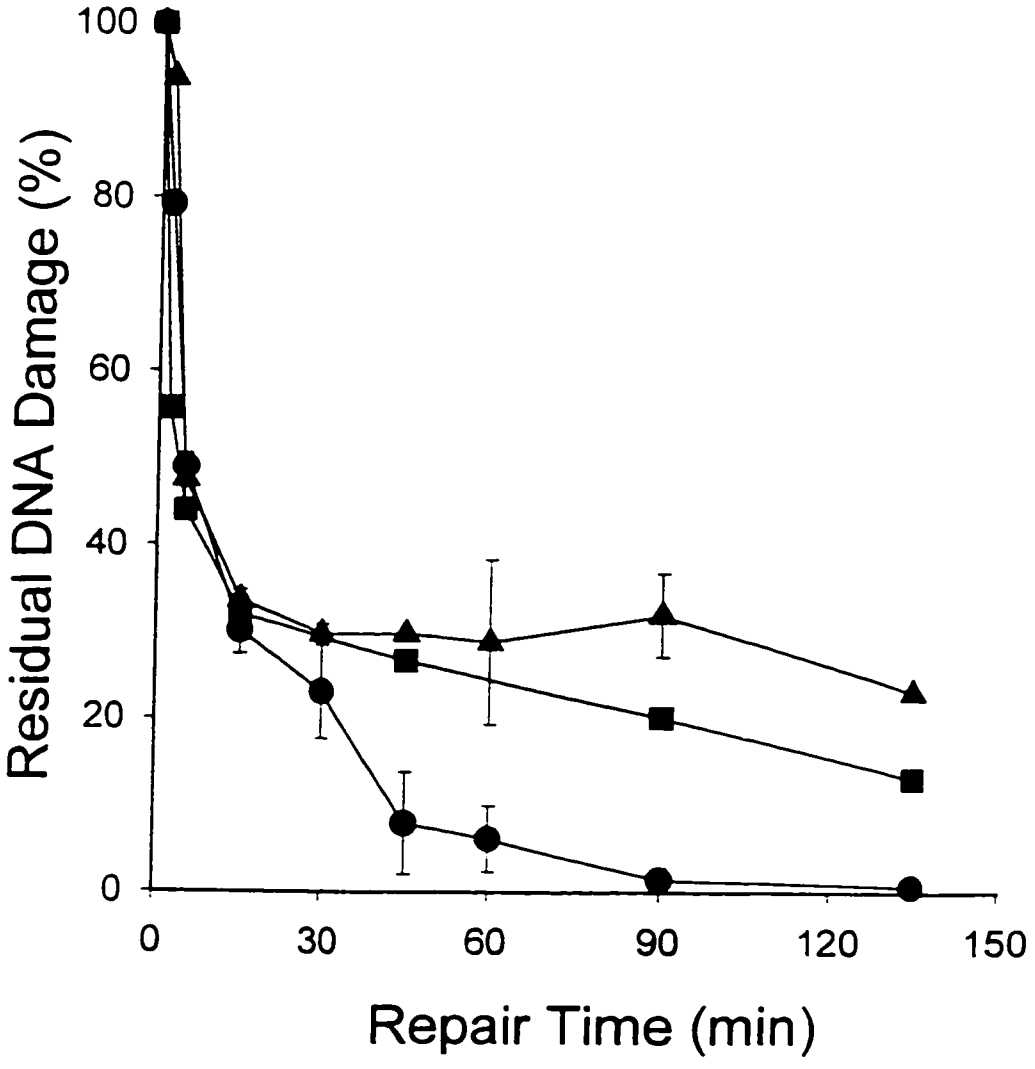


Figure 1.1. DNA strand break repair in AG1522 cells exposed to 4Gy of ^{60}Co-gamma radiation. Cells were treated with 10 µg/ml cisplatin at 37°C for 30 minutes immediately before irradiation (triangles) or continuously during repair time (squares). Control cells not treated with cisplatin are represented by circles.

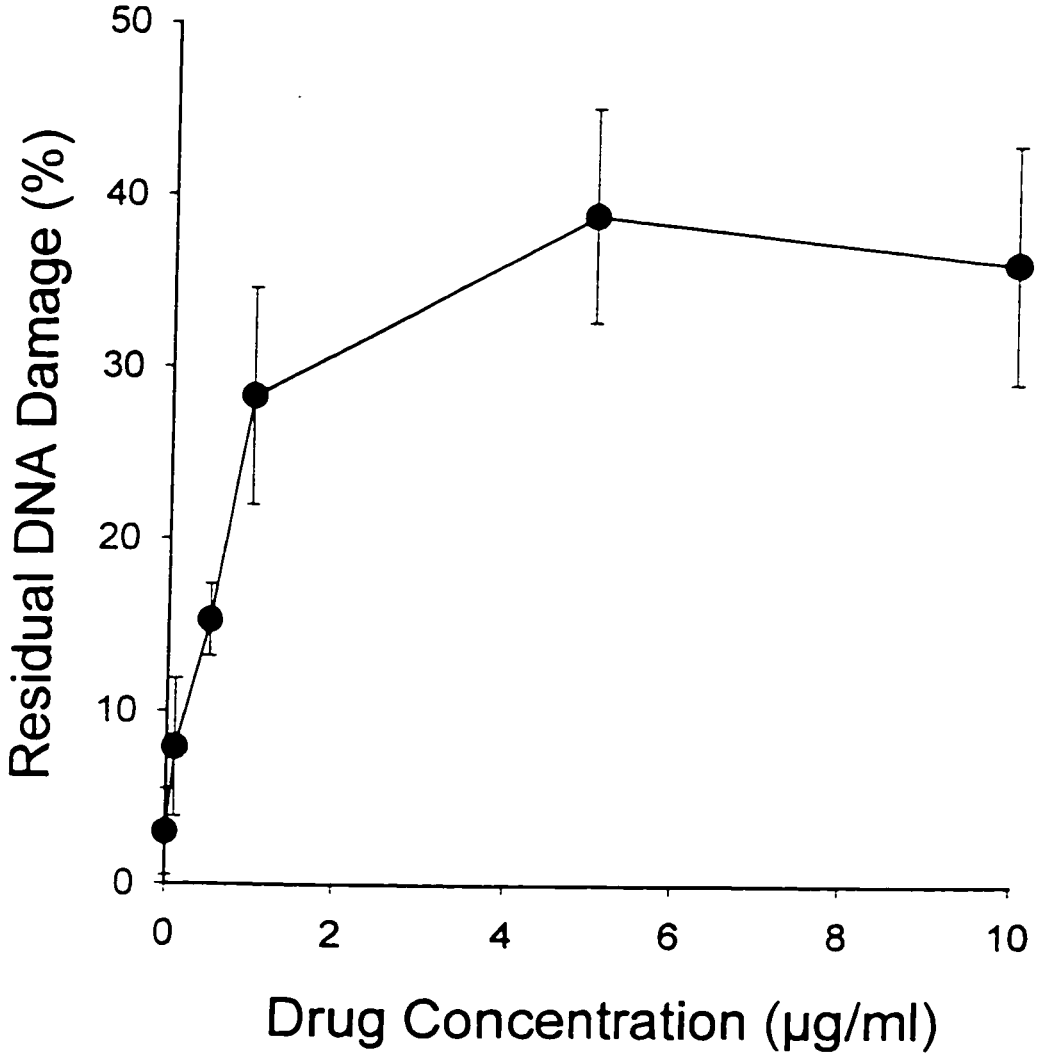


Figure 1.2. DNA damage remaining in AG1522 cells after 60 minutes of repair at 37°C following exposure to 4 Gy ^{60}Co-gamma radiation. Cells were treated with increasing concentrations of cisplatin (for 30 minutes at 37°C) immediately before exposure to radiation.

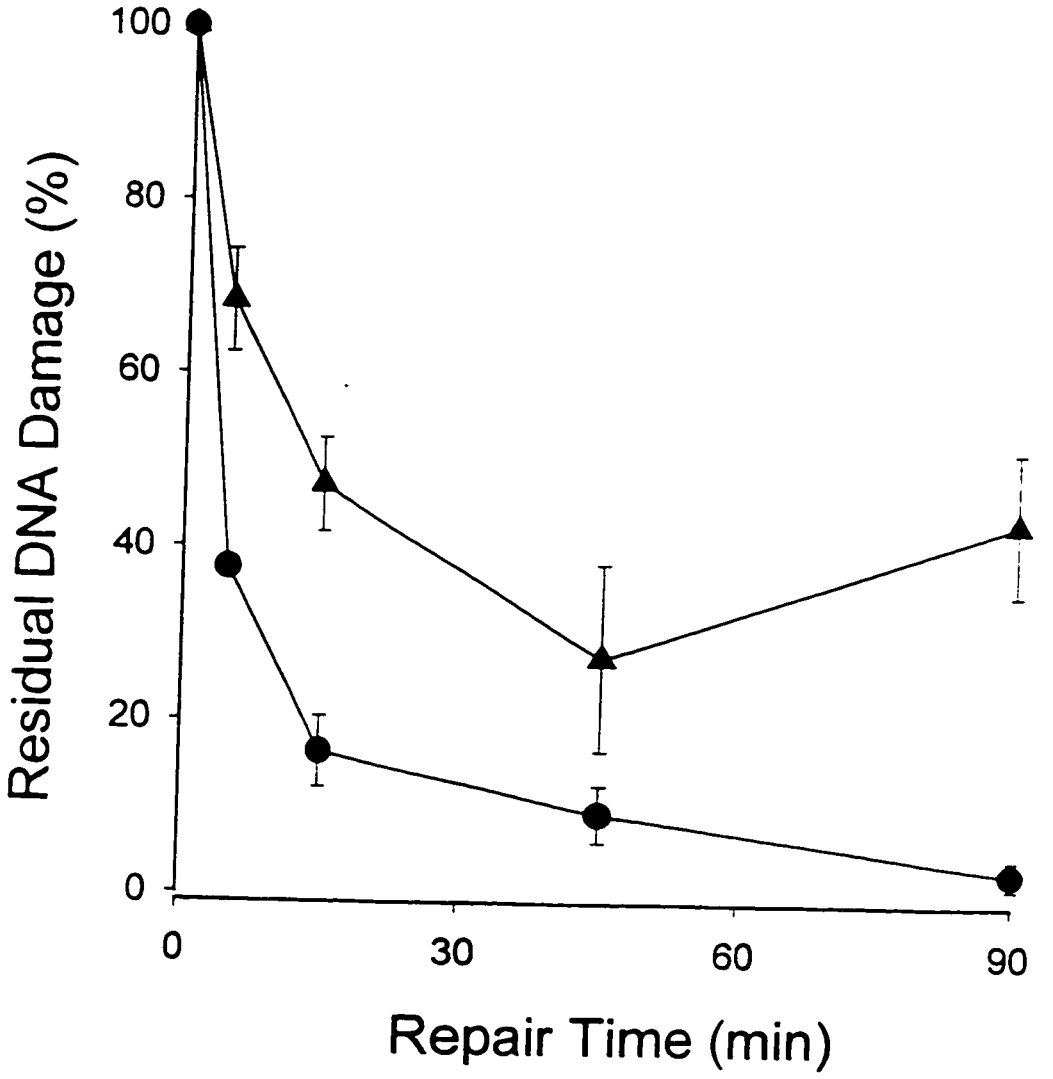


Figure 1.3. DNA strand break repair in AG1522 cells. Cells were treated with10 µg/ml of cisplatin (for 30 minutes at 37°C) 24 hours before (triangles) exposure to 4 Gy ^{60}Co-gamma radiation. Control cells (circles) were not treated with cisplatin before irradiation.

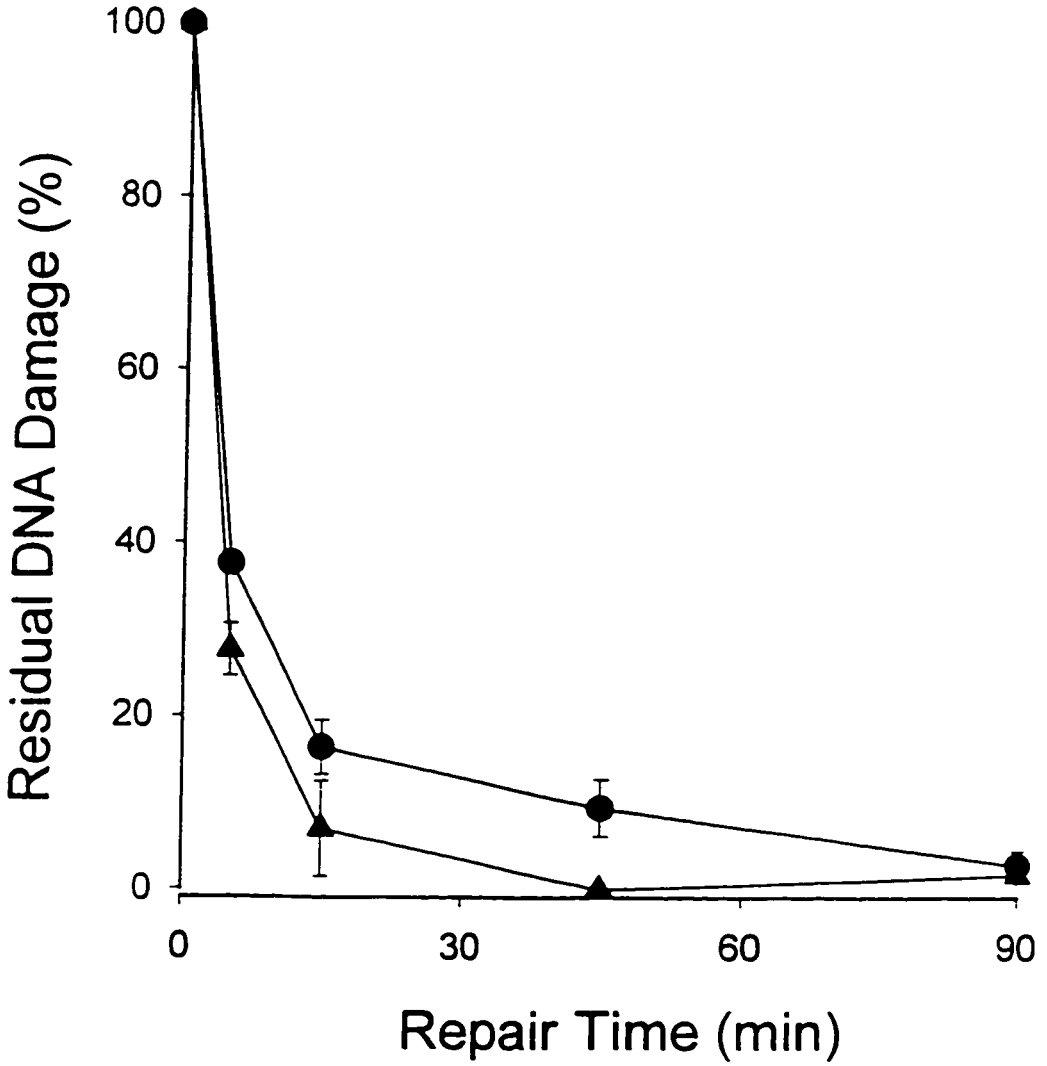


Figure 1.4. DNA strand break repair in AG1522 cells. Cells were treated with1 µg/ml of cisplatin (for 30 minutes at 37°C) 24 hours before (triangles) exposure to 4 Gy ^{60}Co-gamma radiation. Control cells (circles) were not treated with cisplatin before irradiation.

Discussion

DNA is considered to be the critical cellular molecule damaged by ionizing radiation (Hall 1988, Elkind 1985). Exposure to radiation causes a variety of DNA lesions including single and double strand breaks (Frankenberg-Schwager 1989, Ward 1988). The rejoining kinetics for radiation-induced total DNA strand breaks in a variety of mammalian cell lines has been compiled (Dikomey and Franzke 1986). Taken together, the data show three components of strand break repair. The first component represents rejoining of 70 - 90% of all single strand breaks with a $t_{1/2}$-value between two and 10 minutes. The second component is thought to comprise single strand break rejoining and formation of new single strand breaks due to excision of damaged bases, with a $t_{1/2}$-value of 10 minutes and more. The third component is thought to represent rejoining of DNA double strand breaks with a $t_{1/2}$-value of greater than one hour (reviewed in Frankenberg-Schwager 1989). In the present study, the rejoining of strand breaks in AG1522 cells caused by 4 Gy ^{60}Co gamma radiation appeared to be biphasic with an initial fast component (up to 15 minutes of repair time) followed by a slower component after 15 minutes of repair to completion of rejoining (Figure 1.1, closed circles). These results are not inconsistent with the published data as it is possible that the fast component encompasses single stand break repair and formation of new breaks, and the slow component represents mainly the repair of DNA double strand breaks. To delineate more precisely the various components of the rejoining kinetics, more data is required.

Cisplatin treatment immediately before irradiation had no effect on the fast-rejoining component but inhibited the slow component (Figure 1.1). Single strand breaks are thought not to contribute significantly to cell lethality, mainly because they are repaired rapidly and efficiently (Frankenberg-Schwager 1989, Ward 1988). However, it has been postulated that excision of a platinum-DNA adduct, in the vicinity of a single strand break in the opposite strand, would

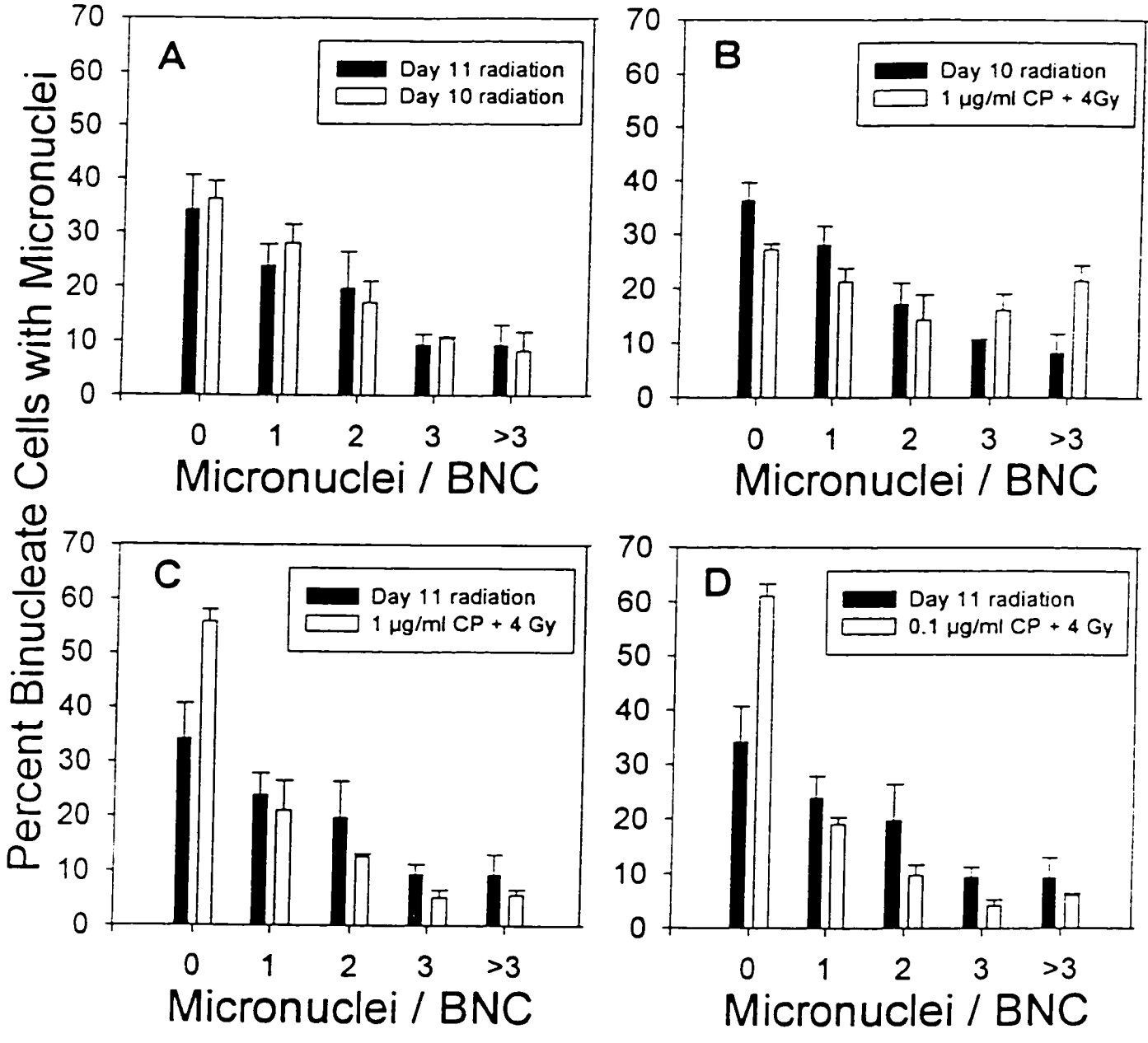


Figure 1.5. Micronucleus frequency in AG1522 cells after exposure to 4 Gy ^{60}Co gamma radiation with or without a cisplatin (CP) treatment. Irradiation of cells 10 days after seeding cells is compared to 11 days post seeding (Panel A). Cells were treated with cisplatin immediately before irradiation (Panel B), or 24 hours prior to irradiation (Panels C,D).

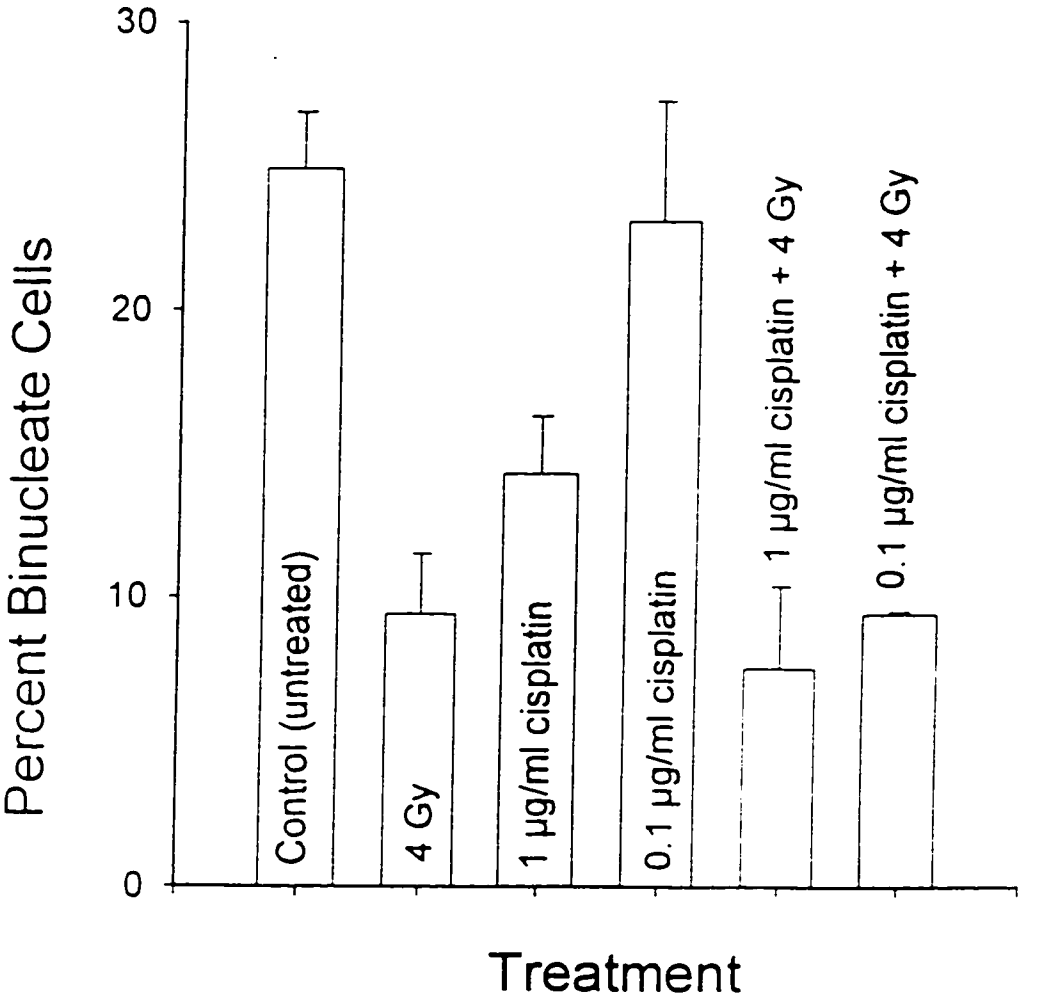


Figure 1.6. Formation of binucleate cells 48 hours after treatment. Cells were treated with cisplatin (30 minutes at 37°C) 24 hours prior to radiation.

create a potentially lethal double strand break which requires more time to repair (Chadwick et al. 1976, Yang et al. 1995, Douple 1990). These double strand breaks may also be more difficult to repair than double strand breaks caused by radiation, because the excision repair machinery processing the cisplatin adduct may block recombinational repair protein access to the nascent double strand breaks. Exposure of cells to cisplatin during the course of repair also inhibited the slow component of strand break repair but to a lesser extent (Figure 1.1). This diminished inhibitory effect may have been due to the amount of single-strand break repair achieved before the formation of reactive cisplatin species, the binding of reactive cisplatin to DNA and the formation of potentially lethal double strand breaks via the excision repair mechanism (Dewitt 1987).

As shown in Figure 1.2, the inhibitory effect of cisplatin on the repair of radiation-induced strand breaks, measured at 60 minutes of repair time, increased as the drug concentration increased. The effect leveled off with no further inhibition above 1.0 - 5.0 μg/ml cisplatin, indicating that for the number of strand breaks induced by 4.0 Gy (approximately 4000 single- and 160 double strand breaks), all remaining at 60 minutes of repair time may have been in close proximity to repair-inhibiting cisplatin adducts and were consequently more difficult to rejoin.

It is also possible that cisplatin molecules may have indirectly influenced the amount of DNA damage caused by radiation by binding to and scavenging intracellular thiols such as glutathione. Thiols protect cells from radiation-induced damage by scavenging free radical species which would otherwise damage DNA. The depletion of glutathione by cisplatin could therefore lead to a reduction in chemical repair of radiation-induced DNA lesions and a net increase in the number of DNA lesions to be repaired enzymatically (Yang et al. 1995, Odenheimer and Wolf 1982). Based on the experiments presented here, however, depletion of intracellular thiols by cisplatin is probably not a major factor in radiosensitization since the amount of initial DNA strand breaks caused

by radiation, as measured by the FADU assay, was not significantly different in cells pretreated with cisplatin compared to control cells which were exposed only to radiation. The similar levels of initial DNA damage in control and cisplatin treated cells also indicates that the overall distribution of radiation-induced DNA damage was unchanged by cisplatin adducts even though at sites of cisplatin attachment a change in DNA topology may have created sites that were more or less susceptible to attack by radiation-induced free radicals.

The inhibitory effect of cisplatin adducts on the repair of radiation-induced strand breaks is further demonstrated in Figure 1.3. Cisplatin treatment (10 µg/ml, 30 minutes) 24 hours prior to irradiation inhibited both the slow- and fast-rejoining of strand breaks (Figure 1.2), whereas cisplatin treatment immediately before irradiation only inhibited the slow component of repair (Figure1.1). It has been shown that with short incubation times (30 minutes or less at 37°C), cisplatin monofunctional adducts are a dominant DNA lesion (Eastman 1986), but with increasing time, monofunctional adducts rearrange to bifunctional adducts. The rate of cisplatin binding to DNA is governed by the rate of hydrolysis of the chloride ligands (Bruhn et al. 1990). At 37°C, the reported half life for initial binding of cisplatin to DNA and closure of the monofunctional to bifunctional adduct is 1.9 and 2.1 hours respectively. A 30 minute cisplatin treatment immediately before irradiation would, therefore, yield a significantly higher ratio of monofunctional to bifuntional adducts than a 24 hour incubation in between the cisplatin and radiation treatments. The results of the present study (Figure 1.1,1.3) therefore may indicate that bifunctional crosslinks (inter- and intrastrand DNA adducts, DNA-protein crosslinks) which are produced slowly over time (Jones et al. 1991) and repaired very slowly (Fraval and Roberts 1979, Dijt et al. 1988, Jones et al. 1991, Larminat et al. 1993, Hill et al. 1994) are able to inhibit the fast component of repair (repair of single strand breaks) and possibly also the slow component (double strand breaks), while monofunctional

cisplatin adducts are capable only of inhibition of the slow component of repair of radiation-induced DNA strand breaks.

Decreasing the cisplatin exposure to 1.0 μg/ml for 30 minutes, 24 hours prior to irradiation did not seem to inhibit the repair of radiation-induced single strand breaks (Figure 1.4). In contrast, at every repair time point analyzed, the amount of residual DNA damage was less (although not significant at all points) in cells treated with cisplatin 24 hours prior to irradiation as compared to control cells. It is possible that at this drug concentration, either all of the cisplatin- DNA adducts had been removed by the cellular excision and recombinational repair processes (Calsou and Salles 1993, Sancar and Sancar 1988, Hannan et al. 1984, Cole 1973) by the time of irradiation or, the cisplatin- DNA adducts were sparse enough so as not to interact with radiation-induced single strand breaks.

Alternatively, the number of cisplatin- DNA adducts produced by this cisplatin treatment may have invoked an "adaptive" cellular response whereby cells become resistant to the detrimental effects of an agent (e.g. radiation) via a prior treatment with another stress (e.g. cisplatin). Azzam et al. (1996, 1994) have demonstrated that adaptive responses to radiation exist in AG1522 fibroblast cells. AG1522 cells preexposed to between 0.1 and 4.25 Gy chronic gamma radiation (dose rate .0024 Gy/min) led to a significant reduction in the number of micronuclei induced by a subsequent challenge exposure of gamma radiation. Since micronuclei are mainly derived from acentric fragments which result from unrejoined DNA double strand breaks, the reduction of micronuclei in cells preexposed to radiation indicated induction of a DNA double strand break repair mechanism.

Cisplatin appeared to induce a similar cellular response (Figure 1.5C and 1.5D). Cisplatin treatment of cells 24 hours prior to irradiation resulted in a reduced number of radiation-induced micronuclei. At drug concentrations of 1 μg/ml and 0.1 μg/ml there were significantly more binucleate cells without micronuclei. It seems plausible, therefore, that cisplatin-DNA adducts signal the

induction of a DNA double strand break repair system. Taken together with results from the repair of strand breaks using the FADU assay (Figure 1.4), the data support the contention that exposure of cells to low concentrations of cisplatin led to an enhanced cellular capacity for strand break repair either by inducing transcription and/or translation of repair proteins, or by increasing the activity of constitutive repair proteins.

The adaptive response exhibited by AG1522 cells may also involve a cell cycle division delay during which DNA damage is repaired (Azzam 1995). Cisplatin pretreatment did not appear to cause a significant delay as measured by the frequency of binucleate cell formation. The same number of irradiated cells reached the binucleate cell stage whether of not they received a prior cisplatin treatment (Figure 1.6).

In summary, the time interval between a cisplatin treatment and a subsequent radiation exposure differentially influenced the response of AG1522 cells. Mild cisplatin treatments 24 hours prior to irradiation led to increased repair capacity and a reduction in unrepaired DNA double strand breaks. In contrast, cisplatin treatment immediately before radiation exposure resulted in radiosensitization by interfering with the repair of radiation-induced lesions.

Since cisplatin appears to interfere with repair of radiation-induced DNA damage, the combination of cisplatin and radiation may be most beneficial in cancer treatment regimes where low dose rate irradiation (brachytherapy) is utilized (Wilkins et al. 1996, Raaphorst et al. 1996).

References

Alvarez M.V., G. Cobreros, A. Heras and M.C. Lopez Zumel. Studies on cis-dichlorodiammineplatinum(II) as a radiosensitizer. Br. J. Cancer 37:68-72 (1978).

Azzam, E.I. Adaptive responses to ionizing radiation in normal human fibroblasts. Thesis, Ottawa, (1995)

Azzam, E.I., S.M. deToledo, G.P. Raaphorst and R.E.J. Mitchel. Low dose ionizing radiation decreases the frequency of neoplastic formation to a level below the spontaneous rate in C3H 10T1/2 cells. Radiat. Res. 146: 369-373 (1996).

Azzam, E.I., G.P. Raaphorst and R.E.J. Mitchel. Radiation-induced adaptive response for protection against micronucleus formation and neoplastic transformation in C3H 10T1/2 mouse embryo cells. Radiat. Res. 138: S28-S31 (1994).

Azzam, E.I., S.M. deToledo, G.P. Raaphorst and R.E.J. Mitchel. Reponse adaptativeau rayonnement ionisant des fibroblastes de peau humaine. Augmentation de la vitesse de reparation de l'AND et variation de l'expression des genes. J.Chim. Phys. 91: 931-936 (1994).

Begg, A.C. Additivity versus repair inhibition in fractionated treatments combining drugs and X-rays: A theoretical approach. Int. J. Radiation Oncology Biol. Phys. 13: 921-927 (1987).

Begg, A.C., P.J. Vander Kolk, L. Dewitt and H. Bartelink. Radiosensitization by cisplatin of RIF1 tumour cells *in vitro*. Int. J. Radiat. Biol. 50: 871-884 (1986).

Birnboim, H.C. and J.J. Jevcak. Fluorometric method for detection of DNA strand breaks in human white blood cells produced by low doses of radiation. Cancer Res. 41: 1889-1892 (1981).

Bruhn, S.L., J.H. Toney and S.J. Lippard. Biological processing of DNA modified by platinum compounds. Progress in Inorganic Chemistry: Bioinorganic Chemistry 38:477-517 (1990).

Calsou, P. and B. Salles. Role of DNA repair in the mechanisms of cell resistance to alkylating agents and cisplatin. Cancer Chemother. Pharmacol. 32:85-89 (1993).

Carde,P. and F. Laval. Effects of cis-dichlorodiammine platinum II and X-rays on mammalian cell survival. Int. J. Radiation Oncology Biol. Phys. 7:929-933 (1981).

Chadwick, K.H., H.P. Leenhouts, I. Szumiel and A.H.W. Nias. An analysis of the interaction of a platinum complex and radiation with CHO cells using the molecular theory of cell survival. Int. J. Radiat. Biol. 30: 511-525 (1976).

Cole, R.S. Repair of DNA containing interstrand crosslinks in *Escherichia coli*: Sequential excision and recombination. Proc. Nat. Acad. Sci. 70: 1064-1068 (1973).

Dewitt, L. Combined treatment of radiation and cisdiamminedichloroplatinum (II): A review of experimental and clinical data. Int. J. Radiation Oncology Biol. Phys. 13: 403-426 (1987).

Dijt, F.J., A.M. Fichtinger-Schepman. F. Berends and J. Reedijk. Formation and repair of cisplatin-induced adducts to DNA in cultured normal and repair-deficient human fibroblasts. Cancer Res. 48: 6058-6062 (1988).

Dikomey, E. and J. Franzke, J. DNA repair kinetics after exposure to X-irradiation and to internal β rays in CHO cells. Radiation and Environmental Biophysics 25: 189-194 (1986).

Douple, E.B. Interactions between platinum coordination complexes and radiation. In Antitumor Drug-Radiation Interactions (B.T. Hill and A.S. Bellamy, Eds.) pp171-190 CRC Press, Boca Raton, Florida (1990).

Dritschilo A., A.J. Piro and A.D. Kelman. The effect of cisplatinum on the repair of radiation damage in plateau phase Chinese hamster (V-79) cells. Int. J. Radiat. Oncol. Biol. Phys. 5: 1345-1349 (1979).

Eastman, A. Reevaluation of interaction of cis-dichloro(ethylenediammine)-platinum(II) with DNA. Biochem. 25: 3912-3915 (1986).

Elkind, M.N. DNA damage and cell killing. Cause and effect? Cancer 56: 2351-2362 (1985).

Evans, H.J., G.J. Neary and F.S. Williamson. The relative biological efficiency of single sodes of fast neutrons and gamma-rays on *Vicia faba* roots and the effect of oxygen. Part II Chromosome danage: the production of micronuclei. Int. J. Radiat. Biol. 3: 216-229 (1959).

Fenech M. and A. Morley. Solutions to the kinetic problem in the micronucleus assay. Cytobios. 43: 233-246 (1985).

Fenech, M. The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations. Mutat. Res. 285: 35-44 (1993).

Frankenberg-Schwager, M. Review of repair kinetics for DNA damage induced in eukaryotic cells in vitro by ionizing radiation. Radiotherapy and Oncology 14: 307-320 (1989).

Fraval, H.N.A. and J.J. Roberts. Excision repair of cis-diamminedichloroplatinum(II)-induced damage to DNA of Chinese hamster cells. Cancer Res. 39:1793-1797 (1979).

Hall, E.J. Radiobiology for the Radiobiologist, Third Edition. J.B. Lippincott Company, Philadelphia (1988).

Hannan, M.A. S.G. Zimmer and J. Hazle. Mechanisms of cisplatin (cis-diamminedichloroplatinum II)-induced cytotoxicity and genotoxicity in yeast. Mutat. Res. 127: 23-30 (1984).

Hill, B.T. S.A. Shellard, A.M.J. Fichtinger-Schepman, H.J. Schmoll and A. Harstrick. Differential formation and enhanced removal of specific cisplatin-DNA adducts in two cisplatin-selected resistance human testicular teratoma sublines. Anti-Cancer Drugs 5:321-328 (1994).

Jones, J.C., W. Zhen, E. Reed, R.J. Parker, A. Sancar and V.A. Bohr. Gene-specific formation and repair of cisplatin intrastrand adducts and interstrand cross-links in Chinese hamster ovary cells. J. Biol. Chem. 266:7101-7107 (1991).

Larminat, F., W. Zhen and V.A. Bohr. Gene-specific DNA repair of interstrand cross-links induced by chemotherapeutic agents can be preferential. J. Biol. Chem. 268: 2649-2654 (1993).

Odenheimer, B. and W. Wolf. Reactions of cisplatin with sulfur-contating amino acids and peptides I. Cysteine and glutathione. Inorganica Chimica Acta 66: 141-143 (1982).

Prosser, J.S., J.E. Moquet, D.C. Lloyd and A.A. Edwards. Radiation induction of micronuclei in human lymphocytes. Mutat. Res. 199: 37-45 (1988).

Raaphorst, G.P., G. Wang, D. Stewart and C.E. Ng. Concomittant low dose-rate irradiation and cisplatin treatment in ovarian carcinoma cell lines sensitive and resistant to cisplain treatment. Int. J. Radiat. Biol. 69: 623-631 (1996).

Sancar, A. and G.B. Sancar. DNA repair enzymes. Ann. Rev. Biochem. 57: 29-67 (1988).

Schwachofer, J.H.M., R.P.M.A. Crooijmans, J. Hoohenhout, H.B. Kal and A.G.M. Theeuwes. Effectiveness in inhibition of recovery of cell survival by cisplatin and carboplatin: Influence of treatment sequence. Int. J. Radiation Oncology Biol. Phys. 20:1235-1241 (1991).

Sibghat-Ullah, H. Intisar, W. Carlton and A. Sancar. Human nucleotide excision repair in vitro: Repair of pyrimidine dimers, psoralen and cisplatin adducts by HeLa cell-free extract. Nuc. Acids Res. 17:4471-4484 (1989).

Skov, K., Mathews J. and H. Zhou. Further studies at low doses: factors relevant to hypersensitivity and increased resistance. Abstract P14-265, 44th Annual Meeting of the Radiation Research Society, Chicago, Illinois (April 1996).

Ward, J.F. DNA damage produced by ionizing radiation in mammalian cells: Identities, mechanisms of formation, and reparability. Progress in Nucleic Acid Research and Molecular Biology 35: 95-125 (1988).

Ward, J.F., E.I. Joiner and W.F. Blakely. Effects of inhibitors of DNA strand break repair on HeLa cell radiosensitivity. Cancer Res. 44: 59-63 (1984).

Wilkins, D.E., C.E. Ng and G.P. Raaphorst. Cisplatin and low dose rate irradiation in cisplatin resistant and sensitive human gliome cells. Int. J. Radiat. Oncol. Biol. Phys. 36: 105-111 (1996).

Wilkins, D.E., D.P. Heller and G.P. Raaphorst. Inhibition of potentially lethal damage recovery by cisplatin in a brain tumor cell line. Anticancer Res. 13: 2137-2142 (1993).

Yang, L., E.B. Douple, J.A O'Hara and H-J. Wang. Production of DNA double-strand breaks by interactions between carboplatin and radiation: a potential mechanism for radiopoteniation. Radiat. Res. 143: 309-315 (1995).

Zak, M. and J. Drobnik. Effect of cis-dichlorodiammine platinum (II) on the post-irradiation lethality in mice after irradiation with X-rays. Strahlentherapie 142: 112-115 (1971).

Chapter 2. Cisplatin Sensitization of Yeast DNA Repair Mutants to the Killing Effect of Ionizing Radiation.

Introduction

The supra-additive cell killing effect of combined cisplatin and radiation has been observed in both prokaryotic (Richmond and Powers 1976, Richmond et al. 1977) and eukaryotic systems (Zak and Drobnik 1971, Wodinsky et al. 1974, Douple and Richmond 1978, Alvarez et al. 1978, Overgaard and Khan 1981, Korbelik and Skov 1989, Skov and MacPhail 1991, Raaphorst et al. 1996). The exact mechanism of radiosensitization by cisplatin has not been established, but it has been postulated that the supra-additive effect of cisplatin and radiation on cells is a consequence of cisplatin inhibition of repair of radiation-induced DNA damage.

The goal of this research was to use repair deficient yeast mutants to determine whether defects in repair processes could modify the radiosensitizing effect of cisplatin. A differential response for cell survival in repair mutants compared to wild type repair proficient cells would indicate that cisplatin radiosensitization may be a function of lesion processing and not lesion formation.

Two DNA repair pathways that are known to respond to radiation-induced DNA damage, and have been extensively characterized in yeast, are excision repair and recombinational repair (Kiefer and Muller 1991). The genes involved in excision and recombinational repair pathways belong to the Rad3 and the Rad52 epistasis groups, respectively. Rad3 mutants are UV-light sensitive and unable to remove pyrimidine dimers and other bulky adducts from their DNA (Unrau 1971, Kiefer and Muller 1991), whereas rad52 mutants are sensitive to ionizing radiation and deficient in meiotic and mitotic recombination. Rad52 mutants are also unable to repair DNA double strand breaks, resulting in an

extremely ionizing radiation sensitive phenotype (Frankenberg-Schwager and Frankenberg 1990).

The experiments in this work were designed to investigate the involvement of recombinational repair and/or excision repair processes in the mechanism of radiosensitization induced by cisplatin. Mutant yeast strains of rad52 (MS32) and rad3 (STX432) were treated with various combinations of radiation and cisplatin. Cisplatin-induced radiosensitization in both mutants was compared to wild type cells and the involvement of these two distinct DNA repair processes in the mechanism of radiosensitization was assessed.

Methods and Materials

Strains. Diploid strains of the yeast *Saccharomyces cerevisiae* were used as the test organisms in these experiments. Strain MS33 was wild type for DNA repair capacity (rec+/rec+, exc+/exc+). Strain MS32 was homozygous for rad52-1 and was defective in recombinational repair (rec-/rec-, exc+/exc+). Strain MS33 is the isogenic parental strain of MS32. Both MS33 and MS32 are homozygous for the his1-7 mutation. Strain STX432 was homozygous for rad3-2 and was defective in excision repair (rec+/rec+, exc-/exc). Yeast strains MS33 and MS32 were gifts from Dr. D.P. Morrison, AECL, Chalk River Laboratories. Strain STX432 was purchased from the Yeast Genetic Stock Center (Berkley, Ca.)

Cell Growth. Cell cultures were grown at 23°C to mid-exponential phase (2-5 x 10^6 cells/ml) in Yeast Extract / Succinate (YES) liquid nutrient medium containing 0.1% yeast extract, 1% yeast nitrogen base with amino acids, 1% sodium succinate, and 2% glucose.

Radiation Survival. For irradiation, yeast cells were washed three times and suspended (at 0°C) in 20 mmol dm^{-3} (mM) sodium phosphate buffer, pH 7.0, at approximately 2 x10^7 cell/ml. These suspensions were equilibrated by bubbling the cell suspension at 0°C with O_2 or N_2 (Matheson ultrahigh purity) for

10 minutes before irradiation and continuously during irradiation. Cells were irradiated at 0°C with ^{60}Co gamma-rays (Gamma cell 220, Atomic Energy of Canada) at a dose rate of about 1 Gy/sec. Survival was determined by plating suitable cell dilutions on solid agar plates of Yeast/Peptone/Dextrose (YPD) containing 0.1% yeast extract, 1% yeast nitrogen base with amino acids, 1% sodium succinate, 2% peptone, 2% glucose, followed by growth at 23°C. Colony formation was scored following five days of incubation at 23°C.

Cisplatin Survival. Cells ($2x10^7$) were treated for 30 minutes with cisplatin (Bristol Myers Squibb) diluted to the appropriate concentration in 23°C sodium phosphate buffer. After cisplatin treatment, cells were washed three times with 0°C buffer and survival was determined by plating suitable dilutions on solid YPD agar plates.

Data and Figures. All figures shown represent the combined results from at least three independent experiments. Standard error bars are shown except where the error is less than or equal to the symbol size. Student's T-test and one-way analysis of variance were performed to determine the statistical significance between various treatments, and p-values are given when the data were significantly different.

Results

Radiation Survival. Wild type MS33, recombinational repair deficient MS32 and excision repair deficient STX432 yeast cells were irradiated with increasing doses of gamma rays in the presence of N_2 (Figure 2.1). MS32 cells were sensitive to killing by gamma radiation relative to wild type MS33 cells. STX432 showed an intermediate level of sensitivity at doses greater than 1 kGy.

Cisplatin Survival. All three yeast strains were tested for survival against increasing concentrations of cisplatin (Figure 2.2). The results are similar to radiation survival curves with wild type MS33 demonstrating resistance to killing

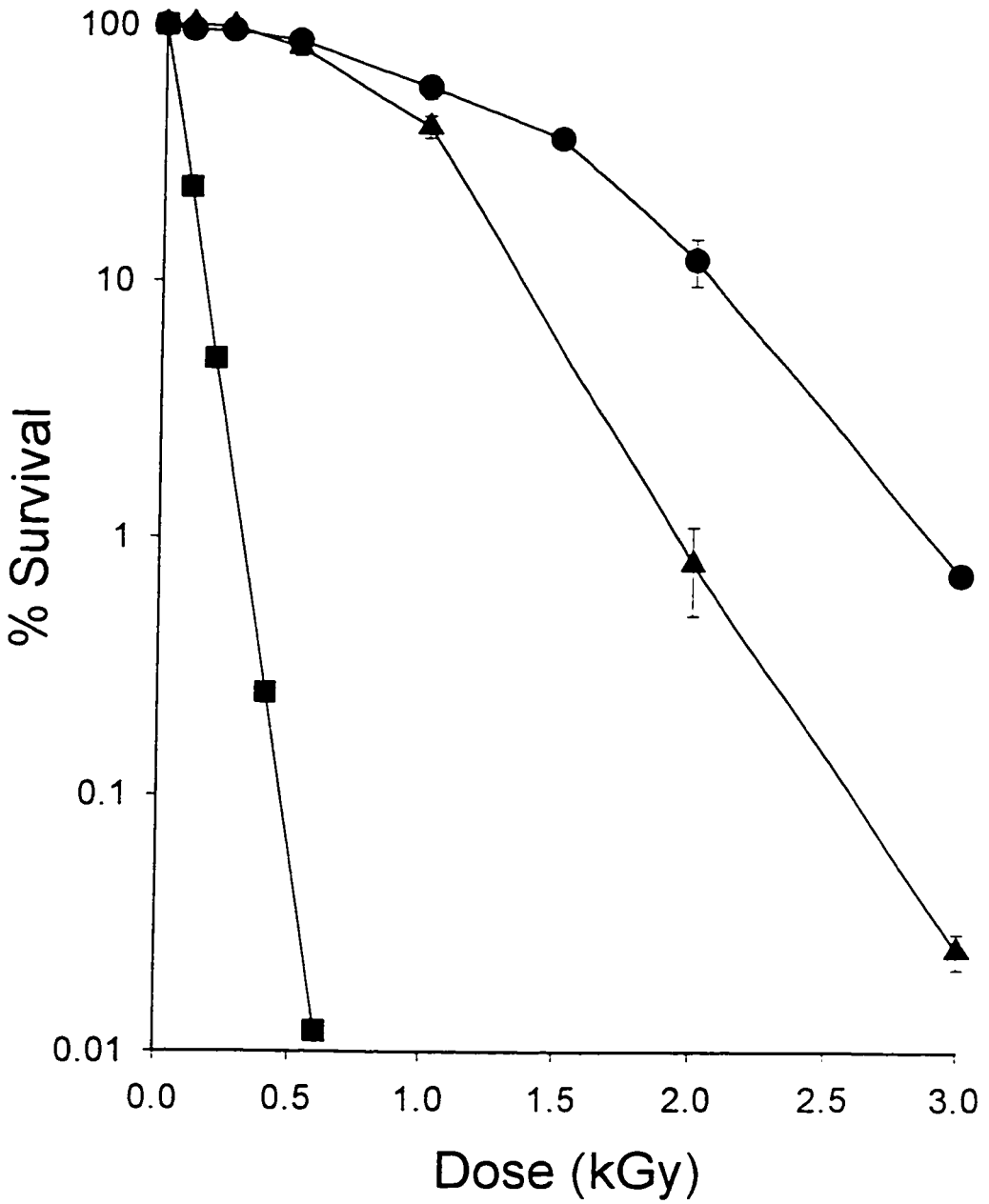


Figure 2.1. Survival of wild type MS33 (circles), recombinational repair deficient MS32 (squares) and excision repair deficient STX 432 (triangles) yeast cells after ^{60}Co gamma irradiation delivered in the presence of nitrogen. Data for survival of MS33 cells to irradiation in nitrogen is also shown in Fig. 2.3.

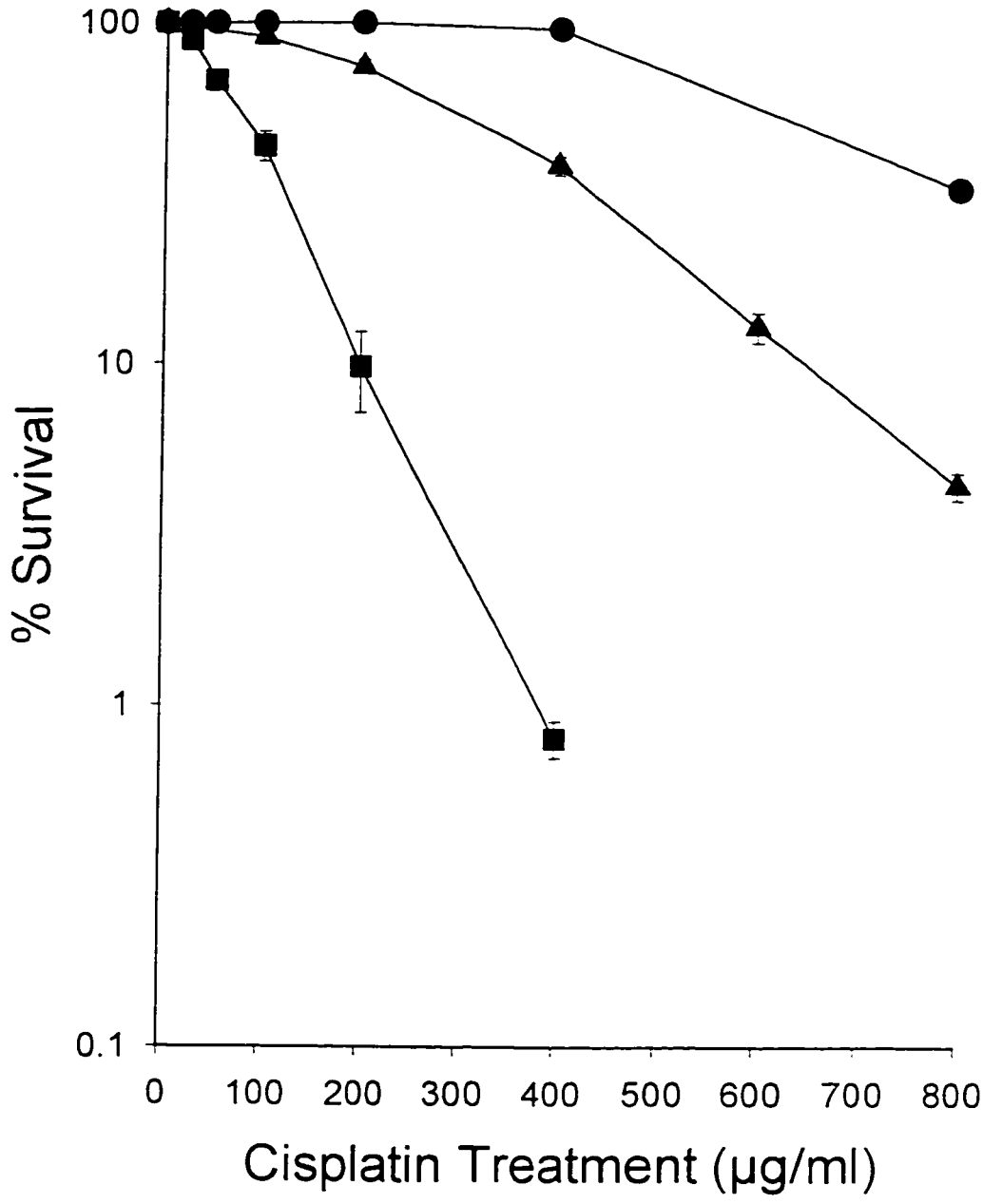


Figure 2.2 Survival of wild type MS33 (circles), recombinational repair deficient MS32 (squares) and excision repair deficient STX 432 (triangles) after cisplatin treatment at 23°C for 30 minutes in air.

by cisplatin, MS32 demonstrating sensitivity and STX432 showing an intermediate response.

Combined Treatment of Cisplatin and Radiation. Survival of wild type MS33 yeast cells to gamma radiation after a cisplatin treatment (400 µg/ml, 30 min.) is shown in Figure 2.3. Cisplatin treatment resulted in radiosensitization when the gamma radiation was delivered in oxygen or nitrogen. At a survival level of 10% (D_{10}) the enhancement ratio was 1.25 and 1.3 for oxic and anoxic irradiation conditions respectively. In Figure 2.4, survival of MS33, MS32 and STX432 yeast cells to gamma radiation (delivered in the presence of N_2) after a cisplatin treatment of 400 µg/ml for 30 minutes is shown. Cisplatin treatment resulted in radiosensitization of wild type MS33 and excision repair deficient STX432 cells ($p<0.01$) but not recombinational repair deficient MS32 cells. At a survival level of 10% (D10), the enhancement ratio was similar for MS33 and STX432 cells (1.3 and 1.4 respectively). Figure 2.5 shows that cisplatin, at any concentration tested, did not change the survival of recombinational repair deficient MS32 cells at any dose of radiation which allowed .01% or greater survival (see also Figure 2.4).

Discussion

The results of the present study showed that recombinational repair deficient MS32 (rad52) cells were very radiosensitive compared to wild type MS33 cells (Figure 2.1). This was not unexpected since DNA double strand break repair, via Rad52 dependent recombinational repair, is known to be the major pathway which confers radiation resistance in yeast (Frankenberg-Schwager and Frankenberg 1990). The radiation sensitivity of excision repair deficient STX432 cells (rad3) (Figure 2.1) showed that excision repair may also be an important pathway which repairs radiation damage. Radiosensitivity of another strain defective for rad3 (strain D7-3) has been previously demonstrated (R.E.J. Mitchel, unpublished data), but in both studies the isogenic

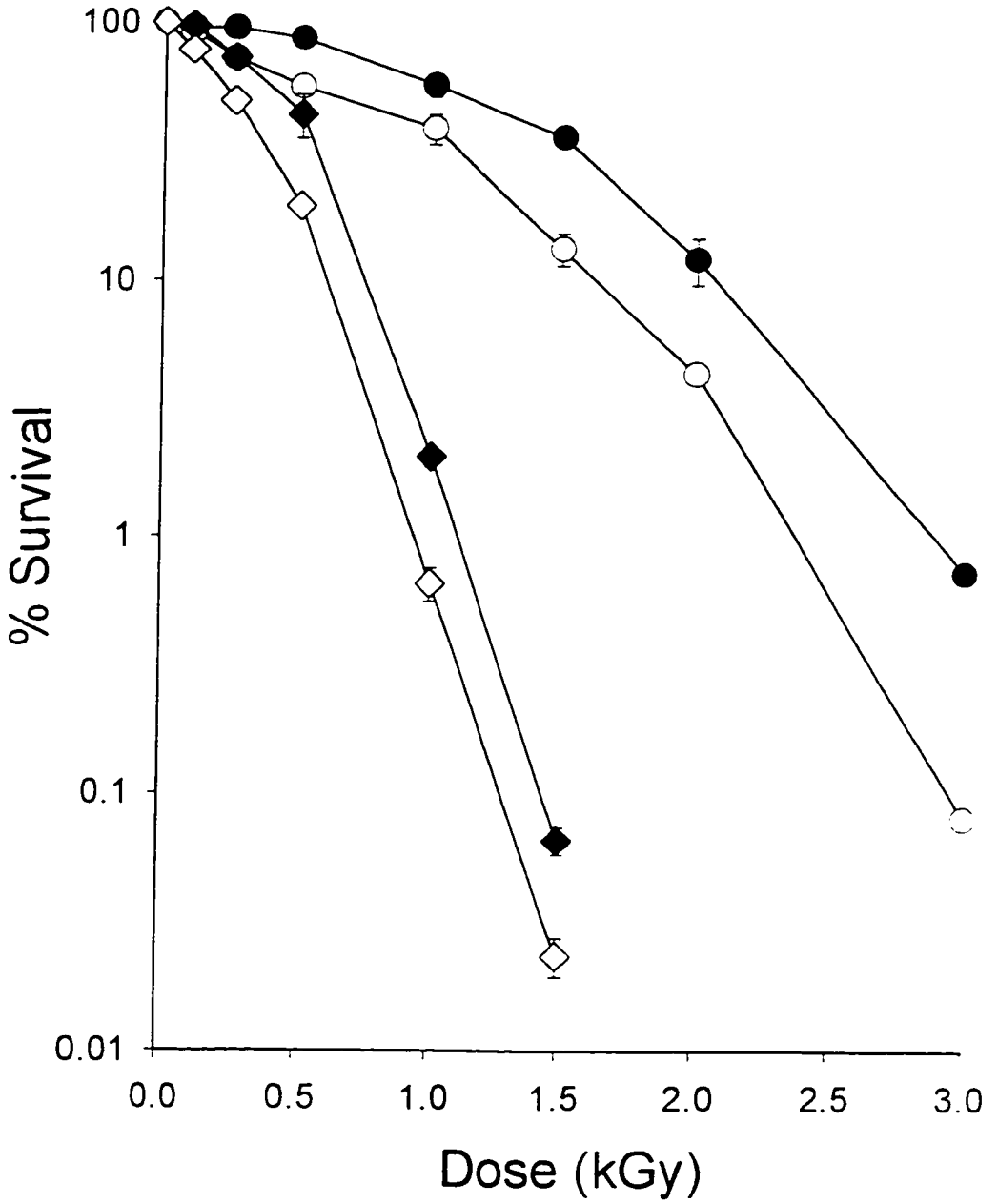


Figure 2.3. Survival of wild type MS33 yeast cells after ^{60}Co gamma irradiation delivered in the presence of nitrogen (circles) or oxygen (diamonds), with (open symbols) or without (closed symbols) a cisplatin treatment at 23°C for 30 minutes immediately before irradiation. Data for irradiation alone in nitrogen are the same data as shown in Fig. 2.1.

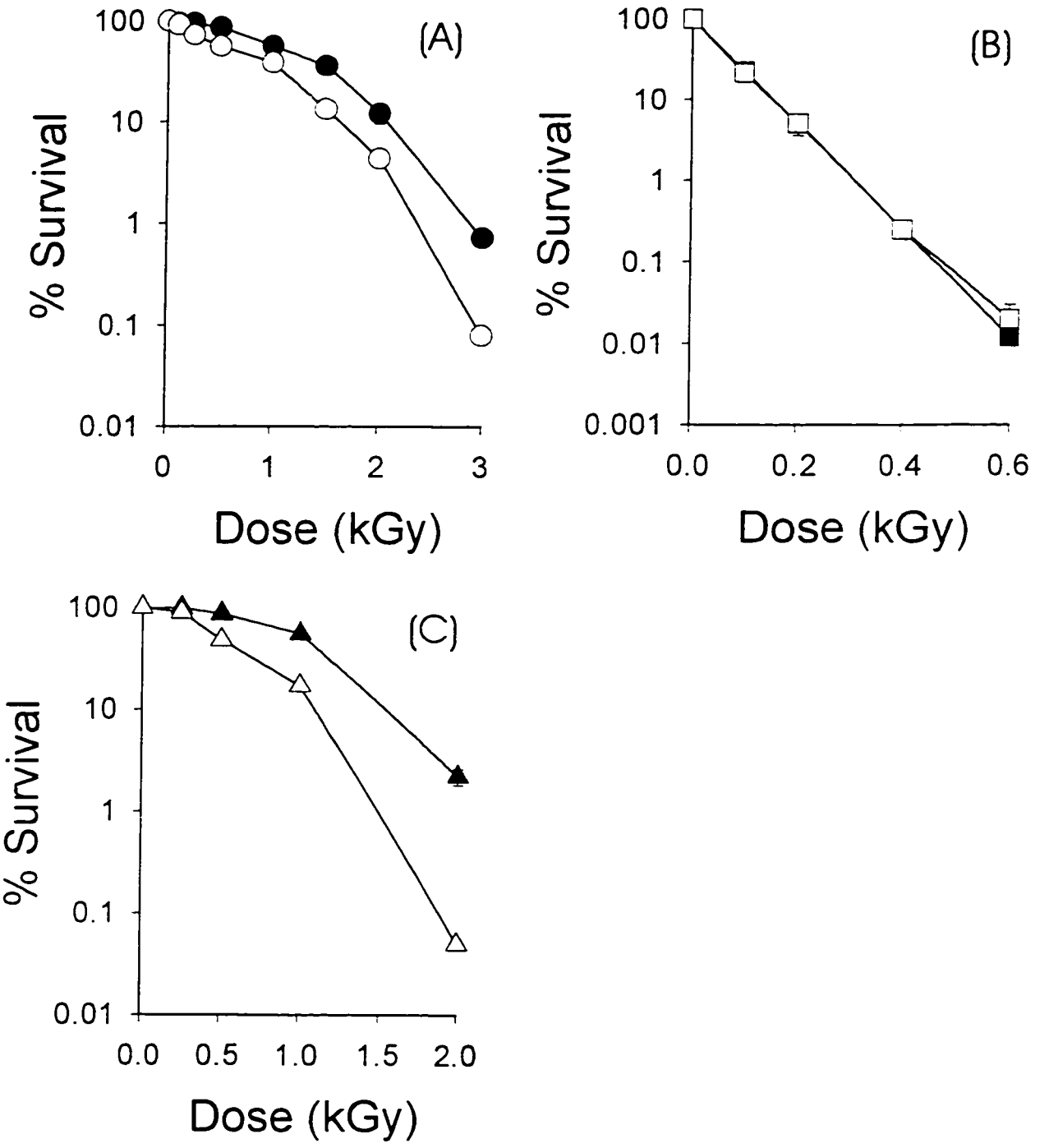


Figure 2.4. Survival of (A) wild type MS33, (B) recombinational repair deficient MS32 and (C) excision repair deficient STX 432 yeast cells after ^{60}Co gamma irradiation in the presence of nitrogen, with (open symbols) or without (closed symbols) a cisplatin treatment at 23°C for 30 minutes immediately before irradiation. Fig. 2.4A represents data from Fig. 2.3.

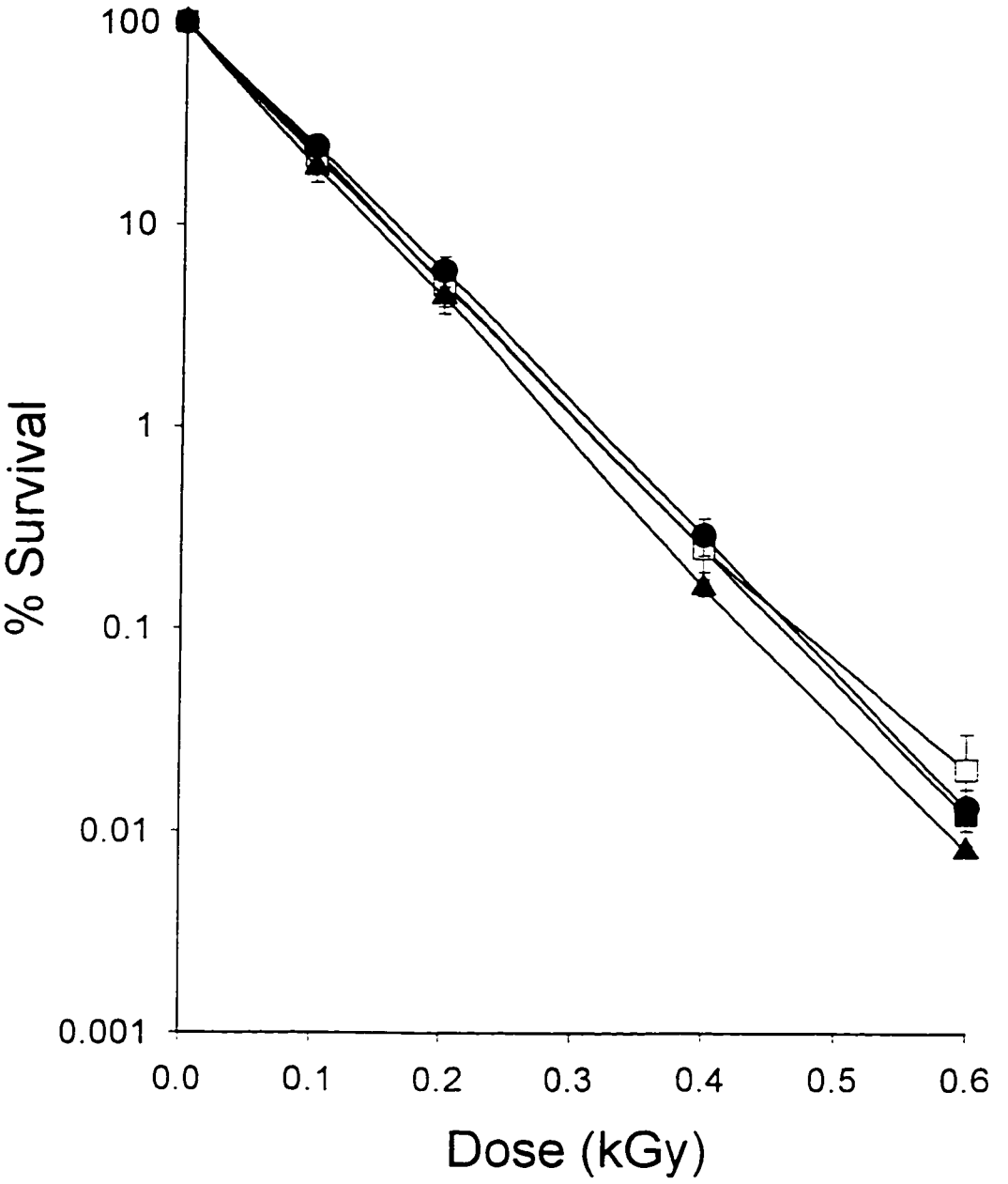


Figure 2.5. Survival of recombinational repair deficient MS32 yeast cells after ^{60}Co gamma irradiation in the presence of nitrogen, after a prior cisplatin treatment of 10 µg/ml (circles), 50 µg/ml (triangles) or 400 µg/ml (open squares) at 23°C for 30 minutes. Survival of cells not pretreated with cisplatin are shown as closed squares. Data shown have been normalized for cisplatin toxicity.

wild type parent strain was not available to make direct comparisons. Therefore, the relative radiosensitivity of excision repair deficient STX432 may be due to some other intrinsic characteristic of the strain.

The results for cisplatin sensitivity of the different strains showed that recombinational repair deficient MS32 and excision repair deficient STX432 cells were also sensitive compared to wild type MS33 cells (Figure 2.2). These data support previous results (Hannan et al. 1984) and demonstrate that both excision and recombinational repair processes are involved in repairing cisplatin DNA adducts. The extreme sensitivity of recombinational repair deficient MS32 cells to both DNA damaging agents indicates that in yeast cells, for the treatments employed, recombinational repair involving proteins in the Rad52 gene series (Rad51,55,57) (Hays et al. 1995) is a dominant mode of repair. Radiation-induced DNA double strand breaks and cisplatin-induced DNA interstrand crosslinks are two potentially lethal lesions that are most likely repaired via a recombinational process since in both cases, correct genetic information from a duplicate source (e.g. homologous chromosome or sister chromatid) is required for restoring the damaged sites successfully.

Wild type MS33 cells were sensitized by cisplatin to killing by radiation under oxic and anoxic conditions (Figure 2.3). Radiosensitization under both conditions has been previously reported (Richmond and Powers 1976, Richmond et al. 1977, Overgaard and Khan 1981, Alvarez et al. 1978, Douple and Richmond 1978, Begg 1987, Skov and MacPhail 1991). However, several studies have shown that the synergistic effect for cell killing is diminished or absent when cells are irradiated in air (Richmond and Powers 1976, Richmond et al. 1977, Korbelik and Skov 1989). Compared to wild type MS33 cells, excision repair deficient STX432 cells were sensitive to radiation (Figure 2.2) but could be further radiosensitized by cisplatin to an extent similar to the wild type cells (enhancement ratios of 1.3 and 1.4 respectively) (Figure 2.4). In contrast, recombinational repair deficient MS32 cells were sensitive to radiation but could

not be made more sensitive by cisplatin treatments (Figure 2.4 and 2.5). It is possible that in MS32 cells, a consequence of a defective recombinational repair genotype is the constitutive upregulation of stress proteins. Stress proteins such as metalothioneins or other thiol containing molecules might scavenge cisplatin, thereby decreasing the possibility of an interaction of these adducts with radiation-induced lesions and/or repair processes and abrogate the radiosensitizing effect of cisplatin. It has been shown in some cisplatin resistant cell lines, the resistance is due in part to thiol scavenging of cisplatin (Zamble and Lippard 1995, Lazo and Pitt 1995). However, since MS32 cells were being killed at all concentrations of cisplatin tested (Figure 2.2), it is certain that at least some cisplatin is interacting with DNA and therefore the lack of radiosensitization by cisplatin in MS32 cells cannot be due to total scavenging of cisplatin. To test for this possibility, MS32 cells could be treated with thiol scavengers to deplete the intracellular thiol pool, and thus allow binding of cisplatin to DNA.

It is also possible that cisplatin sensitization of wild type cells involves the coupling of repair processes to gene transcription. It is known that bulky adducts such as cisplatin crosslinks and pyrimidine dimers (Jones et al. 1991, Selby and Sancar 1993, Larminat et al. 1993) are preferentially repaired when present in transcriptionally active DNA compared to inactive DNA. The preferential repair is carried out by a nucleotide excision repair process which is coupled to transcription. In *Escherichia coli*, the coupling of these two processes is carried out by a protein which is thought to recognize and bind to sites of stalled transcription and recruit the nucleotide excision repair machinery (Drapkin et al. 1994). Through this mechanism, actively transcribed gene sequences are preferentially repaired compared to the rest of the genome. A similar protein called ERCC6 and a transcription-coupled nucleotide repair system has been identified in cells derived from humans with Cockayne's syndrome (Mellon and Hanawalt 1989). In yeast, no known mutant with a defect in gene specific

nucleotide excision repair has been found, although Rad16 has been suggested as a candidate gene (Drapkin et al. 1994).

Transcription-coupled machinery has been associated with nucleotide excision repair of bulky adducts but not recombinational repair of complex lesions. It is possible, however, that a system similar to the one suggested for nucleotide excision repair also exists for recombinational repair. One could postulate that in yeast, transcriptionally coupled recombinational repair (TCRR) exists, and a defect in the Rad52 protein might act to uncouple recombinational repair processes from transcriptionally active genes. In this case, repair of any damaged DNA sequence would be carried out without bias for the status of whether it was undergoing transcription or not. This would effectively reduce repair of damaged sites within active genes because the priority of repairing active genes would be lost.

The idea of TCRR is only speculative and there is only indirect evidence that the Rad52 gene product may be involved. Wild type MS33 yeast cells are resistant to killing by a chemical mutagen N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG) but are sensitive to MNNG generated mutations in a histidine gene which is not transcribed under conditions when yeast are grown in complete media. In contrast, recombinational deficient MS32 yeast cells are sensitive to killing by MNNG but resistant to MNNG generated mutations in the histidine gene (Mitchel and Morrison 1987). Under normal conditions if the Rad52 recombinosome were coupled to transcription, repair of transcriptionally active (essential) genes by recombinational repair could be preferential and lead to increased survival, while non-transcriptionally active (non-essential) genes would not be repaired leading to mutation. If the processes become uncoupled in a rad52 mutant, then all DNA may be repaired at the same rate and efficiency, resulting in relatively more mutations in transcriptionally active genes. This could result in enhanced cell death, and relatively less mutations in non-essential genes [for example, when histidine is supplied in the media, genes such as his1

(ATP phosoribosyl transferase) are not essential]. Thus in MS32 cells, cisplatin may not interfere with the processing of lesions since the defect i.e. no preferential repair of transcribed genes, already exists by virtue of the defective rad52 gene. In contrast, cisplatin DNA adducts may interfere with repair of radiation-induced DNA damage in cell lines which have a functional recombinosome, such as wild type MS33 and excision repair defective STX432 cells.

In summary, yeast cells may have a Rad52 dependent TCRR pathway which serves to preferentially repair lesions, which require recombinational repair, in transcriptionally active genes compared to lesions contained in inactive regions of DNA. When recombinational repair proficient yeast cells are treated with a combination of cisplatin and radiation, the cisplatin lesions may uncouple recombinational repair from transcription and sensitize the cells to radiation-induced DNA damage.

Therefore, in conclusion, cisplatin damage repair requires both functional excision and recombinational repair systems but radiosensitization by cisplatin seems to act primarily by stalling or altering recombinational repair processes. It is possible that cisplatin-DNA adducts inhibit the preferential and rapid repair of transcriptionally active genes by uncoupling the recombinational repair system from transcription, leaving the damage to be processed by the global repair system. Such uncoupling of recombinational repair from transcription would result in radiosensitization of cells which have a functional recombinational repair system.

References

Alvarez M.V., G. Cobreros, A. Heras and M.C. Lopez Zumel. Studies on cis-dichlorodiammineplatinum(II) as a radiosensitizer. Br. J. Cancer 37:68-72 (1978).

Begg, A.C. Additivity versus repair inhibition in fractionated treatments combining drugs and X-rays: A theoretical approach. Int. J. Radiation Oncology Biol. Phys. 13: 921-927 (1987).

Douple, E.B. and R.C. Richmond. Platinum complexes as radiosensitizers of hypoxic mammalian cells. Br. J. Cancer 37:98-102 (1978).

Drapkin, R., A. Sancar and D. Reinberg. Where transcription meets repair. Cell 77: 9-12 (1994).

Frankenberge-Schwager, M., and Frankenberge, D. DNA double starnd breaks: their repair and relationship to killing in yeast. Int. J. Radiat. Biol. 58: 569-76.

Hannan, M.A., S.G. Zimmer and J. Hazle. Mechanisms of cisplatin (cis-diammine dichloroplatinum II)-induced cytotoxicity and genotoxicity in yeast. Mutat. Res. 127: 23-30 (1984).

Hays, S.L., A.A. Fermenich and P. Berg. Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55, and Rad57 proteins. Proc. Natl. Acad. Sci. USA 92: 6925-6929 (1995).

Jones, J.C., W. Zehn, E. Reed, R.J. Parker, A. Sancar and V.A. Bohr. Gene specific formation and repair of cisplatin intrastrand adducts and interstrand croos-links in Chinese hamster ovary cells. J. Biol. Chem. 266: 7101-7107 (1991).

Kiefer, J. and Muller, J. Yeast as a Model Object to Study DNA Repair, In: Yeast Molecular Biology and Biotechnology, Ed. R. Prasad, Omega Scientific Publishers, New Delhi, India, pp. 172-187 (1991).

Korbelik, M. and K.A. Skov. Inactivation of hypoxic cells by cisplatin and radiation at clinically relevant doses. Radiat. Res. 119: 145-156 (1989).

Larminat, F., W. Zehn and V.A. Bohr. Gene specific repair of intersrtand cross-links induced by chemotherapeutic agents can be preferential. J. Biol. Chem. 268: 2649-2654 (1993).

Lazo, J.S. and B.R. Pitt. Metallothioneins and cell death by anticancer drugs. Annu. Rev. Pharmacol. Toxicol. 35: 635-53 (1995).

Mellon, I. and P.C. Hanawalt. Induction of the *Escherichia coli* lactose operon selectively increases repair of its transcribed DNA strand. Nature 342: 95-98 (1989).

Mitchel, R.E.J. and D.P. Morison. Inducible DNA repair systems in yeast: competition for lesions. Mut. Res. 183: 149-159 (1987).

Overgaard, J. and A.R. Khan. Selective enhancement of radiation response in a C3H mammary carcinoma by cisplatin. Cancer Treat. Rep. 65: 501-503 (1981).

Raaphorst, G.P., G. Wang, D. Stewart and C.E. Ng. Concomittant low dose-rate irradiation and cisplatin treatment in ovarian carcinoma cell lines sensitive and resistant to cisplain treatment. Int. J. Radiat. Biol. 69: 623-631 (1996).

Richmond, R.C. and E.L. Powers. Radiation sensitization of bacterial spores by cis-dichlorodiammineplatinum(II). Radiat. Res. 68:251-257 (1976).

Richmond, R.C., J.D. Zimbrick and D.L. Hykes. Radiation-induced DNA damage and lethality in *E. Coli* as modified by the antitumor agent cis-dichlorodiammineplatinum(II). Radiat. Res. 71: 447-460 (1977).

Schwachofer, J.H.M., R.P.M.A. Crooijmans, J. Hoohenhout, H.B. Kal and A.G.M. Theeuwes. Effectiveness in inhibition of recovery of cell survival by cisplatin and carboplatin: Influence of treatment sequence. Int. J. Radiation Oncology Biol. Phys. 20:1235-1241 (1991).

Selby, C.P. and A. Sancar. Molecular mechanism of transcription-repair coupling. Science 260: 53-58 (1993).

Skov, K. and S. MacPhail. Interaction of platinum drugs with clinically relevant X-ray doses in mammalian cells: A comparison of cisplatin, carboplatin, iproplatin, and teraplatin. Int. J. Radiation Oncology Biol. Phys. 20: 221-225 (1991).

Unrau, P., Wheatcroft, R., Cox, B.S. The excision of pyrimidine dimers from DNA of ultraviolet irradiated yeast. Mol. Gen. Genet. 354: 359-62 (1971).

Wodinsky, I., J. Swiniarski, C.J. Kensler and J.M. Venditti. Combination radiotherapy and chemotherapy for P388 lymphocytic leukemia in vivo. Cancer Chemotherapy Rep. 4: 73-97 (1974).

Zak, M. and J. Drobnik. Effect of cis-dichlorodiammine platinum (II) on the post-irradiation lethality in mice after irradiation with X-rays. Strahlentherapie 142: 112-115 (1971).

Zamble, D.B. and S.J. Lippard, Cisplatin and DNA repair in cancer chemotherapy. Trends in Biochem. Sci. 20: 435-439 (1995).

Chapter 3. Induction of Recombinational Repair in Yeast by Cisplatin

Introduction

In prokaryotes and eukaryotes, DNA repair capacity can be elevated in response to a variety of stresses including ionizing radiation (Mitchel and Morrison 1984), heat shock (Mitchel and Morrison 1982, 1984), mutagenic chemicals (Mitchel and Morrison 1987) and chemotherapeutic drugs (Lazo and Pitt 1995). In yeast, it has been shown that different stresses induce specific sets of genes (McClanahan and McEntee 1984, Ruby and Szostak 1985, McClanahan and McEntee 1986, Lazo and Pitt 1995). A critical set of genes induced in yeast by DNA damaging agents is the Rad50 -57 gene series (Game 1993). These proteins form the complex machinery (Hays et al. 1995) used to repair DNA lesions via an error free recombinational process (Mitchel and Morrison 1987, Game 1993).

Recombinational repair is essential for successful repair of lesions involving both DNA strands since the damage in either strand can not be resynthesized using the genetic information encoded by the damaged complementary strand. Instead, the damaged or missing genetic information is copied from a duplicate source (i.e. homologous chromosome or sister chromatid) (Resnick 1976, Szostak et al. 1983, Sancar and Sancar 1988). Lesions requiring the recombinational repair system include double strand breaks and interstrand crosslinks. Recombinational repair is not, however, restricted to repair of these relatively complex lesions. For example, it has been shown that recombinational repair can restitute chemically generated N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) DNA lesions (Mitchel and Morrison 1987, Boreham and Mitchel 1991). MNNG is a chemical mutagen that methylates DNA at various sites including the N-7 and O-6 positions of guanine (Maga and McEntee 1985). If the methylated guanine is not repaired, it pairs incorrectly with thymine instead of cytosine during replication, resulting in a GC to AT transition (Yarosh 1985). It has been suggested that MNNG exposure induces an error

prone system which is responsible for essentially all MNNG-generated mutations. The number of MNNG-generated mutations, however, has been shown to be reduced in yeast cells pre-exposed to ionizing radiation (Mitchel and Morrison 1987, Boreham and Mitchel 1991) or heat (Mitchel and Morrison 1986) but not by pretreatment with MNNG itself (Polakowska et al. 1986). In fact, MNNG induces an error prone system (Mitchel and Morrison 1986) which increases the mutagenic effect of a subsequent treatment with other chemical mutagens like ethyl methanesulfonate (Mitchel and Morrison 1987). It has been proposed that preexposure of cells to radiation and heat induces an error free recombinational system which competes with the error prone system for repair of these lesions (Mitchel and Morrison 1986). An increase in cellular error free repair capacity would lead to a decrease in the number of MNNG lesions processed by the error prone system and result in a net suppression of mutation (see Figure 1).

It is not known whether DNA crosslinks are effective signals for the induction of error free recombinational repair, although in yeast, crosslinks caused by furocoumarins resulted in upregulation of RAD54 transcription (Averbeck and Averbeck 1994). In addition, there is evidence to indicate that the basic signal for induction of recombinational repair is perturbation of DNA topology (Boreham et al. 1990). Therefore it is likely that DNA crosslinks which cause base destacking and strand kink angles of 40 to 60° (Bruhn et al. 1990), could (a) invoke a general stress or heat shock-like response and (b) lead specifically to an increased error free repair capacity. We have tested these postulates in yeast using a DNA inter- and intrastrand crosslinking agent, cisdiamminedichloroplatinum II (cisplatin) and compared the results to ionizing radiation- and heat-induced cellular repair capacity.

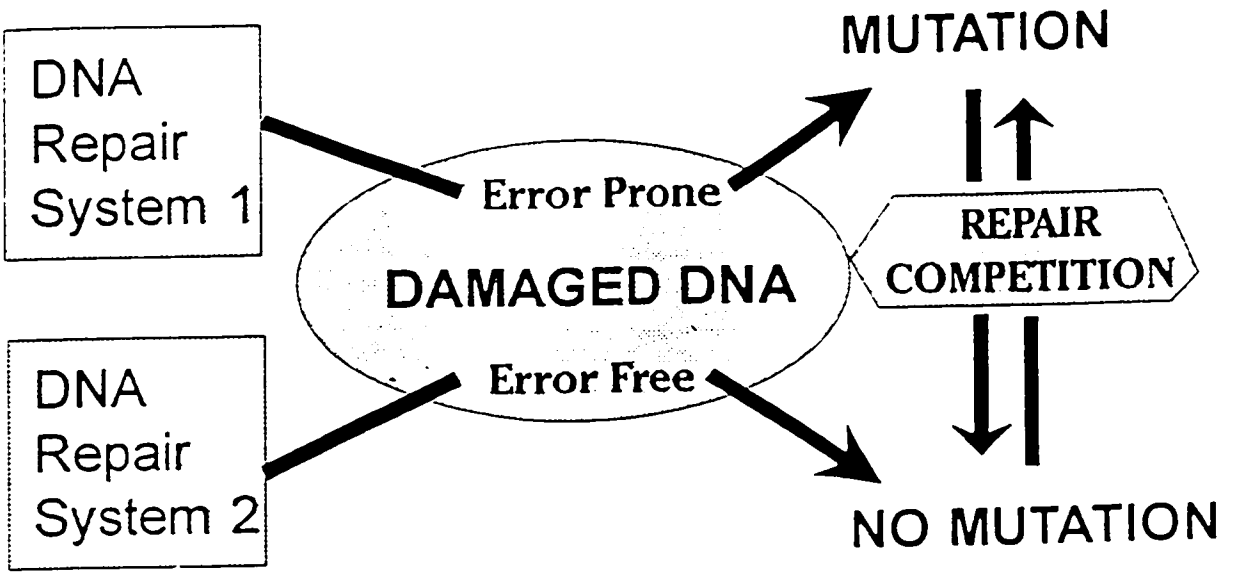


Figure 3.1. Model for Suppression of MNNG-generated mutations. DNA damage caused by MNNG induces an error prone repair system (system1) and results in mutation. DNA damage caused by radiation induces an error free repair system (system 2) and results in few mutations. Under different conditions, both systems can be induced and both compete to repair the DNA damage. Increasing doses of radiation induces higher levels of error free repair and consequently out competes the error prone system for processing lesions, resulting in less mutation.

Materials and Methods

Strains. Diploid strains of the yeast *Saccharomyces cerevisiae* were used as the test organisms in these experiments. Strain MS33 was wild type for DNA repair capacity (rec+/rec+, exc+/exc+). Strain MS32 was homozygous for *rad 52-1* and was defective in recombinational repair (rec-/rec-, exc+/exc+). Yeast strains MS33 and MS32 were gifts from Dr. D.P. Morrison, AECL, Chalk River Laboratories.

Cell Growth. Cell cultures were grown at 23°C to mid-exponential phase (2-4 x 10^6 cells/ml) in liquid nutrient medium containing 0.1% yeast extract, 1% yeast nitrogen base with amino acids, 1% sodium succinate, and 2% glucose (called Yeast Extract/ Succinate medium or YES).

Thermal Tolerance. Thermal survival was determined by placing 2.5 ml cell aliquots (2x10^6 cells/ml in YES medium at 0°C) in 16 x 100 mm glass test tubes and submerging the glass test tubes into a 52°C shaking water bath. Exposure to 52°C served as the challenge heat temperature. At specified time intervals individual tubes were removed, rapidly cooled to 0°C, and the cells were plated for survival on YPD plates.

Radiation Resistance. Cells were washed twice and suspended (at 0°C) in 20 mmol dm^{-3} (mM) sodium phosphate buffer, pH 7.0, at approximately 2 x10^6 cell/ml. These suspensions were equilibrated by bubbling the cell suspension at 0°C with N_2 (<1 ppm O_2) or O_2 (Matheson ultrahigh purity) for 10 minutes before irradiation and continuously during irradiation. Cells were irradiated with ^{60}Co gamma-rays (Gamma cell 220, Atomic Energy of Canada) at a dose rate of about 1 Gy/sec. Survival was determined by plating suitable dilutions on solid agar (2%) plates containing 0.1% yeast extract, 1% yeast nitrogen base with amino acids, 1% sodium succinate, 2% peptone, 2% glucose (called Yeast/Peptone/Dextrose media or YPD), followed by growth in an incubator at 23°C.

Induction of Thermal Tolerance and Radiation Resistance by Heat Shock. Cells were resuspended in fresh YES medium ($2x10^6$ cells/ml) at 23°C and heat shocked by an abrupt transfer of the culture (50 ml in a 250ml flask) to a shaking water bath at 37°C for 30 minutes. At the end of 30 minutes the culture was rapidly cooled to 0°C. All strains showed 100% survival to this inducing heat treatment. The heat shocked cells were then tested for thermal tolerance and radiation resistance. Non-heat shocked control cells were kept at 23°C for 30 minutes and then treated in the same way as cisplatin treated samples.

Induction of Thermal Tolerance by Radiation Exposure. Cell suspensions at 0°C in phosphate buffer were equilibrated for 10 minutes by bubbling N_2 through the suspensions 10 minutes prior to and during the exposure. ^{60}Co gamma-ray irradiations (50 and 200 Gy) were performed in a Gamma-Cell 220 (Atomic Energy of Canada Limited) at a dose rate of about 1 Gy/sec. The anoxically irradiated cells were resuspended in fresh YES medium at $2x10^6$ cells/ml and incubated at 23°C. Samples were removed thereafter every two hours for 6 hours, washed three times in cold sodium phosphate buffer, resuspended in YES and tested for thermal tolerance. Cells were plated for survival on YPD plates. Control cells were not irradiated prior to incubation for zero to six hours, but were then handled in the same way as irradiated samples.

Induction of Thermal Tolerance and Radiation Resistance by Cisplatin Treatment. Cells were treated for 30 minutes at 23°C with cisplatin (Bristol Myers Squibb) diluted to the appropriate concentration in sodium phosphate buffer. After cisplatin treatment, cells were washed three times with 0°C buffer and tested for thermal tolerance and radiation resistance. Control cells were suspended in sodium phosphate buffer without cisplatin, for 30 minutes at 23°C and then treated in the same way as cisplatin treated cell samples.

Mutation and Suppression of MNNG Induced Mutation by Cisplatin. Cisplatin-induced mutation was determined by measuring reversion to histidine independence. Revertant cells able to form colonies were counted after

incubation at 23°C for 6 days on solid medium lacking histidine. Total survival after the various treatments was measured by scoring colony formation on YPD plates.

Chemical mutagenesis was performed at 23°C in 20 mmol dm^{-3} (mM) phosphate buffer (pH 7.0) at a cell density of 1 x 10^7 cells/ml. Cells were treated with 20 µg/ml of MNNG (Aldrich Chemical Co.) for 30 minutes either immediately after or at the same time as a cisplatin treatment. After MNNG exposure, cells were washed once with 10% (w/v) sodium thiosulphate to inactivate the MNNG and then twice with phosphate buffer, all at 0°C. Reversion to histidine independence and survival to treatments was determined as described above.

Data and figures. Each experiment was repeated at least three times from independent cultures. All figures shown represent the combined results of all the experiments. Standard error bars are shown except where the error is less than or equal to the symbol size. Student's T-test and one-way analysis of variance were performed to determine the statistical significance between the various treatments and p-values are given.

Results

Thermal Tolerance and Heat-Induced Thermal Tolerance. Wild type MS33 cells were more sensitive to the lethal effects of a 52°C heat shock than recombinational repair deficient MS32 cells (Figure 3.2). After one minute of 52°C heat shock, all paired means are significantly different ($p<0.01$). A prior heat treatment of 37°C for 30 minutes induced thermal tolerance in both MS33 and MS32 cells. The maximum level of thermal tolerance achieved was the same for both strains.

Radiation-Induced Thermal Tolerance. Radiation exposure induced thermal tolerance in both wild type MS33 (Figure 3.3A) and recombinational repair deficient MS32 cells (Figure 3.3B) as previously reported (Mitchel and Morrison 1982). However, wild type MS33 cells developed significantly more

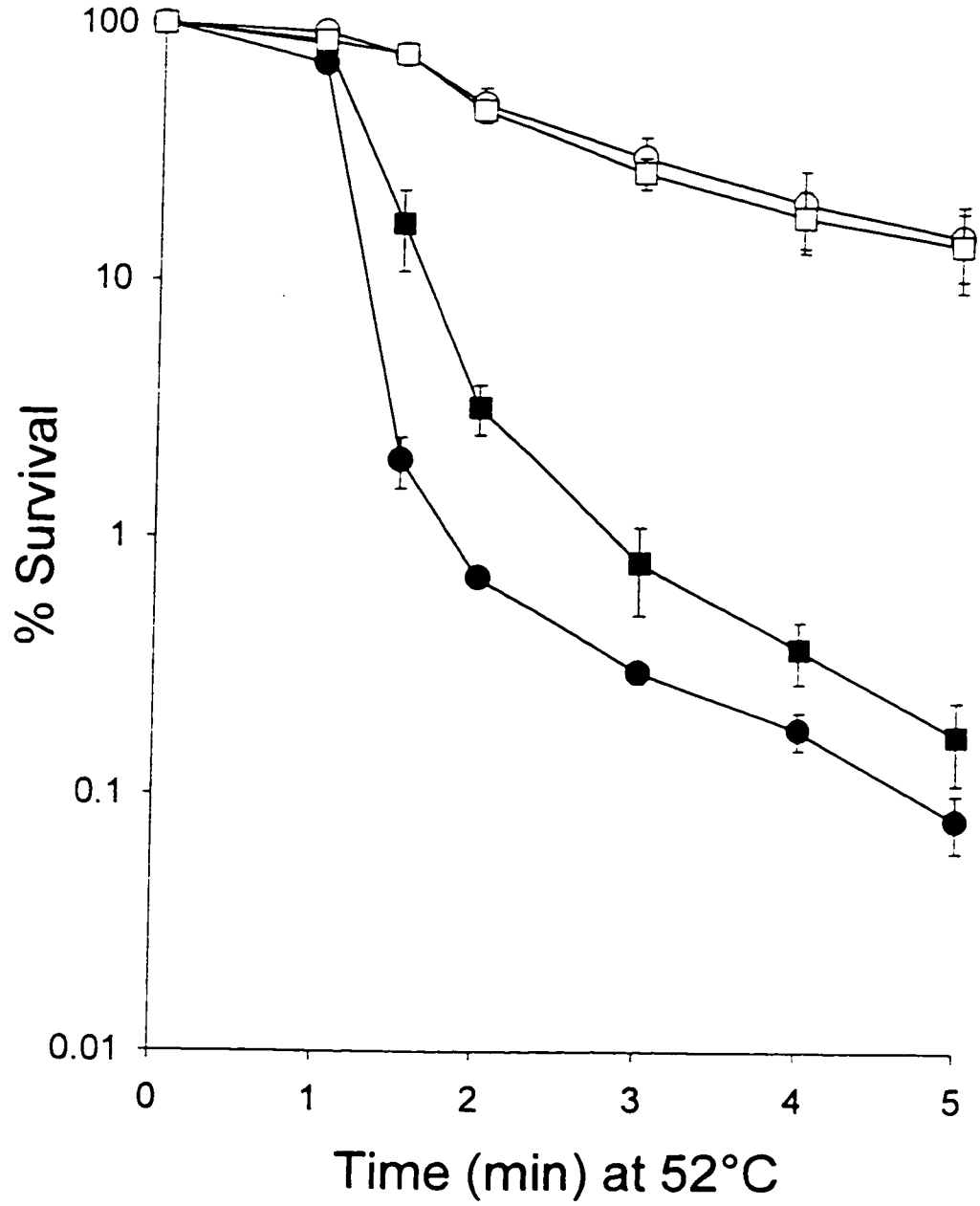


Figure 3.2. Survival of wild type MS33 (circles) and recombinational repair deficient MS32 (squares) to 52°C heating for various times, before (closed symbols) and after (open symbols) a 30 minute, 37°C heat treatment.

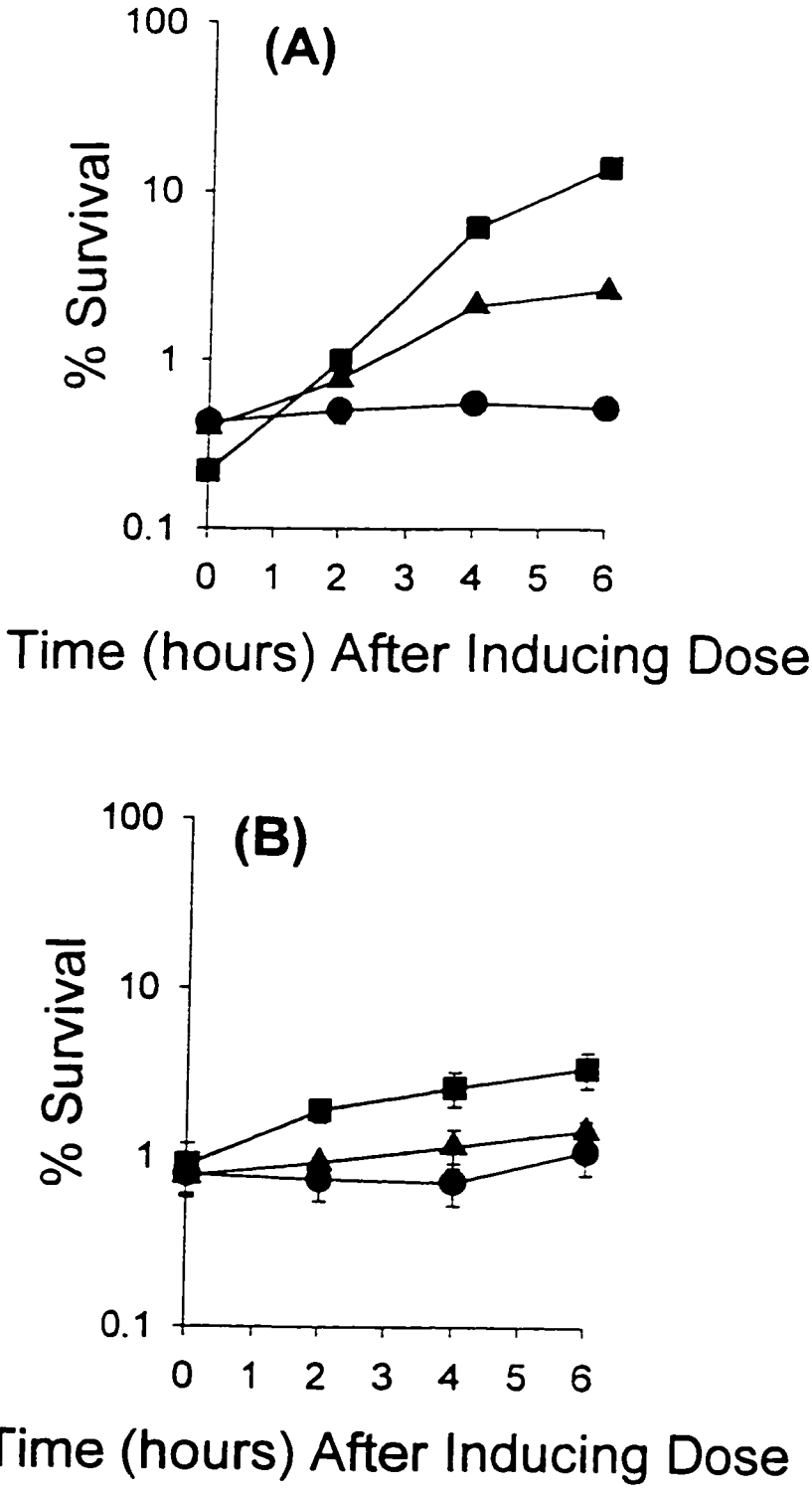


Figure 3.3. Radiation-induced thermal tolerance in (A) wild type MS33 and (B) recombinational repair deficient MS32 yeast cells. Cells exposed to 50 Gy (triangles) or 200 Gy (squares) inducing dose prior to a 52°C, three minute heat treatment. Control cells are represented by circles.

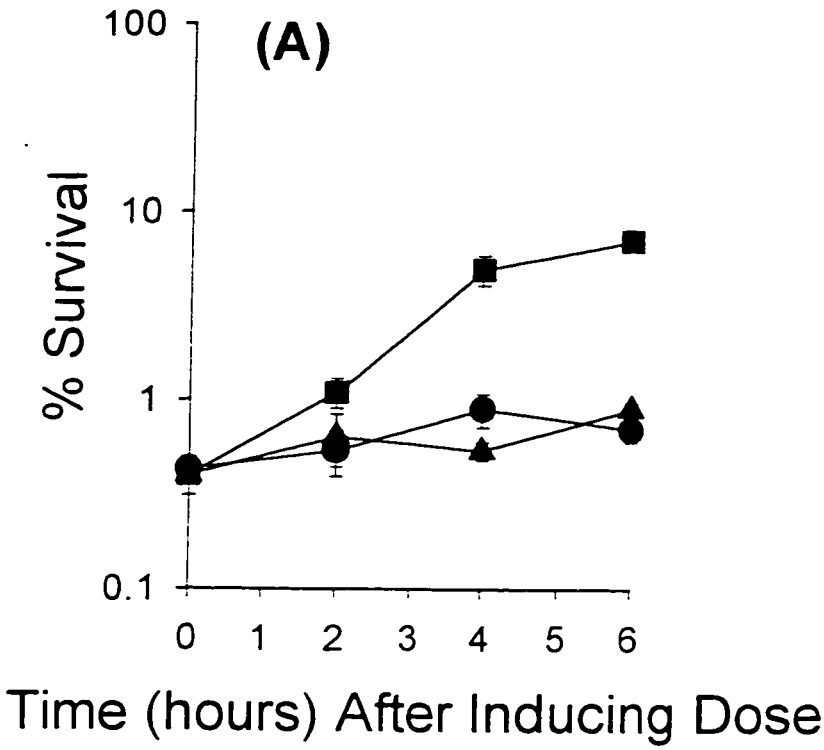
thermal tolerance compared to recombinational repair deficient MS32 cells after induction by 50 and 200 Gy gamma radiation ($p<0.01$).

Cisplatin-Induced Thermal Tolerance. Cisplatin (400 µg/ml, 30 minutes) induced thermal tolerance in both wild type MS33 (Figure 3.4A) and recombinational repair deficient MS32 cells (Figure 3.4B). The level of thermal tolerance reached at each time point was similar for both yeast strains. A smaller dose of cisplatin (50 µg/ml, 30 minutes) had no effect on thermal survival of either yeast strain.

Radiation Resistance and Heat-Induced Radiation Resistance. Wild type MS33 cells were constitutively more resistant than recombinational repair deficient MS32 cells to the lethal effects of gamma radiation at all doses and wild type cells developed a radiation resistant phenotype after exposure to a mild heat shock (37°C, 30 minutes) (Figure 3.5) ($p<0.001$). In contrast, radiation resistance did not develop in recombinational repair deficient MS32 cells as previously reported (Mitchel and Morrison 1982).

Cisplatin-Induced Radiation Resistance. Cisplatin, at 50 or 400 µg/ml at 23°C for 30 minutes did not induce radiation resistance in recombinational repair deficient MS32 cells (Figure 3.6). However, in wild type MS33 cells, radiation resistance was induced by cisplatin treatment (Figure 3.7). Induction of radiation resistance by cisplatin (400 µg/ml at 23°C for 30 minutes) was evident at one hour after cisplatin treatment and was maximal at two hours post cisplatin treatment in wild type cells (Figure 3.8). When the radiation test dose immediately preceded the cisplatin inducing treatment, wild type MS33 cells were sensitized to cell killing by radiation (Figure 3.8, 0 hour after inducing dose). Cisplatin-sensitization to killing by radiation was not evident in recombinational repair deficient cells (Figure 3.6, 0 hour after inducing dose).

Cisplatin Suppression of MNNG-Generated Mutations. Wild type MS33 cells showed a cisplatin dose dependent reduction in mutations generated by



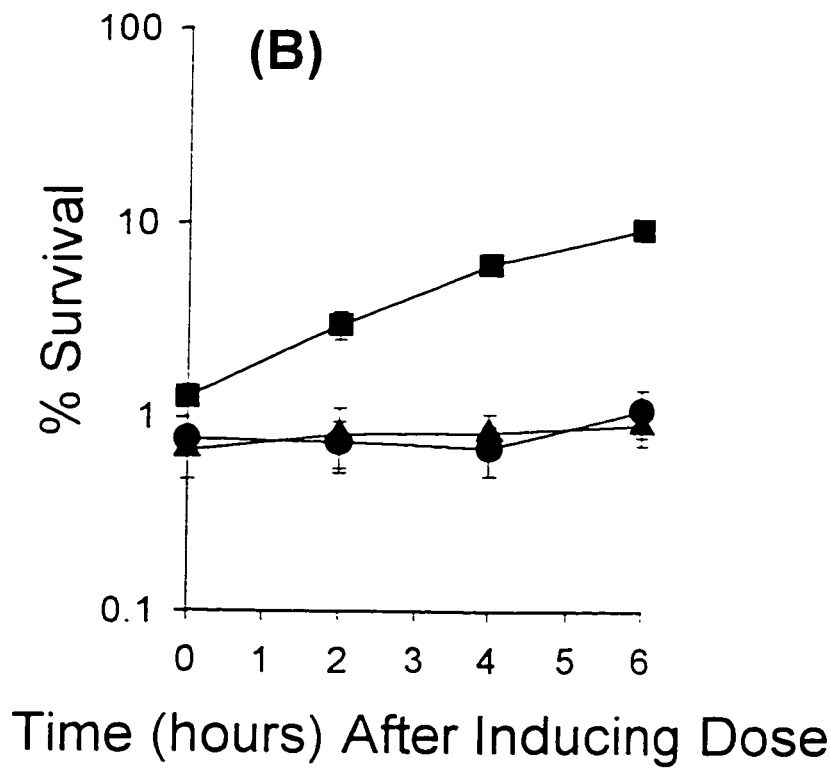


Figure 3.4. Cisplatin-induced thermal tolerance in (A) wild type MS33 and (B) recombinational repair deficient MS32 yeast cells. Cells were treated with 50 µg/ml (triangles) or 400 µg/ml (squares) cisplatin, incubated for various time intervals and then heated at 52°C for three minutes. Control cells are represented by circles.

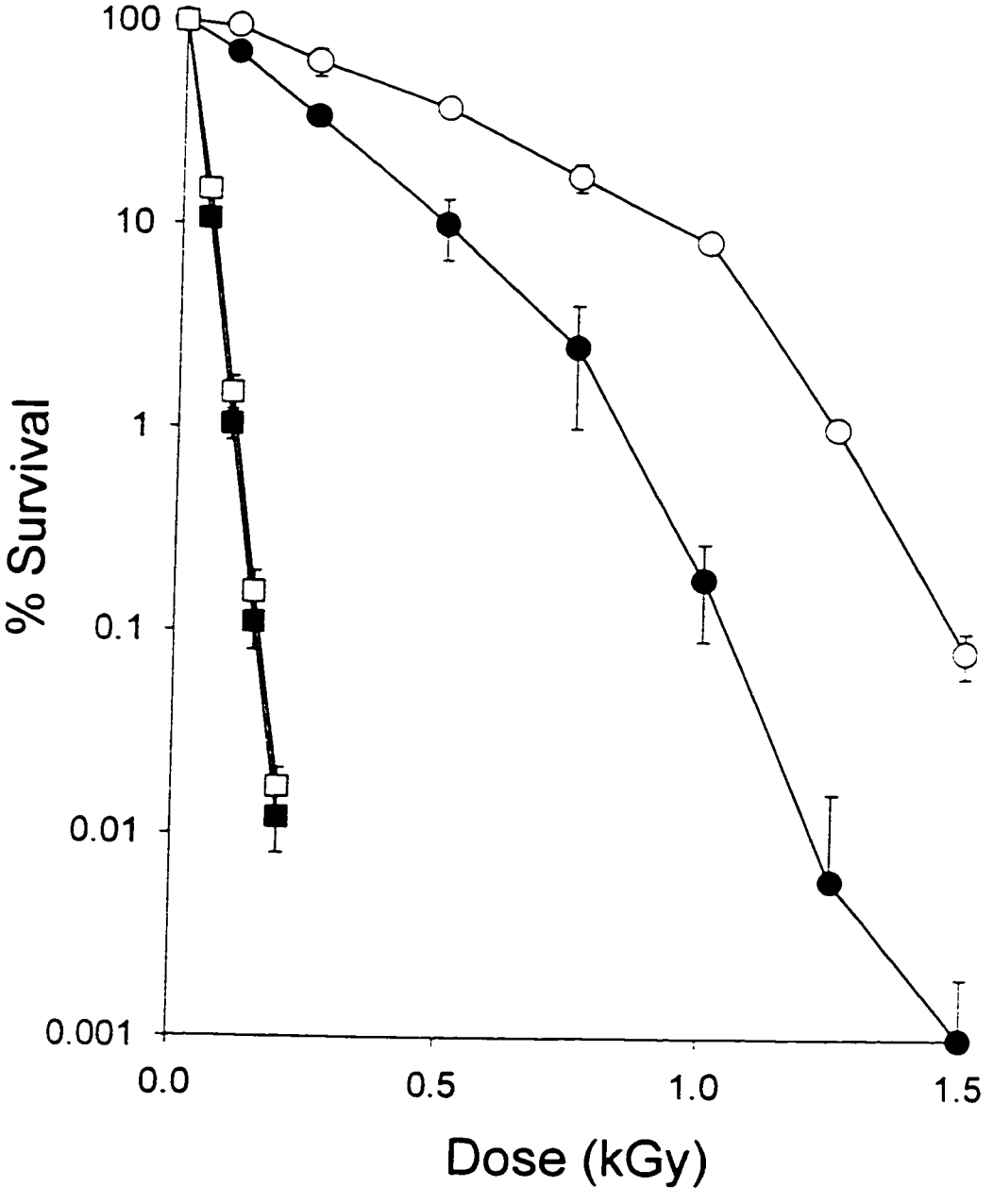


Figure 3.5. Survival of wild type MS33 (circles) and recombinational repair deficient MS32 (squares) to ^{60}Co gamma radiation in the presence of oxygen, before (closed symbols) and after (open symbols) a 30 minute 37°C heat shock.

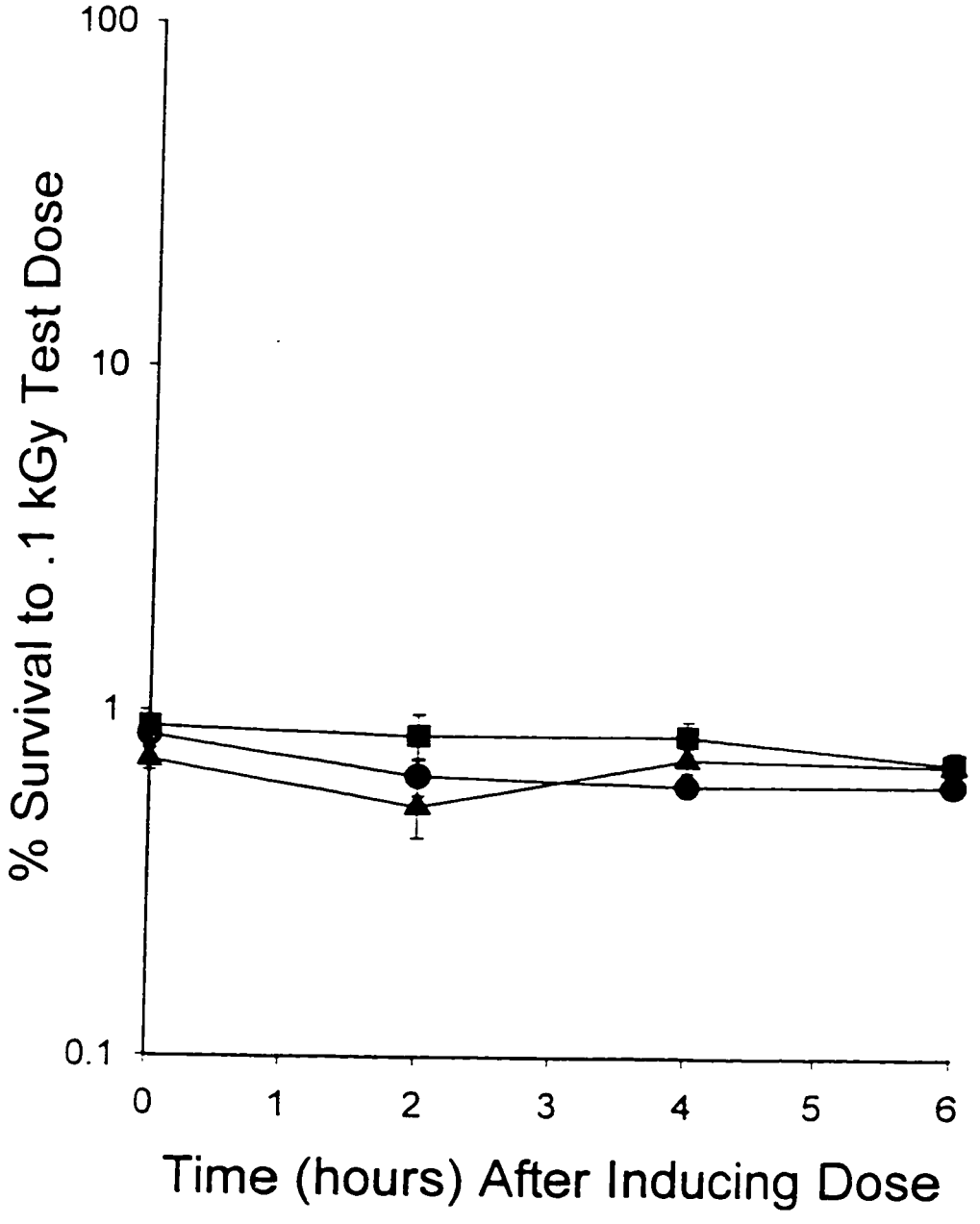


Figure 3.6. Lack of cisplatin-induced radioresistance in recombinational repair deficient MS32 yeast. Cells were treated for 30 minutes at 23°C with 50 µg/ml (triangles) or 400 µg/ml (squares) cisplatin, incubated for various time intervals and then irradiated (0.1 kGy in the presence of oxygen). Control cells not treated with cisplatin are represented by circles.

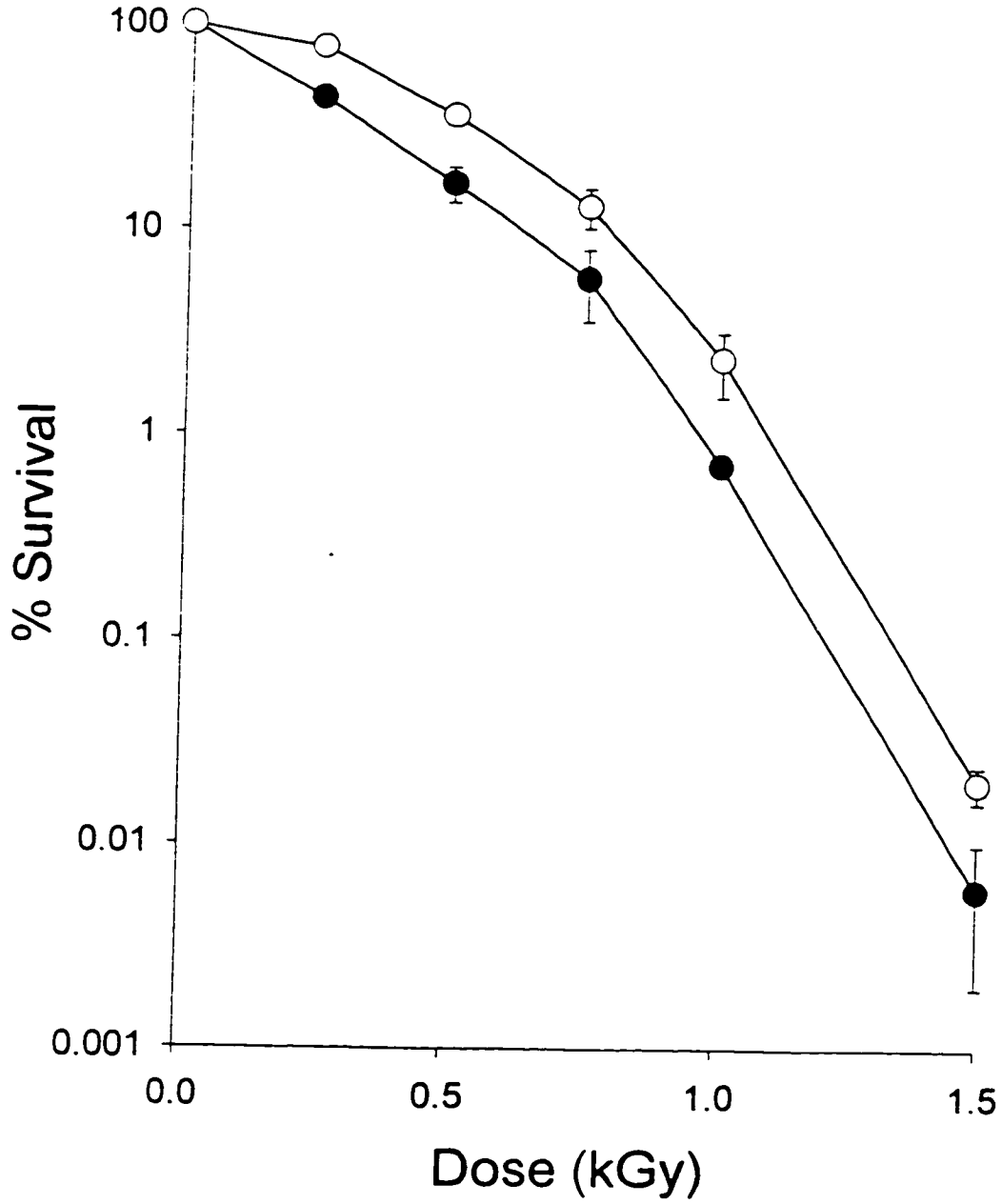


Figure 3.7. Survival of wild type MS33 to ^{60}Co gamma radiation in the presence of oxygen, two hours after treatment at 23°C for 30 minutes with 400 µg/ml cisplatin (open circles). Radiation survival of untreated control cells is represented by closed circles.

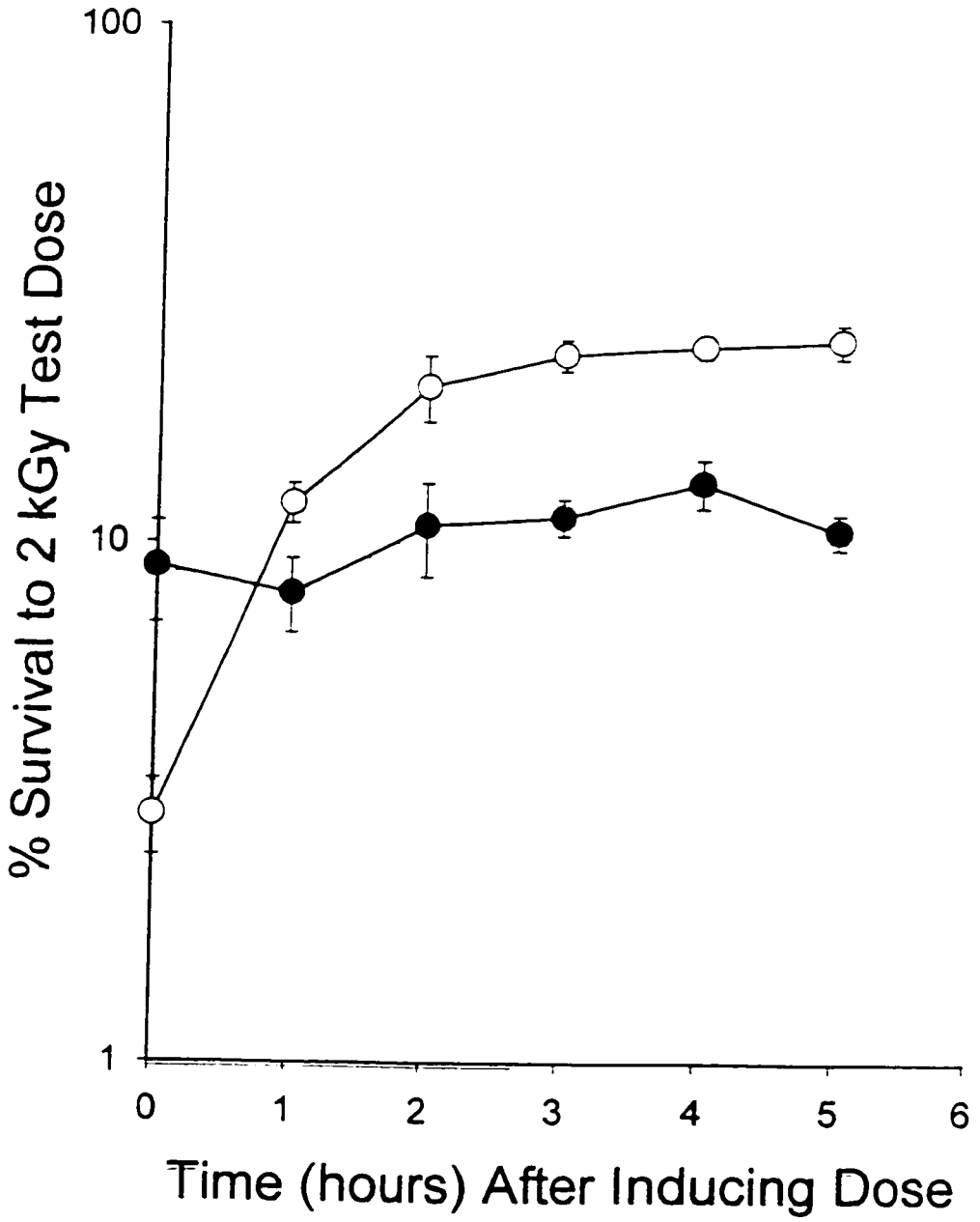


Figure 3.8. Cisplatin-induced radioresistance in wild type MS33 yeast. Cells were treated with 0 µg/ml (closed circles) or 400 µg/ml (open circles) cisplatin, incubated for various time intervals and then irradiated (2 kGy in the presence of nitrogen).

MNNG (Figure 3.9). Simultaneous treatment of cisplatin and MNNG did not result in reduction of MNNG-generated mutation until concentrations of cisplatin exceeded 100 μg/ml. Treatment of cisplatin immediately before MNNG resulted in reduction of mutation at all cisplatin concentrations tested although the reduction was not statistically significant until the concentration exceeded 50 μg/ml. The largest reduction measured (400 μg/ml, 30 minute pretreatment reduced the number of mutations from 1230 ± 100 to 386±91 per 10^6 survivors) was still significantly higher than the spontaneous mutation rate of 4 per 10^6 survivors for wild type MS33 cells. Under the conditions of the cisplatin treatments used in this study, cisplatin alone did not produce any mutations above the spontaneous level in wild type MS33 cells. In contrast, cisplatin treatment alone produced mutations in recombinational repair deficient MS32 cells (Figure 3.10, Table 1).

The combined exposures of cisplatin and MNNG in MS32 cells did not result in a reduction in the number of MNNG-generated mutations (Figure 3.10). Lack of suppression of MNNG-generated mutations was found when the two treatments were given simultaneously and when cisplatin treatment (100 μg/ml for 30 minutes at 23°C) immediately preceded the MNNG treatment (5 or 20 μg/ml for 30 minutes at 23°C) (Table 1). The largest MNNG exposure (20 μg/ml, 30 minutes) generated 148 ± 11.2 mutations per 10^6 survivors. This frequency is significantly less than was observed for MS33 cells (1230 ± 108 mutations per 10^6 survivors) ($p<0.001$).

Discussion

The Rad52 protein forms a recombinational repair complex with at least three other proteins from the Rad52 epistasis group (Hays et al 1995). A primary function of this complex is to mediate an error free recombinational process to repair damaged DNA. This process is critical for repair of potentially lethal DNA double strand breaks or interstrand crosslinks in which the missing or

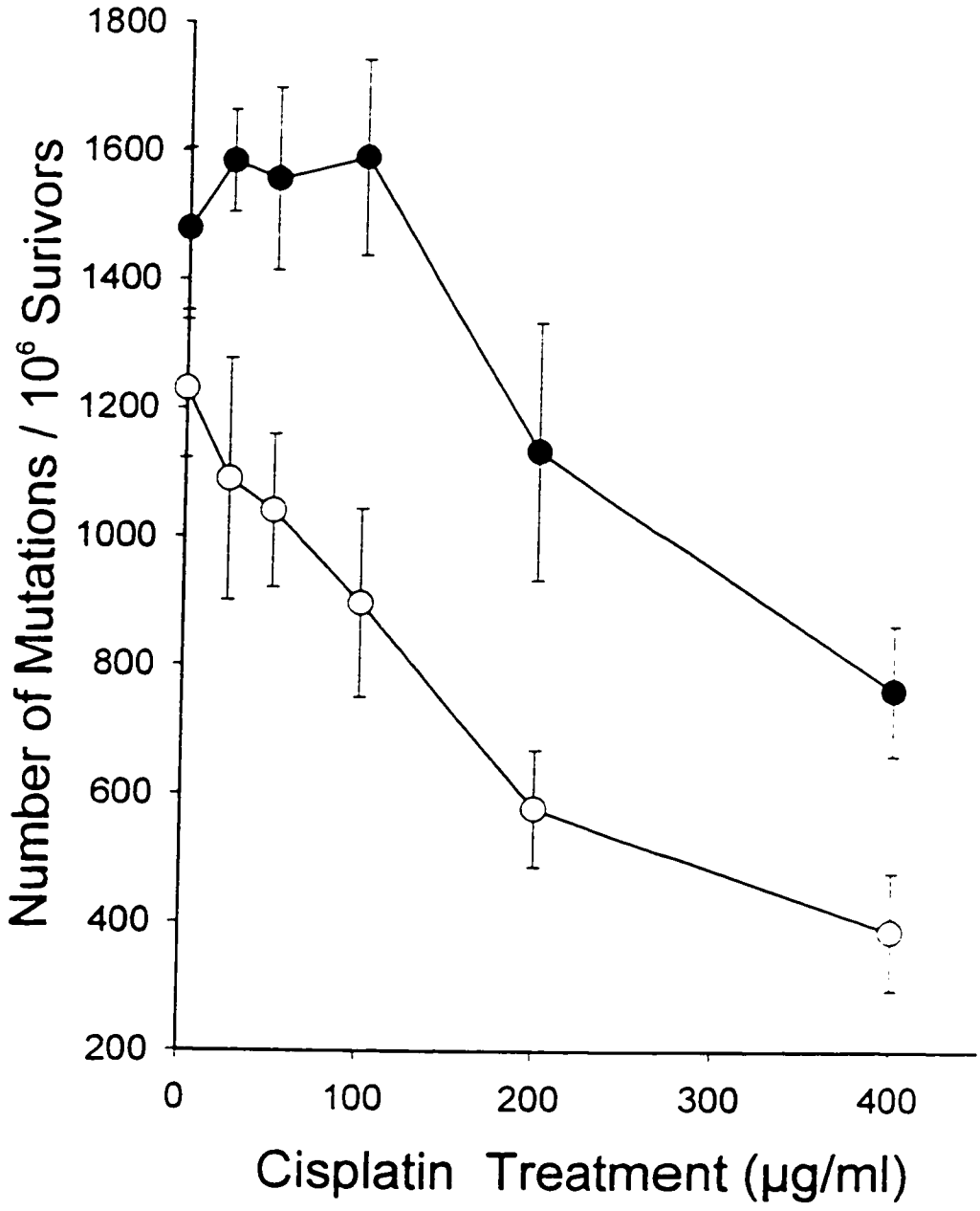


Figure 3.9. Cisplatin suppression of MNNG-generated mutations in wild type MS33 yeast. Cells were treated simultaneously with cisplatin and MNNG (20 µg/ml) (closed circles) or sequentially with cisplatin followed by MNNG (20 µg/ml) (open circles).

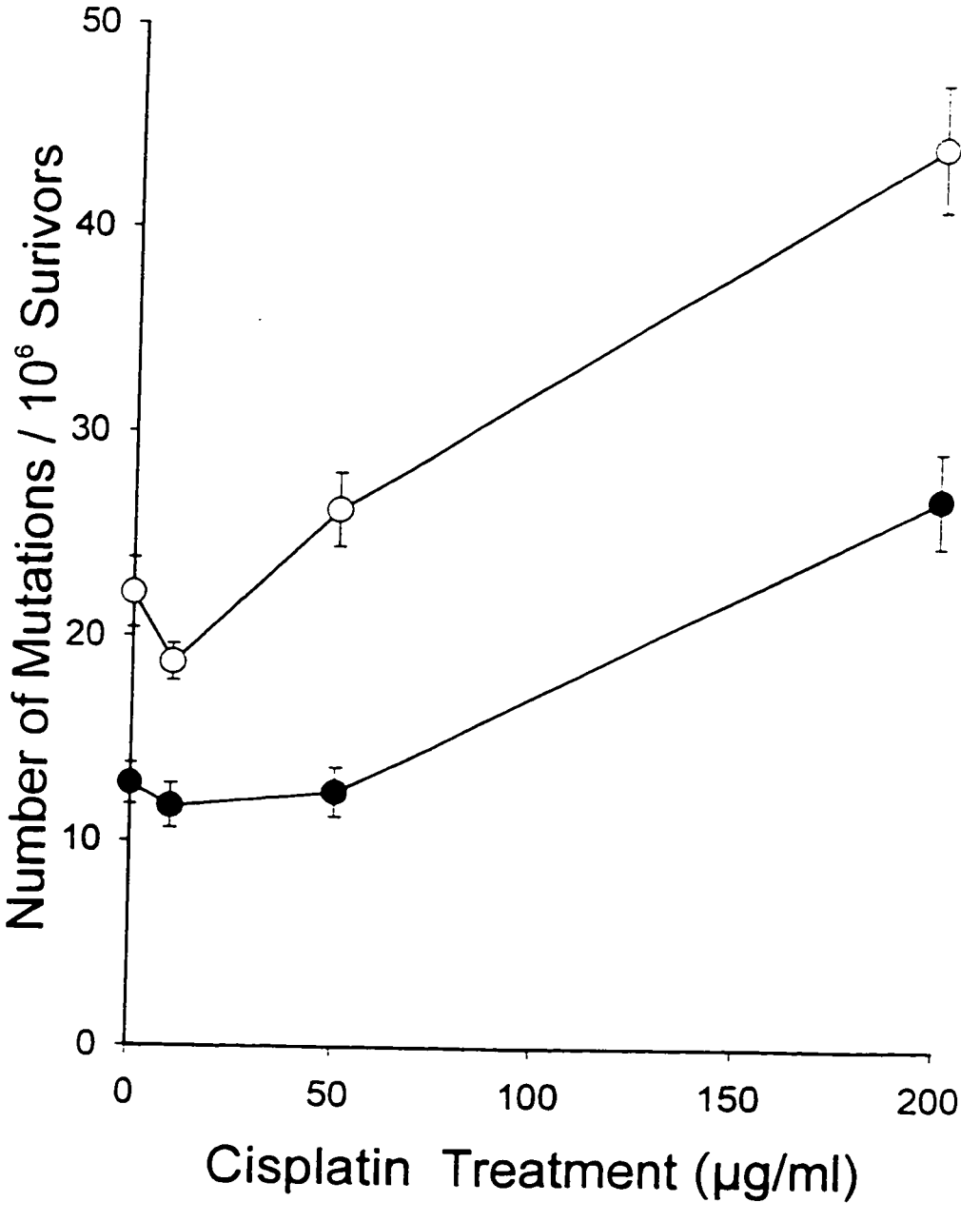


Figure 3.10. Lack of Cisplatin suppression of MNNG-generated mutations in recombinational repair deficient MS32 yeast. Cells were treated with cisplatin alone at 23°C for 30 minutes (closed circles) or simultaneously with MNNG (5 µg/ml) (open circles).

Table 1. Lack of Cisplatin suppression of MNNG-generated mutations in recombinational repair deficient MS32 yeast when cells are treated sequentially with cisplatin followed immediately by MNNG.

Treatment	Number mutations / 10^6 survivors
Control (spontaneous)	15.0 ± 1.7
100 µg/ml cisplatin	20.4 ± .05
5 µ/ml MNNG	23.0 ± 3.4
100 µg/ml cisplatin followed by 5 µg/ml MNNG	32.5 ± 3.0 *
Control (spontaneous)	15.0 ± 1.7
100 µg/ml cisplatin	20.4 ± .05
20 µg/ml MNNG	148.0 ± 11.2
100 µg/ml cisplatin followed by 20 µg/ml MNNG	152.0 ± 1.7 *

* cisplatin treatment did not suppress MNNG-generated mutation ($p < 0.01$)

damaged genetic information must be copied from a duplicate source within the genome.

A defect in Rad52 inhibited induction of radiation resistance by heat (Figure 3.5) as previously shown (Mitchel and Morrison 1982), and cisplatin (Figure 3.6), indicating that Rad52 protein is required to confer radiation resistance and that the mechanism of induced resistance is dependent upon a recombinational repair mechanism. A defective Rad52 conferred a partially thermal tolerant phenotype (Figure 3.2, closed squares) and did not prevent induction of thermal tolerance by heat and radiation (Figures 3.2, 3.3) as previously shown (Mitchel and Morrison 1984) and cisplatin (Figure 3.4), demonstrating that Rad52 is not an essential part of the mechanism of thermal tolerance. Cisplatin adducts signaled induction of thermal tolerance in both MS33 and MS32 to the same extent, but DNA lesions caused by radiation seemed to be more effective for induction of thermal tolerance in MS33 compared to MS32 cells. These results indicate that, for maximal thermal tolerance induction, the signaling mechanism for cisplatin-induced thermal tolerance does not require Rad52, whereas the signaling mechanism for radiation-induced thermal tolerance does. The mechanism of thermal tolerance is known to involve induction of a set of heat shock proteins (hsp) which function to chaperone proteins to cellular compartments, ensure correct protein folding and prevent protein denaturation by heat (Morimoto et al. 1990). Hsp72 is a member of the heat shock protein family and is known to be induced by a variety of stressors including cisplatin (Matsumoto et al. 1996). Therefore, induction of hsp72 and other hsps by cisplatin could account for the thermal tolerant phenotype exhibited by both yeast strains tested.

In wild type MS33, but not in recombinational repair deficient MS32 cells, a cisplatin treatment (400 µg/ml, 30 minutes) resulted in increased resistance to radiation. Increased resistance was observed when the radiation was delivered under oxic (Figure 3.7) or anoxic (Figure 3.8) conditions, indicating that lesions

produced under both conditions were processed by the same inducible repair process. It is possible that the radiation resistant phenotype induced by cisplatin treatment may be the result of enhanced expression of cellular thiols such as metalothioneins or glutathione, which sequester platinum and block the formation of cisplatin-monofunctional DNA adducts and the conversion of mono- to bifunctional DNA adducts (Odenheimer and Wolf 1982, Zamble and Lippard 1995, Lazo and Pitt 1995). However, since recombinational repair deficient MS32 cells are not deficient in thiol containing compounds, it is unlikely that thiols play a dominant role in cisplatin-induced radiation resistance.

Cisplatin-induced radiation resistance in wild type yeast cells is most likely due to the upregulation of their recombinational repair capacity. This interpretation is supported by the suppression of MNNG-generated mutations by a prior exposure to cisplatin in MS33 cells (Figure 3.9) and not in recombinational repair deficient MS32 cells (Figure 3.10, Table 1). Suppression of MNNG-generated mutations is believed to be the result of competition between error-free and error-prone repair systems (Figure 3.1) (Mitchel and Morrison 1986, 1987). MNNG lesions induce the error-prone system and cause mutations. If cisplatin adducts signal induction of an error-free repair system, which is capable of repairing cisplatin and MNNG lesions, then fewer MNNG-generated mutations would result. In this study, increasing doses of cisplatin resulted in more MNNG lesions being processed by the error-free repair pathway and a reduction in the frequency of MNNG-generated mutations (Figure 3.9).

In wild type MS33 cells, both cisplatin/MNNG treatment regimes (simultaneous or sequential treatment of cisplatin and MNNG) resulted in a reduction in the number of mutations, indicating that cisplatin adducts had caused upregulation of recombinational repair capacity. At the highest dose of cisplatin, the number of mutations was still significantly higher than the spontaneous level of 4 mutations per 10^6 MS33 survivors. Induction of an error-free system is paralleled by a relative reduction, but not elimination, in the

number of lesions processed by the error-prone system. Therefore, it is likely that even at maximal induction of the error-free system, some MNNG lesions will be repaired by the error-prone system (Mitchel and Morrison 1987). It is also possible that some cisplatin adducts can be processed by the error-prone repair pathway and contribute to the pool of mutations observed.

Exposure of wild type MS33 cells to cisplatin before MNNG led to suppression of MNNG-generated mutations at a lower cisplatin concentration than simultaneous treatment of cells with cisplatin and MNNG. The sequential treatment schedule also reduced the number of mutations to a greater extent. These differences may be due to the different spectrums of cisplatin adducts at the time of MNNG treatment. When cisplatin and MNNG were delivered simultaneously, induction of an error-free system can be attributed mainly to cisplatin monofuctional adducts. In contrast, when MNNG was delivered after the cisplatin treatment, there was more time for conversion of cisplatin mono- to bifunctional adducts and it is possible that the presence of bifunctional adducts resulted in a more effective induction of error-free recombinational repair capacity. The conversion of monofuctional to bifunctional adducts has been studied (Eastman 1986, Bruhn et al. 1990) and results show that for short cisplatin treatments (15 or 30 minutes) the ratio of mono- to bifunctional adducts is approximately 50:50. During longer incubation times (60 minutes) the ratio changes to 30:70, mono- to bifunctional adducts, and at longer incubation times (2 hours or more) monofuntional adducts constitute only a few percent of the total cisplatin adducts. Further, it was reported that bifunctional, but not monofuctional, cisplatin adducts produce dramatic topological perturbations in the DNA helix by creating kink angles of 40 to 60° (Bruhn et al. 1990). In the present study, during the 30 minute cisplatin exposure and subsequent 30 minute MNNG treatment, some cisplatin monofuctional adducts, which do not appear to distort the DNA helix (Bruhn et al. 1990), would have been converted into helix distorting, bifunctional adducts, and may have resulted in more

effective signaling of an error free recombinational repair system repair system. These perturbations in DNA topology may serve as the signal for induction of a radiation resistance mechanism as has been suggested by a study comparing topoisomerase mutants to wild type yeast cells for induction of radiation resistance (Boreham et al. 1991).

Exposure of wild type MS33 cells to MNNG alone resulted in significantly more mutations than was observed for recombinational repair deficient MS32 cells. This result has been previously reported (Mitchel and Morrison 1986) but the reason for fewer mutations in MS32 cells is unknown. It has been proposed that the reduced number of MNNG-generated mutations is due to a Rad52-independent process, although this mechanism has not been characterized (Boreham et al. 1991). It is also possible that Rad52 functions to couple a recombinational repair system to repair of lesions in actively transcribed DNA, and that in recombinational repair deficient (rad52) cells, this connection is non-existent and the repair of lesions in actively transcribed DNA is carried out by the global repair system. In recombinational repair deficient (rad52) cells, MNNG lesions in the histidine gene (the marker used for expression of mutations) are not preferentially repaired but are repaired at the same rate as actively transcribed genes. Therefore, the overall mutation frequency in non-transcribed DNA is lower in rad52 cells than in wild type cells in which damage is preferentially repaired in actively transcribed genes (see chapter 2 for discussion).

In summary, cisplatin adducts reduced the number of MNNG-generated mutations in wild type MS33 cells, but not in recombinational repair deficient MS32 cells. The inability to suppress MNNG-generated mutations in MS32 cells demonstrated that Rad52 protein was required, thus implicating recombinational repair and indicates that cisplatin-DNA adducts signal the induction of an error free recombinational repair process as well as a general stress or heat shock-like response.

References

Averbeck, A. and S. Averbeck. Induction of the genes RAD54 and RNR2 by various DNA damaging agents in *Saccharomyces cerevisiae*. Mut. Res. 315: 123-138 (1994).

Boreham, D.R. and R.E.J. Mitchel. DNA lesions that signal the induction of radioresistance and DNA repair in yeast. Radiat. Res. 128: 19-28 (1991).

Boreham, D.R., A. Trivedi, P. Weinberger and R.E.J. Mitchel. The involvment of topoisomerases and DNA polymerase I in the mechanism of induced thermal tolerance and radiation resistance in yeast. Radiat. Res. 123: 203-212 (1990).

Bruhn, S.L., J.H. Toney and S.J. Lippard. Biological processing of DNA modified by platinum compounds. Progress in Inorganic Chemistry: Bioinorganic chemistry 38: 477-517 (1990).

Eastman, A. Reevaluation of interaction of cis-dichloro(ethylenediammine)-platinum(II) with DNA. Biochem. 25: 3912-3915 (1986).

Game, J.C. DNA double-strand breaks and the RAD50-57 genes in *Saccharomyces*. Seminars in Cancer Biol. 4: 73-83 (1993).

Hays, S.L., A.A. Fermenich and P. Berg. Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55, and Rad57 proteins. Proc. Natl. Acad. Sci. USA 92: 6925-6929 (1995).

Lazo, J.S. and B.R. Pitt. Metallothioneins and cells death by anticancer drugs. Annu. Rev. Pharmacol. Toxicol. 35:635-653 (1995).

Maga, J.A. and K. McEntee. Response of S. cerevisiae to N-methyl-N'-nitro-N-nitrosoguanidine: mutagenesis, survival and DDR gene expression. Mol. Gen. Genet. 200: 313-321 (1985).

Matsumoto, H., S. Hagashi, H. Shioura, T. Ohtsubo, T. Ohnishi and E. Kano. Suppression of heat-induced hsp72 accumulation by cisplatin in human glioblastoma cells. Cancer Letters 110: 253-257 (1996).

McClanahan, T. and K. McEntee. DNA damage and heat shock dually regulate genes in Saccharomyces cerevisiae. Mol. Cell. Biol. 6: 90-96 (1986).

McClanahan, T. and K. McEntee. Specific transcripts are elevated in *Saccharomyces cerevisiae* in response to DNA damage. Mol. Cell. Biol. 4: 2356-2363 (1984).

Mitchel, R.E.J. and D.P. Morrison. An oxygen effect for gamma-radiation induction of radiation resistance in yeast. Radiat. Res. 100: 205-210 (1984).

Mitchel, R.E.J. and D.P. Morrison. Heat-shock induction of ionizing radiation resistance in *Saccharomyces cerevisiae*, and correlation with stationary growth phase. Radiat. Res. 90: 284-291 (1982).

Mitchel, R.E.J. and D.P. Morrison. Inducible DNA repair systems in yeast: competition for lesions. Mut. Res. 183: 149-159 (1987).

Mitchel, R.E.J. and D.P. Morrison. Inducible error-prone repair in yeast. Suppression by heat shock. Mut. Res. 159: 31-39 (1986).

Morimoto, R.I., A. Tissieres and C. Georgopoulos. The stress response, function of the proteins, and perspectives, I. In Stress Proteins in Biology and Medicine (Eds. R.I. Morimoto, A. Tissieres and C. Georgopoulos) Cold Spring Harbor Laboratory Press, New York (1990).

Odenheimer, B. and W. Wolf. Reactions of cisplatin with sulfur-containing amino acids and peptides I. Cysteine and glutathione. Inorganica Chimica Acta 66: 141-143 (1982).

Polakowska, R., G. Perozzi and L. Prakash. Alkylation mutagenesis in *Saccharomyces cerevisiae*: lack of evidence for an adaptive response. Curr. Genet. 10: 647-655 (1986).

Resnick, M.A. The repair of double-strand breaks in DNA: A model involving recombination. J.Theor. Biol. 59: 97-106 (1976).

Ruby, S.W. and J.W. Szostak. Specific *Saccharomyces cerevisiae* genes are expressed in response to DNA-damaging agents. Mol. Cell. Biol. 5: 75-84 (1985).

Sancar, A. and G.B. Sancar. DNA repair enzymes. Ann. Rev. Biochem. 57: 29-67 (1988).

Szostak, J.W., T.L. Orr-Weaver, R.J. Rothstein and F.W. Stahl. The double-strand break model for recombination. Cell 33: 25-35 (1983).

Yarosh, D.B. The role of O^6-methylguanine-DNA methyltransferase in cell survival, mutagenesis and carcinogenesis. Mut. Res. 145: 1-16 (1985).

Zamble, D. and S. Lippard. Cisplatin and DNA repair in cancer chemotherapy. TIBS 20: 435-439 (1995).

Chapter 4. Rearrangement of Human Cell Homologous Chromosome Domains in Response to Ionizing Radiation[1]

Introduction

Studies have shown that human chromosomes within the interphase nucleus are spatially organized as territories or domains (Cremer et al., 1988; Pinkel et al., 1986; Manuelidis and Borden, 1988, Trask et al., 1988, Zorn et al., 1979). These studies have also examined the positions of homologous and non-homologous chromosome domains relative to each other. Homologous and non-homologous chromosome domains do not appear to be located in rigid positions at set distances from each other (Emmerich et al., 1989, Rappold et al., 1984). Instead, a large range of distances between chromosome domains in interphase nuclei has been reported (Emmerich et al., 1989, Ferguson and Ward, 1992, Haaf and Schmid, 1991, Lichter et al., 1988, Popp et al., 1990, Rappold et al., 1984, Vourc'h et al., 1993) and it has been suggested that this indicates a dynamic nuclear environment, influenced by factors such as cell metabolism and gene activity. It is unclear whether the organization of chromosome domains within the interphase nucleus is random or non-random. While the large variability in inter-chromosome domain distances suggests a random arrangement, there are reported examples of non-random nuclear organization. In human sperm nuclei (which may be a special case), chromosomes are looped into a hairpin-like configuration with the ends of each chromosome located at the nuclear periphery and the centromeres located in the interior (Zalensky et al., 1995). Also, depending on cell type, telomeres and centromeres appear to be non-randomly situated in interphase nuclei (Blackburn and Szostak, 1984, Ferguson and Ward, 1992, Walker et al., 1991, Zalensky et al., 1995), and there have been a few reports of homologous chromosome pairing in both prokaryotic

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[1] Dolling, J-A., D.R. Boreham, D.L. Brown, G.P. Raaphorst and R.E.J. Mitchel. Rearrangement of human cell homologous chromosome domains in response to ionizing radiation. Int. J. Radiat. Biol. 72: 303-311 (1997).

(Minton and Daly, 1995) and eukaryotic nuclei (Hadlaczky et al., 1986, Hilliker and Appels, 1989).

Assessing the spatial organization of chromosome domains has been facilitated by fluorescence in situ hybridization (FISH) of specific whole chromosomes (Pinkel et al., 1986), confocal laser scanning microscopy (Oud et al., 1989) and fluorescence microscopy coupled with computerized image analysis (Hilliker and Appels, 1989, Hiraoka et al., 1987). Initial experiments designed to map the interphase positions of chromosome domains using two-dimensional measurements were limited by the inability to resolve domains superimposed within the nucleus. Using computer assisted three-dimensional reconstruction technology, positions of chromosome domains can now be accurately mapped within the interphase nucleus.

The position of a chromosome domain with respect to its homolog has not been linked to any specific functional property. A recent study of homologous chromosome pairing in the radiation resistant bacterium *Deinococcus radiodurans* has raised the possibility of a link between the positioning of homologous chromosomes close to one another and resistance to DNA damage induced by ionizing radiation (Minton and Daly, 1995). The authors proposed a model in which pairing of homologous chromosomes mediated by Holliday junctions along the entire length of the chromosomes would permit efficient repair of potentially lethal radiation-induced DNA double strand breaks (dsbs). We propose that a similar process of homologous chromosome pairing, to facilitate recombinational repair of DNA dsbs, may exist in other organisms including cells derived from mammalian tissues (Hiraoka et al., 1987, Choulika et al., 1995). We have tested this hypothesis in cultured human skin fibroblasts and lung endothelial cells. Using conventional epifluorescence and confocal microscopy, we have examined the relative positions of homologous chromosomes in human cells to determine if homologous chromosomes rearrange within the interphase nucleus in response to a radiation exposure.

Materials and Methods

Cell lines and Culture Conditions. Human diploid skin fibroblast cell lines designated AG1522 (passage 8) and GM38 (passage 10) were purchased from the NIA Cell Repository and NIGMS Cell Repository (Coriell Institute for Medical Research, Camden, New Jersey) respectively. The cell lines were maintained in D-MEM/F-12 (GIBCO) supplemented with 15% fetal bovine serum, 2mM glutamine and 25 mg/ml gentamicin sulfate (GIBCO) and incubated at 37°C in a humidified atmosphere of 2% CO_2 and 98% air. Prior to irradiations, cells were grown to confluence. For the AG1522 cell line, this was achieved by inoculating 2.0 X 10^4 cells per 2.5 cm^2 chambered slide, changing the culture media five and eight days after inoculation and then irradiating cells 48 hours after the last media change. At the time of irradiation the cell cycle distribution of AG1522 was typically 87% G_0/G_1, 7%S and 6% G_2M as determined by flow cytometric analysis. For the GM38 cell line, confluent cells were obtained by carrying out an additional media change 11 days after inoculation and then irradiating 48 hours later. At the time of irradiation, the cell cycle distribution was typically 90% G_0/G_1, 3% S and 7% G_2M.

A human lung endothelial cell line, HMVEC-L (Clonetics) was maintained in Endothelium Cell Basal Medium (Clonetics) supplemented with 2% fetal bovine serum, 10 ng/ml epidermal growth factor, 1 mg/ml hydrocortisone, 4 mg/ml bovine brain extract, 50 mg/ml gentamicin and 50 ng/ml amphotericin-B and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were grown to confluence as per the manufacturer's protocol. Briefly, 5 X 10^4 cells were inoculated into 2.5 cm^2 chambered slides and the media was changed two, five and seven days after inoculation. Irradiations were carried out 48 hours after the last media change. At the time of irradiation, the cell cycle distribution was typically 96% G_0/G_1, 2% S and 2% G_2/M.

Irradiation. Attached, confluent human skin fibroblast cell lines, AG1522 and GM38, were irradiated in a ^{60}Co gammacell 200 (Atomic Energy of Canada

Ltd.) at a dose rate of 0.8 Gy/minute. All irradiations were carried out with the cells at 0°C in phosphate buffered saline (PBS). After irradiation, cells were either immediately fixed or incubated at 37°C in complete media for two hours and then fixed. Cell cultures were fixed for five minutes with a 1:1 mixture of 0°C PBS and methanol:acetic acid (3:1 v/v) followed by 10 minutes with methanol:acetic acid (3:1 v/v) at room temperature. The fixative was then removed and the slides were air dried.

The endothelial cell line HMVEC-L was irradiated in complete media at 37°C using an X-ray unit (Siemens Electric Company, Germany) at a dose rate of 4.0 Gy/min. After irradiation, cells were then either immediately fixed or incubated in complete media at 37°C for one or two hours and then fixed as described above.

Fluorescence in situ Hybridization for Detection of Specific Chromosome Domains in Interphase Nuclei. Slide preparations of cell cultures were prepared for fluorescence in situ hybridization (FISH) detection of chromosome domains using a whole chromosome painting system (Oncor). Slide preparations were treated with RNAase (1.0 mg/ml) in 2X SSC (0.3M sodium chloride, 0.04M sodium citrate); rinsed in 2X SSC; dehydrated sequentially in 70%, 80%, and 95% cold ethanol at 0°C for two minutes each; air dried;denatured for two minutes at 70°C in 70% formamide/2X SSC; dehydrated sequentially in 70%, 80%, 90% and 100% cold ethanol for two minutes each and air dried. The chromosome paint mixture (biotin-labeled chromosome 7 or digoxygenin-labelled chromosome 21) was denatured at 70°C for 10 minutes, partially renatured at 37°C for 2.5 hours and applied to 37°C prewarmed slides. Coverslips were applied and sealed with rubber cement. Hybridization was carried out at 37°C for 16 hours in a humidified chamber. Coverslips were removed and slides were washed in 50% formamide/2X SSC at 43°C for 15 minutes followed by a 0.1X SSC wash at 60°C for 15 minutes. Hybridization of chromosome 7 specific paint to interphase nuclei was detected by first incubating slides with a protein

blocking agent (Oncor kit reagent) for five minutes at room temperature followed by incubation with fluorescein isothiocyanate (FITC)-labeled avidin in a humidified chamber for 20 minutes. Slides were then rinsed in phosphate buffered detergent (PBD) (Oncor kit reagent) and coverslips were mounted with propidium iodide/antifade solution (Oncor). Hybridization of chromosome 21 specific paint to interphase nuclei was detected by incubating the slides at 37°C for five minutes with FITC-labeled anti-digoxygenin antibody. Slides were then rinsed with PBD and coverslips were mounted with propidium iodide/antifade solution.

Determination of Homologous Chromosome Domain Locations Within Interphase Nuclei. Standard epifluorescence microscopy (Zeiss Axiophot, Germany) was used to view homologous chromosome domains within the interphase nuclei of AG1522 and GM38 human skin fibroblast cells. Homologous chromosome domains were defined to be situated 'apart' from each other if they were separated by a distance equal to or greater than the width of one domain. Conversely, the domains were considered to be located 'together' if the distance between them was less than the width of one domain. Experiments were replicated three times and the total number of nuclei examined per treatment is given in Tables I and II.

Scanning confocal fluorescence microscopy (Leica, Germany) was used to view homologous chromosome domains within the interphase nuclei of the HMVEC-L endothelial cells. Each nucleus was scanned from top to bottom with six to eight 0.5 μm optical sections. The images were collected and a composite image was constructed. The distance between centres of homologous chromosome domains, as determined by eye (from an algorithm image) to be the most intense fluorescent signal, was measured using a computerized imaging system and data storage software. The experiment was replicated twice and the total number of nuclei examined per treatment is given in Table III.

Determination of Interphase Nucleus Size. The length and width of HMVEC-L endothelial cell nuclei were determined by measuring images produced by epifluorescence photomicroscopy. Images of FITC-labeled and propidium iodide counter-stained nuclei were photographed at 40X magnification on 35 mm Kodak Ektachrome ASA100 coloured slide film. The projected images were recorded and the length and width of 115 consecutive nuclei were measured using a calibrated micrometer.

Statistics. Data were analyzed using the z-test (Miller and Freund 1977) unless otherwise stated.

Results

The results show that the relative positions of homologous chromosome domains changed with time after irradiation. In unirradiated, control fibroblast cell cultures of AG1522, chromosome 7 domains in about half of the nuclei were considered to be located apart from each other and in the other half, the domains were located together. After 4.0 Gy irradiation and two hours of incubation at 37°C, significantly more domains were located together ($p<0.01$) (Table I). A similar trend was observed for chromosome 21 domains within the nuclei of GM38 fibroblast cells after 0.5 Gy and 4.0 Gy exposures (Table II).

Confocal images of HMVEC-L nuclei were reconstructed (extended focus of six to eight sections) and an example is shown in Figure 4.1. Figure 4.1A is a top view of chromosome 21 domains located apart from each other within a cell nucleus (i.e. the domain centres were equal to or greater than 4.0 mm apart). A side view of the same nucleus is shown in Figure 4.1B. Figures 4.1C and 4.1D are examples of homologous chromosome domains located together (i.e. the domain centres were closer than 4.0 μm) as a top view and side view respectively within a cell nucleus. The distances between homologous chromosome 21 domains were recorded to the nearest tenth of a micrometer. In unirradiated, control cell cultures approximately two thirds of the nuclei had

Figure 4.1. Two dimensional images of chromosome 21 domains in HMVEC-L nuclei. Chromosome domains (indicated by arrows) located apart from each other are depicted in top view (A) and side view (B). Chromosome domains located together are depicted in top view (C) and side view (D).

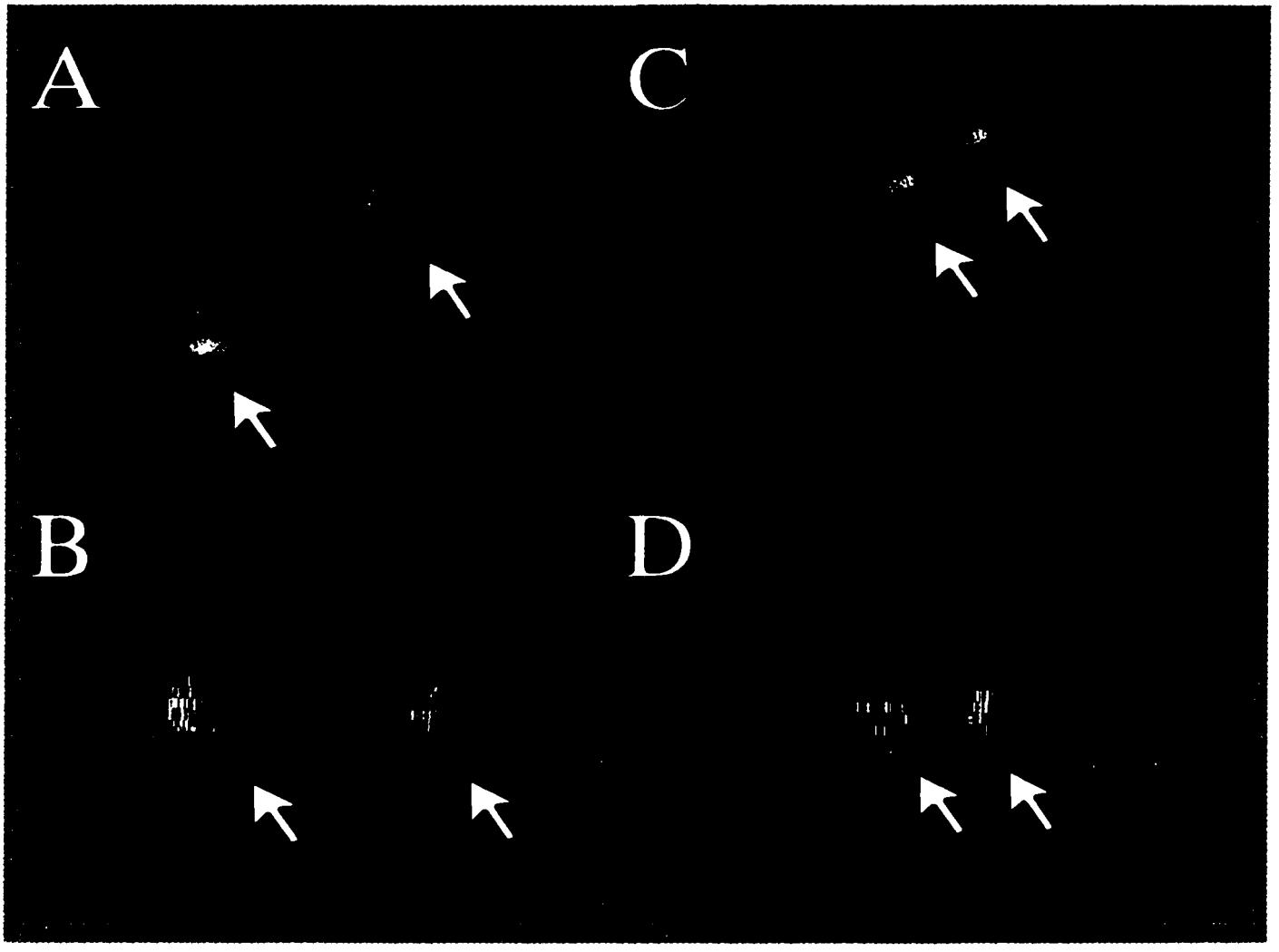
A
C
B
D

Table I. Change in the Relative Positions of Homologous Chromosome 7 Domains in AG1522 Human Fibroblast Cells After Radiation Exposure.

Treatment	Together* (%)	Apart† (%)
0 Gy, (n=151)	48.3 ± 2.9	51.7 ± 2.9
4 Gy, 2 hours at 37°C (n=94)	80.2 ± 6.2	19.3 ± 5.8 ($p<0.01$)

*Chromosome 7 domains were recorded as together if the distance between them was less than the width of one domain.

†Chromosome 7 domains were recorded as apart if the distance between them was greater than or equal to the width of one domain.

'n' represents number of nuclei scored

Table II. Change in the Relative Positions of Homologous Chromosome 21 Domains in GM38 Human Fibroblast Cells After Radiation Exposure.

Treatment	Together* (%)	Apart† (%)
0 Gy, (n=566)	45.8 ± 2.1	54.1 ± 2.3
0.5 Gy, 2 hours at 37°C (n=513)	63.9 ± 4.5	35.8 ± 4.9 ($p<0.01$)
4 Gy, 2 hours at 37°C (n=491)	59.6 ± 0.8	40.3 ± 0.8 ($p<0.01$)

*Chromosome 21 domains were recorded as together if the distance between them was less than the width of one domain.

†Chromosome 21 domains were recorded as apart if the distance between them was greater than or equal to the width of one domain.

Table III. Change in the Relative Positions of Homologous Chromosome 21 Domains in HMVEC-L Human Endothelial Cells After Radiation Exposure.

Treatment	Together* (%)	Apart† (%)
control (n=72)	36.0 ± 1.1	64.0 ± 1.2
4 Gy, no incubation (n=61)	32.9 ± 0.6	67.1 ± 0.6 ($p<0.01$)
4 Gy, 1 hour at 37°C (n=61)	55.6 ± 2.8	44.4 ± 2.8 ($p<0.05$)
4 Gy, 2 hours at 37°C (n=103)	69.6 ± 2.6	30.5 ± 2.6 ($p<0.01$)
1 Gy, 1 hour at 37°C (n=60)	46.5 ± 2.3	53.6 ± 2.3 ($p<0.05$)
1 Gy, 2 hours at 37°C (n=64)	53.7 ± 3.3	46.4 ± 3.3 ($p<0.05$)

*Chromosome domains were recorded as together if the distance between domain centres was less than 4.0 μm.

†Chromosome domains were recorded as apart if the distance between domain centres was greater than or equal to 4.0 μm.

chromosome 21 pairs which were categorized as apart and the remainder were together (Table III). This was not significantly different from cultures fixed immediately after irradiation ($p<0.01$). However, with incubation time after irradiation, the percentage of nuclei with chromosome 21 domains together increased after one hour and was further increased after two hours of post-irradiation incubation at 37°C ($p<0.05$ - $p<0.01$) (Table III). These cells showed an apparent dose response, with a greater change observed at the higher dose.

When the data for each treatment (60-103 nuclei examined per treatment) were plotted as a cumulative frequency relative to the distance between each pair of homologous domains (Figure 4.2), there was no difference between unirradiated cells and cells that were irradiated and fixed immediately after irradiation (Figure 4.2A). The results plotted for a treatment of 1.0 Gy followed by a one hour incubation were not obviously different from irradiated cells fixed immediately after irradiation (Figure 4.2B). However, there were clear differences in domain distances for 4.0 Gy treatments followed by one and two hours post-irradiation incubation and for 1.0 Gy exposures followed by two hours of incubation (Figure 4.2B and 4.2C). For all treatments, the results were significantly different from the control curve at a domain distance of 4.0 μm (Table III).

From cumulative frequency curves (Figure 4.2) it may be difficult to visualize the distribution of chromosome distances, and therefore the data for each treatment were also plotted as histograms to show the range of distances between homologous chromosome 21 domains in HMVEC-L cell nuclei (Figure 4.3). Since measurements from control cell nuclei and from nuclei preserved immediately after irradiation were not different (Figure 4.2A) the data were combined and depict a large range of inter-domain distances with a mean distance of 5.4 μm (Figure 4.3A). The distribution of inter-domain distances for 1.0 Gy followed by one hour of incubation was similar to controls with a mean

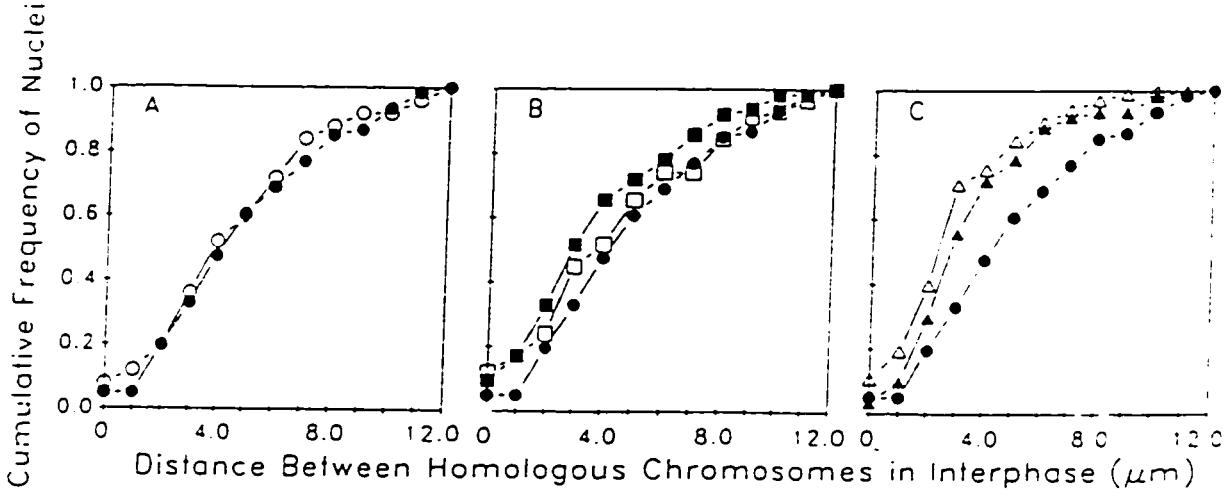

Figure 4.2. Distance between homologous chromosome 21 domains in HMVEC-L nuclei expressed as cumulative frequencies. (A) Unirradiated, control cells (open circles) were compared to irradiated cells preserved with fixative immediately after irradiation (closed circles). (B) Cells were exposed to 1.0 Gy irradiation and incubated for one (open squares) or two (closed squares) hours after irradiation. (C) Cells were exposed to 4.0 Gy irradiation and incubated for one (closed triangles) or two (open triangles) hours after irradiation.

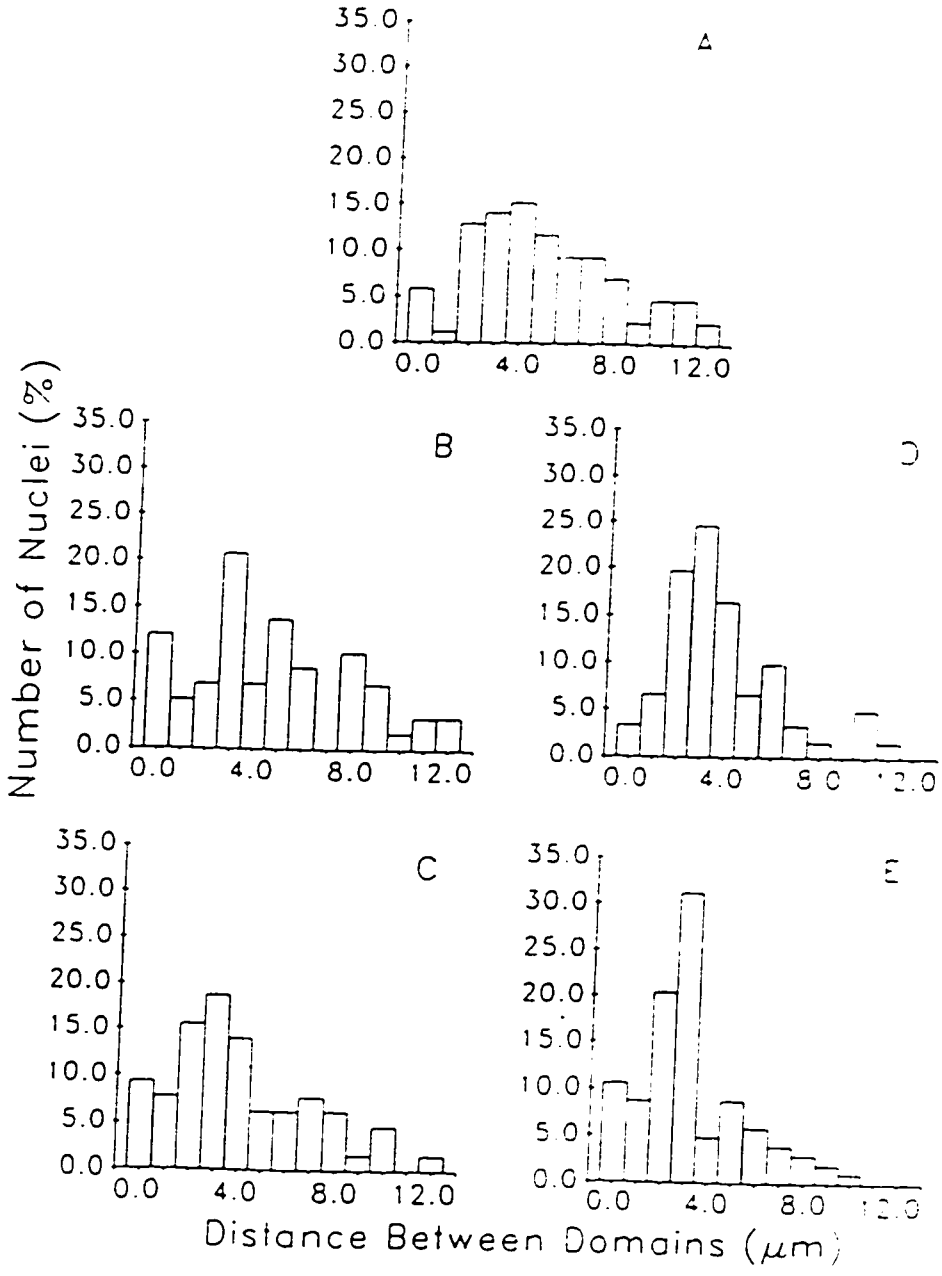

Figure 4.3. Distribution of distances between homologous chromosome 21 domains in HMVEC-L nuclei. (A) Data were combined for unirradiated control cells and cells preserved with fixative immediately after irradiation. (B and C) Cells were exposed to 1.0 Gy and incubated for one and two hours respectively. (D and E) Cells were exposed to 4.0 Gy and incubated for one and two hours respectively.

distance of 5.0 μm (Figure 4.3B), but was skewed towards shorter distances for cell cultures exposed to 1.0 Gy followed by two hours of incubation (Figure 4.3C) or exposed to 4.0 Gy followed by one or two hours post-irradiation incubation (Figure 4.3D and 4.3E). The mean distance was shifted to 4.4 μm, 4.1 μm and 3.6 μm for 1.0 Gy followed by two hours incubation, 4.0 Gy followed by one hour and 4.0 Gy followed by two hours incubation respectively.

The average size of HMVEC-L nuclei from control cells and cells irradiated with 4 Gy and immediately fixed were not different. The size of nuclei in cells that were irradiated and incubated for two hours were significantly larger ($P<0.001$, Student's t-test) than nuclei in control cells. The mean nuclei length of control and irradiated cells followed by two hours of incubation was 7.6 μm± 1.1 and 8.7 μm ± 1.3 respectively. The corresponding widths were 4.6 μm ± 0.6 for control cells and 5.4 μm ± 0.7 for irradiated cells.

Discussion

This paper presents information on the spatial organization of homologous chromosome domains in the interphase nuclei of control and irradiated human cells. Using FISH labeling of whole chromosomes in combination with conventional and confocal microscopy, we observed a large range of distances between homologous chromosome domains in unirradiated cells, from clearly separate and non-overlapping domains to domains which appeared to share some common nuclear space. These observations are in agreement with work by others investigating non-irradiated cells (Rappold et al., 1984, Trask et al., 1993, Vourc'h et al., 1993). This range was the same for cells fixed immediately after irradiation. However, irradiated cells that were incubated under normal growth conditions after irradiation had domains that were repositioned closer to each other. In addition, homologous chromosome domains rearranged within interphase nuclei of all three cell lines tested, indicating that the mechanism may be conserved in various tissue types.

There is a possibility that these domains were closer together because of an overall reduction in the size of nuclei. However, it has been reported that irradiated nuclei may enlarge or swell with incubation time after irradiation (Marelius, 1995). Our results confirmed that after two hours incubation irradiated nuclei were larger. A general increase in the size of nuclei of irradiated cells may be expected to cause a concomitant general increase in the distances between homologous chromosome domains. Our results, however, showed a significant decrease in domain distances. Since post-irradiation rearrangement of homologous chromosome domains was dependent on incubation time and was inversely related to nucleus size, the mechanism probably involved an active process.

We were concerned that cell cycle position may have been a factor in determining chromosome domain distribution and radiation-induced redistribution. It has been reported in mouse lymphocytes, however, that there is no significant difference in the relative distances between homologous chromosomes at any stage in the cell cycle (Ferguson and Ward, 1992). Nevertheless, in an attempt to minimize any possible influence of cell cycle phase, we grew cells to confluence so that the majority of cells would be in G_1/G_0 of the cell cycle. Under our growth conditions, we observed that the AG1522 and GM38 fibroblast cell lines did not seem to form a confluent G_0/G_1 monolayer when cultured in chambered tissue culture slides. Instead, these cells overlapped each other with time in culture, forming multicellular layers. This continued cell division, following formation of a confluent monolayer, resulted in a number of cells in S and G_2/M cell cycle phases. Consequently, HMVEC-L endothelial cells with preferred growth characteristics (cells were more uniform in shape and did not overlap compared to AG1522 and GM 38 cells) were used. Microscopic examination of HMVEC-L cells confirmed the formation of a true confluent monolayer, and cell cycle analysis demonstrated that almost all (96%)

cells were in G_0/G_1. However, regardless of the cell cycle distributions exhibited by the cell lines tested, there was no qualitative difference in results.

It was also possible that the fixation and FISH protocols utilized may have influenced the positions of chromosomes within the interphase nucleus (Emmerich et al., 1989, Trask et al., 1993, Zalensky et al., 1995). The relative two-dimensional positions of interphase chromosome domains in living cells is known to remain unchanged after fixation and hybridization procedures (Cremer et al., 1993, Emmerich et al., 1989) but the three-dimensional structure of nuclei may collapse when cells are preserved with 3:1 methanol:acetic acid (Bacallao et al., 1989). However, the collapse of nuclei is less pronounced in fibroblasts and fibroblast-like cells because fibroblasts tend to be flat when grown in tissue culture, and have flattened nuclei, compared to the spherical nuclei of lymphocytes (Emmerich et al., 1989). Therefore, the steps inherent in the fixation and FISH procedures could have distorted the native state of the chromatin (Trask et al., 1993), but there is no evidence to suggest that these procedures would lead to a rearrangement of chromosome domains (Cremer et al., 1993, Emmerich et al., 1989). Also, there is no evidence to show that exposure to radiation causes changes to the structural integrity of the nuclear architecture and that, when combined with the fixation and FISH techniques, caused domain rearrangement. Since the observed movement of domains appeared to progress with time, we suggest that the rearrangement was not due to radiation-induced physical artifacts, but was due to an active cellular process perhaps involving gene activity and/or DNA repair.

Rearrangement of chromosome domains in response to radiation exposure may have occurred as a result of a change in the spectrum of gene activity. It is known that radiation causes expression of many genes and represses the activity of others (Woloschak and Chang-Liu, 1991, Woloschak et al., 1994, Woloschak et al., 1990). It is possible that changing the overall pattern and level of gene expression may have affected chromatin organization and the

location of a particular chromosome(s) within the nucleus, however to the best of our knowledge this possibility has not been investigated.

A potentially lethal or mutagenic lesion caused by radiation is a DNA double strand break. Chromosome domain movement may be part of the process that occurs to facilitate repair of radiation-induced DNA dsbs during the first several hours after irradiation (Frankenberg-Schwager, 1989). It has been suggested that a mechanism for rejoining radiation-induced dsbs in both prokaryotes and eukaryotes involves a recombinational process (Choulika et al., 1995, Frankenberg-Schwager, 1989, Minton and Daly, 1995, Szostak et al., 1983, Thomson, 1996). During recombinational repair, missing genetic information from a damaged chromosome is copied from a duplicate source. In G_0/G_1 phase cells, the template for repair of a DNA dsb in a chromosome is its homolog. Therefore, homologous DNA sequences must be organized and aligned with each other in order to carry out recombinational repair of DNA dsbs.

To the best of our knowledge, pairing of homologous chromosomes, has not been observed in human somatic cells , although pairing of regions of oppositely imprinted homologous has been reported (LaSalle and Lalande, 1996). However, there is evidence for homologous chromosome pairing in Indian muntjac cells (Hadlaczky et al, 1986, Hilliker and Appels, 1989) and in the bacterium *D. radiodurans* (Minton and Daly, 1995). Further, it has been shown in murine cells that the DNA dsbs induced by the endonuclease I-Sce1 were repaired by recombining with template DNA supplied in the form of a transfected plasmid, demonstrating that recombinational repair does occur in mammalian cells (Choulika et al., 1995). Thus, in mammalian cells exposed to radiation, some DNA lesions may be repaired by a similar recombinational repair process making chromosome domain rearrangement a plausible mechanism in the repair process.

This work has demonstrated that, in response to ionizing radiation, chromosomes 7 and 21 rearranged within the interphase nucleus and the

distance between homologs was reduced. The arrangement of other chromosomes is unknown and there is a possibility that this response is limited to specific chromosome pairs. The response varied in magnitude among the three cell lines studied but demonstrated that a conserved response may exist within different tissue types. Since rearrangement did not occur immediately after irradiation, but required time under growth conditions, the process may involve an active cellular process. The exact reason for homologous chromosome domain rearrangement following exposure to radiation is unknown. However, modifications in chromatin structure, changes in gene activity and induction of DNA repair systems are possible explanations.

References

Bacallao, R., Bomsel, M., Stelzer, E.H.K. and De May, J. Guiding principles of specimen preservation for confocal fluorescence microscopy. In: Handbook of Biological Confocal Microscopy. Edited by: J. B. Prawley. (Plenum Press, New York), pp. 197-205 (1989).

Blackburn, E.H. and Szostak, J.W. The molecular structure of centromeres and telomeres. Annual Review of Biochemistry, 53:163-194 (1984).

Choulika, A., Perrin, A., Dujon, B. and Nicolas, J-F. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of *Saccharomyces cerevisiae*. Molecular and Cellular Biology, 15: 1968-1973 (1995).

Cremer, T., Kurz, A., Zirbel, R., Dietzel, S., Rinke, B., Schrock, E., Speicher, M.R., Mathieu, U., Jauch, A., Emmerich, P., Scherthan, H., Ried, T., Cremer, C. and Lichter, P. Role of chromosome territories in the functional compartmentalization of the cell nucleus. Cold Spring Harbor Symposia on Quantitative Biology, 58: 777-792 (1993).

Cremer, T., Tesin, D., Hopman, A.H. and Manuelidis, L., 1988, Rapid interphase and metaphase assessment of specific chromosomal changes in neuroectodermal tumor cells by in situ hybridization with chemically modified DNA probes. Experimental Cell Research, 176: 199-220 (1988).

Emmerich, P., Loos, P., Jauch, A., Hopman, A.H.N., Wiegant, J., Higgins, M.J., White, B.N., Van Der Ploeg, M., Cremer, C. and Cremer, T. Double in situ hybridization on combination with digital image analysis: a new approach to study interphase chromosome topography. Experimental Cell Research, 181: 126-140 (1989).

Ferguson, M. And Ward, C. Cell cycle dependent chromosomal movement in pre-mitotic human T-lymphocyte nuclei. Chromosoma 101: 557-565 (1992).

Frankenberg-Schwager, M. Review of repair kinetics for DNA damage induced in eukaryotic cells in vitro by ionizing radiation. Radiotherapy and Oncology, 14: 307-320 (1989).

Haaf, T. and Schmid, M. Chromosome Topology in mammalian interphase nuclei. Experimental Cell Research, 192: 325-332 (1991).

Hadlaczky, G.Y., Went, M. and Ringertz, N.R. Direct evidence for the non-random localization of mammalian chromosomes in the interphase nucleus. Experimental Cell Research, 167: 1-15 (1986).

Hilliker, A.J. and Appels, R. The arrangement of interphase chromosomes: structural and functional aspects. Experimental Cell Research, 185: 297-318 (1989).

Hiraoka, Y., Sedat, J.W. and Agard, D.A. The use of a charge-coupled device for quantitative optical microscopy of biological structures. Science, 238: 36-41 (1987).

Lasalle, J.M. and Lalande, M. Homologous association of oppositely imprinted chromosomal domains. Science 272: 725-728 (1996).

Lichter, P. Cremer, T., Borden, J., Manuelidis, L. and Ward, D.C. Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. Human Genetics, 80: 224-234 (1988).

Manuelidis, L. and Borden, J. Reproducible compartmentalization of individual chromosome domains in human CNS cells revealed by in situ hybridization and three- dimensional reconstruction. Chromosoma, 96: 397-410 (1988).

Marelius, J. Autofluorescence imaging of living cells. Thesis, University of Zurich, Zurich Switzerland (1995).

Miller, I. and Freund, J.E. Probability and Statistics for Engineers 2nd Edition. Prentice-Hall Inc., New Jersey (1977).

Minton, K.W. and Daly, M.J. A model for repair of radiation-induced DNA double-strand breaks in the extreme radiophile *Deinococcus Radiodurans*. BioEssays, 17: 457-464 (1995).

Oud, J.L., Brakenhoff, G.J., Van Der Voort, H.T.M., Van Spronsen, E.A. and Nanninga, N. 3-D arrangement of anaphase chromosomes of *Crepis capillaris* root top cells as studied by confocal scanning laser microscopy. Journal of Cell Science, 92: 329-339 (1989).

Pinkel, D., Straume, T. and Gray, J.W. Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. Proceedings of the National Academy of Science, 83: 2934-2938 (1986).

Popp, S., Scholl, H.P., Loos, P., Jauch, A., Stelzer, E., Cremer, C. and Cremer, T. Distribution of chromosome 18 and X centric heterochromatin in the interphase nucleus of cultured human cells. Experimental Cell Research, 189: 1-12 (1990).

Rappold, G.A., Cremer, T., Hager, H.D., Davies, K.E., Muller, C.R. and Yang, T. Sex chromosome positions in human interphase nuclei as studied by in situ hybridization with chromosome specific DNA probes. Human Genetics, 67: 317-325 (1984).

Szostak, J.W., Orr-Weaver, T.L. and Rothstein, R.J. The double-strand break repair model for recombination. Cell, 33: 25-35 (1983).

Thomson, L.H. Evidence that mammalian cells possess homologous recombinational repair pathways. Mutation Research, 363: 77-88 (1996).

Trask, B., Van Den Engh, G., Pinkel, D., Mullikin, J., Waldman, F., Van Dekken, H. and Gray, J. Fluorescence in situ hybridization to interphase cell nuclei in suspension allows flow cytometric analysis of chromosome content and microscopic analysis of nuclear organization. Human Genetics, 78: 251-259 (1988).

Trask, B.J., Allen, S., Massa, H., Fertitta, A., Sachs, R., Van Den Engh, G. and Wu, M. Studies of metaphase and interphase chromosomes using fluorescence in situ hybridization. Cold Spring Harbor Symposia on Quantitative Biology, 58: 767-775 (1993).

Vourc'h, C., Taruscio, D., Boyle, A.L. and Ward, D.C. Cell cycle-dependent distribution of telomeres, centromeres, and chromosome-specific subsatellite domains in the interphase nucleus of mouse lymphocytes. Experiment Cell Research, 205: 142-151 (1993).

Walker, C.L., Cargile, C.B., Floy, K.M., Delannoy, M. and Migeon, B.R., 1991, The barr body is a looped X chromosome formed by telomere association. Proceedings of the National Academy Science, 88: 6191-6195 (1991).

Woloschak, G.E., Liu, C-M., Jones, P.S. and Jones, C.A. Modulation of gene expression in Syrian hamster embryo cells following ionizing radiation. Cancer Research, 50: 339-344 (1990).

Woloschak, G.E. and Chang-Liu, C-M. Expression of cytoskeletal elements in proliferating cells following radiation exposure. International Journal of Radiation Biology, 59: 1173-1183 (1991).

Woloschak, G.E., Chang-Liu, C-M., Panozzo, J. and Libertin, C.R. Low doses of neutrons induce changes in gene expression. Radiation Research, 138: S56-S59 (1994).

Zalensky, A.O., Allen, M.J., Kobayashi, A., Zalenskaya, I.A., Balhorn, R. and Bradbury, E.M. Well-defined genome architecture in the human sperm nucleus. Chromosoma, 103: 577-590 (1995).

Zorn, C., Cremer, C., Cremer, T. and Zimmer, J. Unscheduled DNA synthesis after partial UV irradiation of the cell nucleus. Experimental Cell Research, 124: 111-119 (1979).

Summary

Cisplatin, a DNA crosslinking agent, when used in combination with ionizing radiation can produce greater cell kill than expected if the effects were additive. The mechanism for this radiosensitization is unknown. This work tested the contention that the synergistic effect is due to inhibition of repair of radiation-induced DNA damage by the cisplatin lesions. The work was conducted using higher and lower eukaryotic cells, namely human fibroblasts and yeast cells. Chapters one and two of this thesis investigated DNA repair inhibition caused by cisplatin. Chapter three investigated the effect of cisplatin on the stress response, and the final chapter examined the effect of radiation on homologous domain locations within the interphase nucleus. Together, this thesis provides evidence that DNA repair processes involved in the repair of radiation-induced damage are influenced by the presence of cisplatin DNA adducts.

In chapter one, normal human fibroblasts (AG1522) were irradiated in the presence or absence of cisplatin lesions, and allowed time to repair the radiation-induced DNA damage. Cells irradiated in the presence or absence of cisplatin adducts showed biphasic repair curves. In the absence of cisplatin adducts, the level of radiation-induced DNA damage remaining after 45 minutes of repair time was indistinguishable from unirradiated, control cells. When cells were treated with cisplatin immediately before irradiation, fast repair was not affected but slow repair was inhibited. The rate of the slow repair in the presence of cisplatin adducts was ten fold less than the rate in the absence of cisplatin adducts. Cisplatin treatment 24 hours prior to radiation resulted in inhibition of both fast and slow repair. This may indicate that bifunctional crosslinks which are produced slowly over time in addition to monofunctional adducts are responsible for the cisplatin inhibition of repair of radiation-induced DNA double strand breaks and that DNA single strand break repair is inhibited by bifunctional cisplatin adducts. Chapter two further investigated the effect of cisplatin on

repair processes by examining whether defects in repair mechanisms would modify the radiosensitizing effect of cisplatin. It was determined that cisplatin only sensitized yeast cells with a competent recombinational repair mechanism and I postulate that sensitization was due to cisplatin lesion interference with a transcription coupled recombinational repair process.

Under certain conditions cisplatin may not radiosensitize cells, but may induce a stress response and confer cellular radiation resistance, as is the case for other DNA damaging agents. Since stress response has been well characterized in the yeast *Saccharomyces cerevisiae,* this organism was used as a model system to investigate cisplatin-induced stress response. In chapter three it was found that repair proficient wild type yeast cells became thermal tolerant and radiation resistant two hours after a sublethal cisplatin treatment. Cisplatin pretreatment also suppressed mutations caused by N-methyl-N-nitro-N-nitrosoguanidine (MNNG), a response previously shown in wild type cells with radiation pretreatment. Also, like radiation, the cisplatin-induced stress response did not confer radiation resistance or suppress MNNG mutations in a recombinational repair deficient mutant (rad52). The results from chapter three support the idea that cisplatin crosslinks in DNA can induce a stress response which subsequently confers radiation resistance, thermal tolerance, and mutagen resistance in yeast, and that the mechanism of this cisplatin-induced resistance is dependent on an error free recombinational repair pathway.

The work from the first three chapters in this thesis indicated that error free recombinational repair plays a major role in the interaction between cisplatin and radiation. The error free repair process has been well documented in yeast but evidence for its existence in mammalian cells is limited. In chapter four, using mammalian cells and confocal microscopy, it was shown that after exposure to radiation, the distance between homologous chromosomes was reduced. The rearrangement did not occur immediately after irradiation but required incubation time, indicating involvement of an active cellular process.

The exact reason for homologous chromosome rearrangement following exposure to radiation is unknown, but induction of a recombinational repair system is a possibility. In light of this evidence, it will now be possible to study the effect of cisplatin on this process.

Overall, this work has shown that the radiosensitizing effect of cisplatin was due to inhibition of DNA repair processes involving recombinational repair. However, it was also shown that cisplatin treatment induced a stress response that conferred cellular radiation resistance. Therefore, the effectiveness of cisplatin in combination with radiation may be variable depending upon the cellular process influenced by cisplatin.

Appendix 1

List of Abbreviations

BNC: Binucleate cell - A cell containing two main nuclei as the result of cytochalsin B treatment which permits karyokinesis but not cytokinesis.

ciplatin: cis-diamminedichloroplatinum (II) - A DNA crosslinking agent.

DSB: double strand break - Both strands of DNA are broken

FADU: Fluorescence Analysis by DNA Unwinding - An assay to measure DNA strand breaks.

FISH: Fluorescence *in situ* hybridization

Gy: Gray - SI unit of absorbed dose. Equal to the amount of energy absorbed in joules per kilogram of mass.

MNNG: N-methyl-N'-nitro-N-nitrosoguanidine - A DNA methylating agent.

SSB: single strand break

Saccharomyces cerevisiae Genotypes

MS33: his1-7/1-7 lys1-1/1-1 ade2-1/2-1 hom3-10/3-10

MS32: rad52-1/52-1his1-7/1-7 lys1-1/1-1 ade2-1/2-1 hom3-10/3-10

STX432: rad3-2/3-2 ade2-1/2-1

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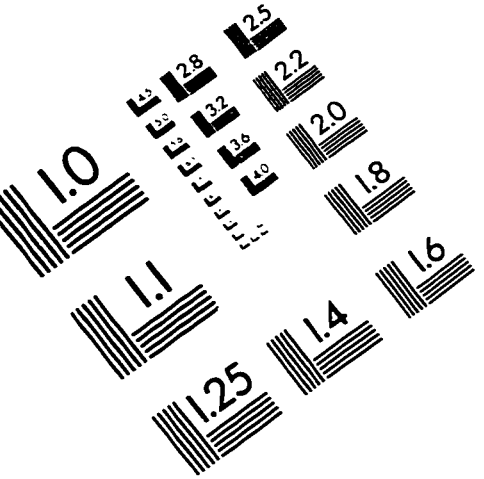

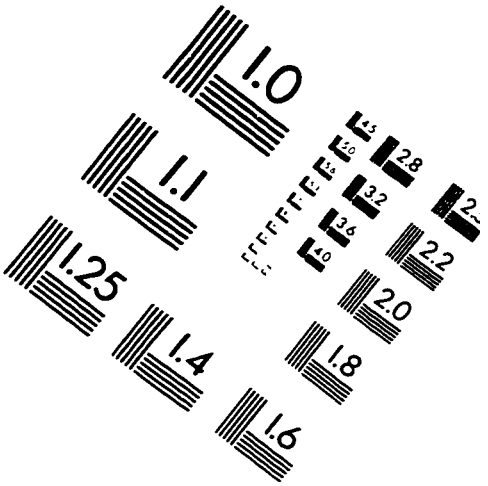

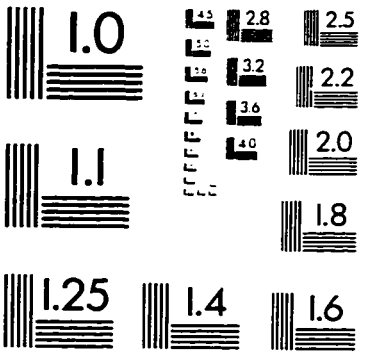

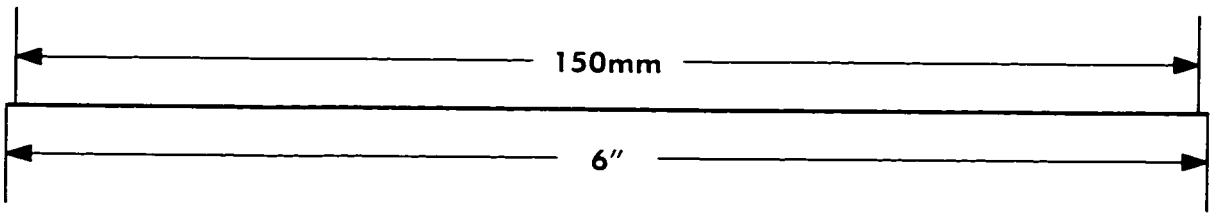

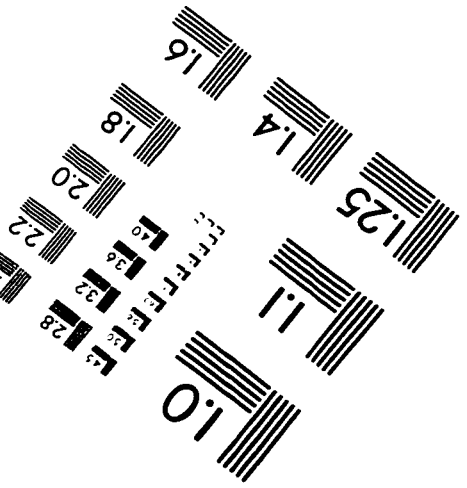

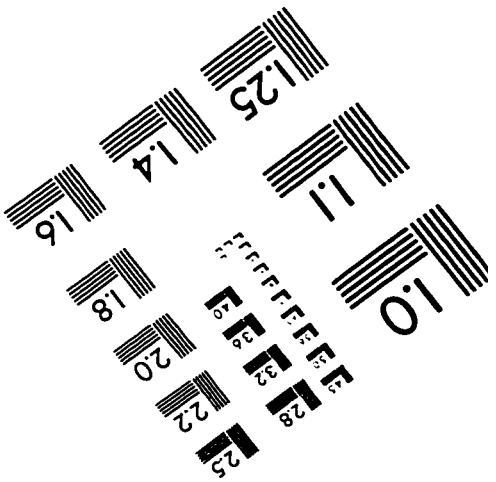

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