ANTICANCER RESEARCH *25*: 53-58 (2005)

A Comparison of Response to Cisplatin, Radiation and Combined Treatment for Cells Deficient in Recombination Repair Pathways

G.P. RAAPHORST, J- MAUDE LEBLANC and L.F. LI

Integrated Cancer Program/The Ottawa Hospital, 501 Smyth Rd., Ottawa, Ontario K1H 8L6, Canada

Abstract. *The responses of cells with mutated DNA repair pathways were compared for cisplatin, radiation and combination treatments. The knockout of the nonhomologous endjoining (NHEJ) pathway resulted in increased radiation sensitivity, but no change in cisplatin response in the mouse cells and increased radiosensitivity but decreased cisplatin sensitivity in chicken cells. The mutation of the homologous recombination repair (HR) pathway through XRCC3 in CHO cells resulted in increased radiation and cisplatin sensitivity and to a lesser extent for the Rad54 knockout in the DT40 chicken cells. The combination treatments of cisplatin and radiation showed that inhibition of the HR repair pathway resulted in super additive effects while the inhibition of the NHEJ pathway in DT40 had no effect. In mouse cells the knockout of the NHEJ pathway resulted in reduced super additivity compared to the parental cell lines. These data show that radiation, cisplatin and combination treatment damage is affected differently by the various DNA repair pathways, which could have a range of effects on combination treatments in tumour cells expressing different levels of DNA repair in the various repair pathways.*

Cisplatin was discovered to be an effective chemotherapy agent in the treatment of cancer (1-10) and has been extensively investigated in cultured mammalian cells. It was shown that cisplatin could form mono and bifunctional adducts on the DNA and that cells had the ability to remove such adducts using DNA repair systems (10-13). One such repair system was shown to be nucleotide excision repair, which when mutated in xeroderma pigmentosa cells could cause enhanced sensitivity to cisplatin and, recently, the homologous recombinational repair pathway has also been implicated (14-18). Now, through gene mutation and gene knockout techniques, it is possible to block cellular DNA repair systems which process DNA single and double strand breaks such as excision repair and nonhomologous, endjoining and homologous recombination repair (HR) (19-26). A number of studies have shown that the inactivation of DNA repair genes can result in radiosensitization (20,22,27-30). It has also been shown that such gene inactivation and knockouts can effect cellular responses to cisplatin (16,17,18,30-32). We and others have shown that combined treatment of cisplatin and radiation can result in additive or superadditive effects, supporting the concept of cisplatin radiosensitization (10,11,33-35). Cisplatin radiosensitization may well be influenced by whether similar or different DNA repair pathways are involved in processing cisplatin and radiation damage in DNA. In order to explore this question further, we set out to compare the responses to radiation, cisplatin and combined treatments in cell lines with the following natural and knockout mutations; nonhomologous endjoining using Ku70 and Ku80 gene knockout cells and homologous recombination using XRCC3 gene mutated and Rad54 knockout cells.

Correspondence to: G.P. Raaphorst, Medical Physics Department, Integrated Cancer Program, The Ottawa Hospital, 501 Smyth Road, Ottawa, Ontario, Canada K1H 8L6. Tel: (613) 737-7700 Ext. 6727, Fax: (613) 247-3507, e-mail: graaphorst@ottawahospital.on.ca

Key Words: Cisplatin, radiation, DNA repair, mutants, recombination repair.

Materials and Methods

The cell lines used in this study are described as follows: The mouse embryo fibroblast line (MEF) and its derivative nonhomologous endjoining knockout (NHEJ) cell line Ku80 were kindly donated by Dr. G.C. Li (21) and their culture details have been previously described (22). The CHO cell line AA8 and its derivative $_{irs}$ISF is a mutant of the XRCC3 gene (XRCC3-/-) and deficient in homologous recombination (HR) DNA repair. They were kindly donated by Dr. L. Thompson and have been previously described in detail (19-20). The DT40 chicken cell lines were developed by Dr. Takeda (30) and kindly donated for these experiments. The DT40 is the parental cell line, the DT40Rad54 is the Rad54 gene knockout cell line deficient in HR repair. The DT40Ku70 is a knockout in the NHEJ pathway and the DT40Rad54Ku70 is a knockout line for both HR and NHEJ

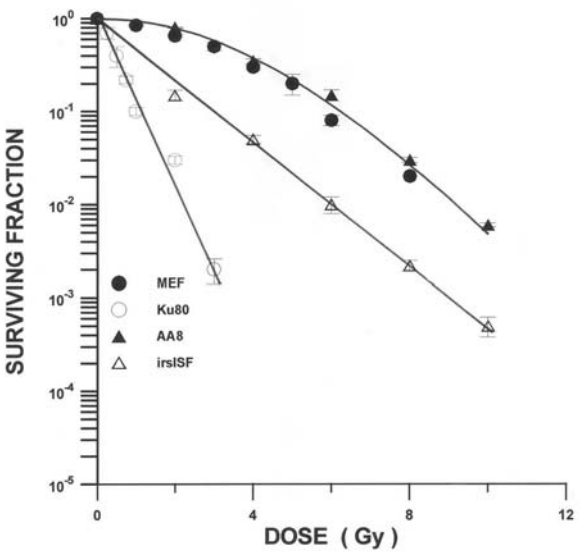

Figure 1. *The radiation response is shown for two cell line pairs consisting of the mouse embryo fibroblast parental line (MEF) and a Ku80 knockout line (Ku80) and a Chinese hamster parental line (AA8) and an XRCC3 mutant line ($_{irs}$ISF).*

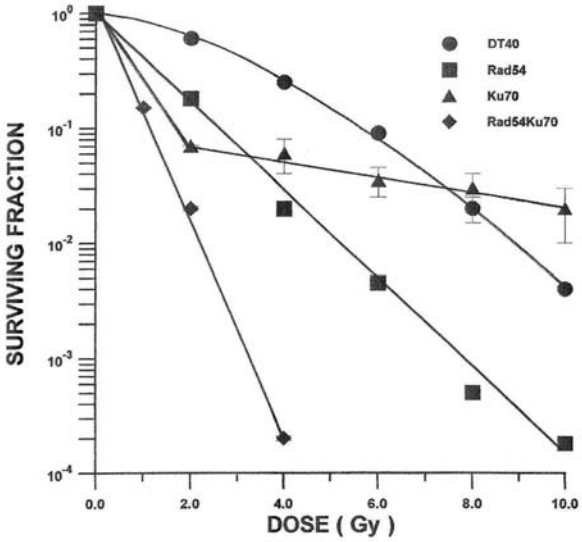

Figure 2. *The radiation response is shown for chicken lymphocyte cells for the parental line DT40 and Rad54 knockout line (DT40Rad54), a Ku70 knockout line (DT40Ku70) and a double knockout of Rad54 and Ku70 (DT40Rad54Ku70).*

pathways. The details of the cells and their culturing have been previously described (30).

The CHO and mouse cell lines were grown in a mixture of 1:1 DMEM and F12 medium supplemented with 10% fetal calf serum and 0.1 mM MEM nonessential amino acids. The cells were grown to plateau phase, then refed and, 48 hours after that, the experiments were performed. The plating efficiencies were 60-90% for the AA8 and $_{irs}$ISF cell lines and 20-25% for the MEF and Ku80 cell lines.

The DT40 cell lines were cultured in DMEM/F12 (Wisent, Montreal, Canada) containing 1% penicillin streptomycin (Wisent), 0.88% tryptose phosphate broth solution (29.5g phosphate broth/L, Sigma, Oakville, ON, Canada), 1% chicken serum (Wisent), 10% fetal bovine serum (Wisent) and 50 μM β-mercaptoethanol (14.3 moles/L Sigma). The cells were grown in suspension in 100-mm petri dishes. Cells were subcultured every two days and the experiments were done in the exponential phase of the cell growth. The day of the experiment, the cells were counted using the hemocytometer. After treatment, the cells were plated in 15-mm petri dishes. The plating efficiencies of the DT40, DT40Rad 54, DT40Ku70 and DTRad54Ku70 cells were 70-90, 50-70, 30-50 and 50-70, respectively.

For cisplatin treatment, cisplatin obtained from David Bull Canada Inc. in isotonic saline (1 mg/ml) was diluted into the culture medium at the required concentration. For the concentration of 1 mg/ml, the dilution factor is 1000 and has no significant effect on the medium. These solutions are pH buffered at 7.2 and added directly to the cells. At the end of treatment, the solutions were removed, cultures rinsed with warm isotonic buffer and then medium was added. Fresh solutions were used for each experiment. For the DT40 cells grown in suspension, cells were centrifuged and the supernatant replaced with isotonic buffer. The cells were then centrifuged one more time and the buffer was replaced with fresh medium.

For irradiation, cells were irradiated in 25-cm^2 tissue culture flasks at room temperature using a Pantak Bipolar Series Model HF320 X-ray, operating at 250 kVp with 1.87 mm base aluminum filtration giving a dose rate of 168 cGy/min.

After treatment, the cells were prepared for the colony survival assay. The mammalian cells were trypsinized, counted and plated at numbers to give about 50 to 100 colonies per 6-cm tissue culture plate. For the DT40 chicken lines, the cells were plated into 6-cm petri dishes at numbers for which they formed 50-100 colonies. Survival was assayed using the suspension colony forming assay in DMEM/F12 media preparation containing 1% methylcellulose, 4000 centipoises (Shin-Etsu Chemical Co. Ltd. Tokyo, Japan). All dishes were placed in a 37˚C incubator until colonies of 50 cells or more were visible. At this point, all colonies were stained and counted. Each experiment was repeated three times and the error bars represent the standard error of the mean.

Results

The radiation response of two cell line pairs is shown in Figure 1. For the inhibition of recombination DNA repair pathways, both the homologous recombination repair mutant ($_{irs}$ISF) and the nonhomologous endjoining knockout (Ku80) showed greater radiosensitivity than the parental wild-type cell lines AA8 and MEF, respectively, indicating both recombination repair pathways as being important in radiation damage repair.

Figure 2 shows the radiation response of four chicken cell lines. The DT40 wild-type cell line was much more radioresistant than the derivative mutants Rad54 (HR gene knockout) and Rad54Ku70 (both HR and NHEJ gene knockout). The results for the Ku70 knockout were more

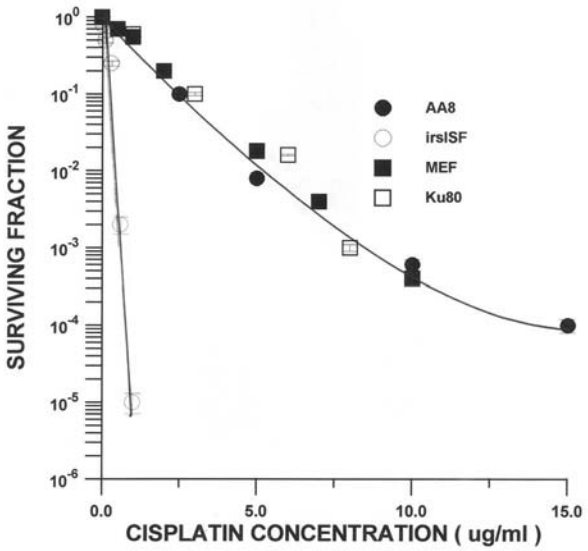

Figure 3. *The cisplatin response is shown for mouse and Chinese hamster parental cells MEF and AA8 and their respective mutants Ku80 and $_{irs}$ISF. Cisplatin exposure was given for 1 hour after which it was removed and cells were rinsed and given fresh medium.*

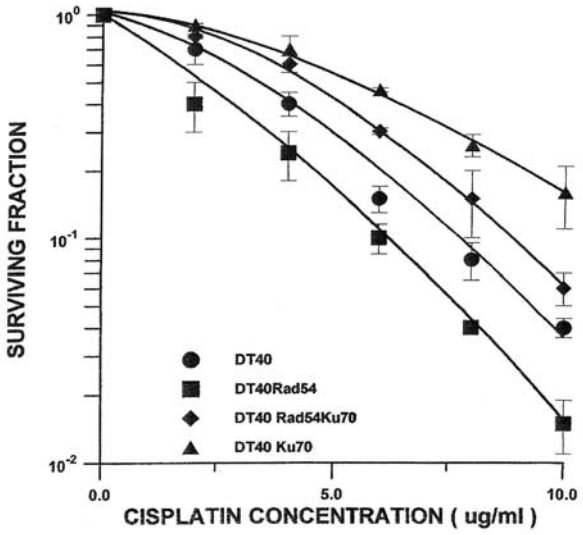

Figure 4. *The response of the four chicken cell lines DT40 wild-type, DT40 Rad54 knockout, DT40Ku70 knockout and the double knockout DT40Rad54Ku70 is shown. Cisplatin exposure was for 1 hour after which it was removed and cells were rinsed and given fresh medium.*

complex, showing low-dose high sensitivity followed by a radiation-resistant plateau for which the cells become more resistant than the parental line. This response was observed earlier (30) and may be related to the up-regulation of the HR system when the NHEJ system is inactivated and a small subpopulation of cells in G1 may remain sensitive if HR is less effective in G_1 cells before chromosomes are replicated.

The recombination repair pathways have profoundly different effects on cisplatin responses. Figure 3 shows that the knockout of NHEJ for the Ku80 cell line had no effect on cisplatin response compared to its normal parental cell line MEF. On the other hand, the mutation in XRCC3 of the HR repair pathway in the $_{irs}$ISF cell line resulted in a large increase in cisplatin sensitivity compared to the parental cell line AA8. These data confirm that the HR pathway is important in repair of cisplatin damage (30,32).

Figure 4 shows the response of DT40 cells and three knockout mutants to cisplatin. The data show that the HR knockout (Rad54) resulted in increased cisplatin sensitivity comparing Rad54 to the wild-type. The knockout of NHEJ for the Ku70 cell line resulted in increased resistance to cisplatin and the knockout of both NHEJ and HR (DT40Rad54 Ku70) reduced this resistance compared to Ku70, but it still remained higher than the parental cell line, DT40.

The combined treatment with cisplatin and radiation was also evaluated in the mutant cell lines in order to determine whether any of these mutations affected the interaction effects of these combined treatments. The results are shown in Table I for cisplatin given for 1h and terminated 5 min before irradiation. While experiments were done for a wide range of cisplatin concentrations, the data are summarized for two concentrations representative of the data and at the level of maximum clinical achievability. For the chicken DT40 cell lines, the parental line DT40 and the mutants DT40K70 and DT40Rad54Ku70 showed additive effects of the two treatments by assessing the interaction ratios (survival of cisplatin alone times survival from X-rays alone divided by survival of the combined treatments). A result of greater than 1.0 indicates super additivity and the data were not significantly different from 1.0. For the DT40Rad54 mutant, the results were significantly greater than 1.0 showing super additivity. For the rodent cell lines, the results for hamster CHO AA8 showed additivity for the low cisplatin concentration and super additivity for the higher concentration, while the $_{irs}$ISF mutant for HR repair showed super additivity for both concentrations. The mouse MEF cell line showed super additivity for both concentrations, while the NHEJ mutant showed super additivity for the low concentration, but just additivity for the higher concentration. It is also of interest to note that, in general, the rodent cell lines showed a greater interaction effect than the chicken cell lines.

Discussion

The data presented in this study clearly show that DNA repair pathways are different for repairing damage stemming from radiation and cisplatin treatments, as reflected in the interesting differences observed for

Table I. *Combined treatment of cisplatin and radiation.*

Cell line	Cis conc μg/ml	Cis alone	Survival response		Interaction ratio[1,2]
			X-rays	Cis + X-ray[3]	
DT40	2	0.65	0.11	0.085	0.85
X = 6 Gy	4	0.10		0.013	0.85
DT40 Rad54	2	0.3	0.14	0.03	1.4*
X = 3 Gy	4	0.04		0.004	1.4*
DT40 Ku70	2	0.9	0.16	0.15	0.96
X = 3 Gy	4	0.5		0.1	0.80
DT40Rad54Ku70	2	0.5	0.018	0.01	0.90
X = 2 Gy	4	0.25		0.005	0.90
AA8	1	0.65	0.031	0.02	1.0
X = 8 Gy	2	0.18		0.004	1.4*
$_{irs}$ISF	0.1	0.37	0.026	0.006	1.60*
X = 6 Gy	0.2	0.15		0.002	1.95*
MEF	1	0.6	0.02	0.0024	5.0*
X = 8 Gy	3	0.22		0.0018	2.5*
Ku80	1	0.5	0.035	0.01	1.75*
X = 2 Gy	3	0.08		0.0032	0.88

[1]Interaction ratio = (survival from cisplatin x survival from X-rays/survival from the combined treatments)
[2]Interaction ratio marked with * show significant difference from 1.0
[3]The standard deviation on the results was less than ± 10%
[4]Cisplatin treatment was for 1 h and was terminated 5 min before irradiation was started.

radiation and cisplatin responses for the recombination repair pathway mutant cell lines. For the NHEJ repair pathway knockout in Ku80, there was a dramatic increase in radiation sensitivity but no change in the cisplatin response, clearly indicating that NHEJ is active in processing radiation damage but not cisplatin damage. On the other hand, the inhibition of the HR pathway through mutation of the XRCC3 gene in the $_{irs}$ISF cell line showed increased sensitivity to both radiation and cisplatin. The increased cisplatin sensitivity was about 10-fold in concentration and confirms earlier results that HR is important in processing cisplatin damage (18). This would indicate that perhaps in S-phase and in G2, where HR is more active (30), the impact of cisplatin would be greater and this is supported in our earlier results showing enhanced numbers of dead S-phase cells through cell cycle analysis (36).

The results for the DT40 cells are very complex. The HR knockout through Rad54 supports the results for the $_{irs}$ISF cell line in that it shows increased cisplatin sensitivity. However, the knockout of the NHEJ pathway shows complex behavior; cells became resistant to cisplatin and resistant to high-dose radiation. We speculate that Ku70 knockout may result in up-regulation of the HR pathway resulting in increased resistance to cisplatin and possibly to high-dose radiation. The low-dose radiation sensitivity may be related to the component of cells in the radiosensitive G_1/G_0 cell cycle phase, where also HR may be less effective. The double knockout DT40Rad54Ku70 showed increased cisplatin sensitivity compared to the Ku70 knockout, but it was still more resistant than the parental cell line. The data in Figure 4 show that the Rad54 knockout caused much less cisplatin sensitization than the XRCC3 mutation shown in Figure 3. Thus, Rad54 knockout may result in only a partial HR inhibition and possibly the additional Ku70 knockout may still allow for some up-regulation in the HR pathway.

We and others have shown that cisplatin radiosensitization can act through the inhibition of cellular recovery of radiation damage (10,35,37). In addition, cisplatin responses and radiosensitization have been shown to affect DNA repair (30,32,38)

Our results, shown in Table I, support the observation that cisplatin radiosensitization can be influenced by DNA repair systems. In the DT40Rad54 HR knockout line, the interaction ratio for cisplatin and radiation was greater than 1.0 and greater than the parental line. Correspondingly, in the CHO cell line the $_{irs}$ISF mutant also showed a greater interaction ratio than the parental line AA8. On the other hand, in the DT40 system the Ku70 NHEJ mutant was not significantly different than the parental line, while in the rodent MEF system Ku80 mutant was less than the parental line. Thus, both the rodent and the chicken lines show that perhaps the HR repair system influences the cellular response to combined cisplatin and radiation treatment. The results for the NHEJ system are less clear since in the chicken system there is no effect, while in the rodent system

the interaction ratio is less than for the parental line. It should be noted that, while direct comparison between all the different cell lines is not possible because of genetic differences, the relative results of mutant to parental in each group is possible and these relative comparisons do show a consistent result for the involvement of the HR repair pathway.

In summary, these data show that altered expression of recombination repair pathways can affect responses to cisplatin, radiation and combined treatments and this could have clinical implications in cancer treatment where tumour cells may have different activity in the various DNA repair pathways.

References

1 Sledge GW: Cisplatin and platinum analogues in breast cancer. Semin Oncol *19*: 78-82,1992.

2 Piver MS: Ovarian carcinoma, a decade of progress. Cancer *54*: 2706-2715, 1984.

3 Taylor SG, Murphy AK, Vannetzel JM *et al*: Randomized comparison of neoadjuvant cisplatin and fluorouracil infusion followed by radiation *versus* concomitant treatment in advanced head and neck cancer. J Clin Oncol *12*: 385-395, 1994.

4 Palazzi M, Calaldo I, Gramaglia A, de Toma D, Milani F and Ravasi G: Preoperative concomitant cisplatin/VP16 and radiotherapy in stage III non-small cell lung cancer. Int J Rad Oncol 27: 621-625, 1993.

5 Zietman AL, Shipley WU and Kaufman DS: The combination of cisplatin based chemotherapy and radiation in the treatment of muscle-invading transitional cell cancer of the bladder. Int J Rad Oncol *27*: 161-170, 1993.

6 Kelland LR: Cisplatin-based anticancer agents. *In*: Uses of Inorganic Chemistry in Medicine. Ed. Farrell NP. pp.109-134, 1999.

7 Wen H, Zu JY, Lin L and Li T: Nasopharyngeal carcinoma (NPC) treated by hyperfractionated irradiation plus alternating chemotherapy. Radiother Oncol *43*: 587, 1997.

8 Teicher BA: The role of platinum complexes in combined modality therapy. *In*: Chemoradiation in Cancer Therapy. Ed. Choy H. Humana Press, Totowa New Jersey, pp. 47-63, 2003.

9 Canal P: Platinum compounds: pharmacokinetics and pharmacodynamics. *In*: A Clinician's Guide to Chemotherapy, Pharmacokinetics and Pharmacodynamics Eds. Grochow LB and Ames MM, Williams and Wilkins, Baltimore, pp. 345-373, 1998.

10 Dewit L: Combined treatment of radiation and cis-disminedichloroplatinum (II): A review of experimental and clinical data. Int J Rad Oncol *13*: 403-426, 1987.

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

12 Sancar A and Sancar GB: DNA repair enzymes. Ann Rev Biochem *57*: 29-67, 1988.

13 Sibghat-Ullah, Intinsar H, Carleton W and Sancar A: Human nucleotide excision repair *in vitro*: repair of pyrimidine dimers, psoralen and cisplatin adducts by HeLa cell-free extract. Nuclear Acids Res *17*: 4471-4484, 1989.

14 Korberle B, Masters JR, Hartley JA and Wood RD: Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours. Current Biol *9*: 273-276, 1999.

15 Zhen W, Evans MK, Haggerty CM and Bohr VA: Deficient gene specific repair of cisplatin lesions in XP and FA cell lines. Carcinogenesis *14*: 919-924,1993.

16 Tanaka K, Nakatsu Y, Sayo M, Kuraoka I, Matsuda T, Kobayashi T, Murai H and Nakane H: Nucleotide excision repair defect and carcinogenesis in XPA-knocked out mice. J Cell Mol Biochem, Supplement *21A*: 272, 1995.

17 Li Q, Yu JJ, Mu C, Yunmbam MK, Slavsky D, Cross CL, Bruton FB and Reed E: Association between the level of ERCC expression and the repair of cisplatin-induced DNA damage in human ovarian cancer cells. Anticancer Res *20*: 645-652, 2000.

18 Zdraveski ZZ, Mello JA, Marinus MG and Essigmann JM: Multiple pathways of recombination define cellular responses to cisplatin. Chem Biol *7*: 39-50, 2000.

19 Liu N, Lamerdin JE, Tebbs RS, Schild D, Tucker JD, Shen MR, Brookman KW, Siciliano MJ, Walter CA, Fan W, Narayana LS, Zhou ZQ, Adamson AW, Sorensen KJ, Chen DJ, Jones NJ and Thompson LH: XRCC2 and XRCC3 new human Rad 51-family members promote chromosome stability and protect against DNA cross links and other damages. Mol Cell *1*: 783-793, 1998.

20 Pierce AJ, Johnson RD, Thompson LH and Jasin M: XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Develop *13*: 2633-2638, 1999.

21 Burgman P, Ouyang H, Peterson S, Chen DJ and Li GC: Heat inactivation of Ku autoantigen: possible role in hyperthermic radiosensitization. Cancer Res *57*: 2847-2850, 1997.

22 Myint WK, Ng C and Raaphorst GP: Examining the non-homologous repair process in cisplatin and radiation treatments. Int J Rad Biol *78*: 417-424, 2002.

23 Sobol RW, Norton JK, Kuhn R *et al*: Requirement of mammalian DNA polymerase β in base excision repair. Nature *379*: 183-186, 1996.

24 Frosina G, Fortini P, Rossi O, Carrozzino F, Raspaglio G, Cox LS, Lane DP, Abbondandolo A and Dogliotti E: Two pathways for base excision repair in mammalian cells. J Biol Chem *271*: 9573-9578, 1996.

25 de Vries A and van Sleeg H: XPA knockout mice. Cancer Biol *7*: 229-240.

26 Hoeijmakers JHJ: Human nucleotide excision repair syndromes: molecular clues to unexpected intricacies. Eur J Cancer *30A*: 1912-1921, 1994.

27 Liang F, Han M, Romanienko PJ and Jasin M: Homology directed repair a major repair pathway in mammalian cells. Proc Nat Acad Science *95*: 5172-5177, 1998.

28 Wachsberge PR, Li WH, Guo M, Chen D, Cheong N, Ling CC, Li G and Iliakis G: Rejoining of DNA double strand breaks in Ku80-deficient mouse fibroblasts. Radiation Res *151*: 398-407, 1999.

29 Utsumi H, Tano K, Takata M, Takeda S and Elkind MM: Requirement for repair of DNA double strand breaks by homologous recombination in split-dose recovery. Radiation Res *155*: 68-686, 2001.

30 Takata M, Sasaki MS, Sonoda E, Monison C, Hasimoto M, Utsumi H, Yamaguchi Y, Wai I, Shinohara A and Takeda S: Homologous recombination and nonhomologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. EMBO J *17*: 5497-5508,1998.

31 Dolling JA, Boreham DR, Brown DL, Raaphorst GP and Mitchell REJ: Cisplatin modification of DNA repair and ionizing radiation lethality in yeast, *Saccharomyces cervisiae*. Mutation Res *433*: 127-136, 1999.

32 Takata M, Sasoki M, Sonada E, Fukushima T, Morisson C, Albala JS, Swagemakers SMA, Kanaar R, Thompson LH and Takeda S: Rad 51 Paralog Rad 51B promotes homologous recombination repair. Mol Cell Biol *20*: 6476-6482, 2000.

33 Raaphorst GP, Wang G and Ng CE: Radiosensitization by cisplatin treatment in cisplatin resistant and sensitive human ovarian carcinoma cell lines. Int J Oncol *7*: 325-330, 1995.

34 Raaphorst GP, Wang G, Stewart DJ and Ng CE: Concomitant low dose rate irradiation and cisplatin treatment in ovarian carcinoma cell lines sensitive and resistant to cisplatin treatment. Int J Rad Biol *69*: 623-631, 1996.

35 Douple EB: Interaction between cisplatin coordination complexes and radiation. *In*: Hill BT, Bellamy AS, eds. Antitumour Drug-radiation Interactions. Boca Raton, Florida: CRC Press 171-190, 1990.

36 Wilkins DE, Ng CE and Raaphorst GP: Cell cycle perturbations in cisplatin sensitive and resistant human ovarian carcinoma cells following treatment with cisplatin and low dose rate irradiation. Cancer Chemother Pharmacol *40*: 159-166, 1995.

37 Wilkins DE, Raaphorst GP and Heller DP: Inhibition of potentially lethal damage recovery by cisplatin in the 9L rat brain cell line. Anticancer Res *13*: 2137-2142, 1993.

38 Dolling JA, Boreham DR, Brown DL, Mitchell REJ and Raaphorst GP: Modulation of radiation-induced strand break repair by cisplatin in mammalian cells. Int J Rad Biol *74*: 61-69, 1998.

Received November 4, 2004
Accepted December 14, 2004