原著

Effect of gemcitabine on mammalian cells by fluorescent immunostaining: examination of cell enlargement and DNA double-strand breaks

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Abstract : The detailed repair pathway by which gemcitabine-induced DNA double-strand breaks（DSBs）has not been elucidated. We evaluated the possible role of gemcitabine by fluorescent immunostaining on a rodent cell line. A repair-deficient clone, the xrs5 cell line, was used in addition to a wild-type rodent cell line（CHO）. We observed changes in the number and size of CHO and xrs5 cells after 24 h of treatment with gemcitabine at a concentration of 5.0 μM. The results showed that the cellular lethality exerted by gemcitabine involves cell enlargement, and the number of DNA DSBs induced by gemcitabine was significantly higher in xrs5 cells than in CHO cells. In terms of changes in DNA DSBs over time, CHO cells showed a decreasing trend, while xrs5 cells showed an increasing one. The findings confirmed that gemcitabine inhibits the repair of DSBs via a pathway other than non-homologous end joining.

Key words: gemcitabine, CHO, xrs5, DNA double-strand break, cell enlargement

1. Introduction

Gemcitabine（4-amino-1-[3,3-difluoro-4-hydroxy-5-（hydroxymethyl）Tetrahydrofuran-2-yl]-1H-pyrimidin-2-one: dFdC）is a deoxycytidine analog that is well known for its antitumor activity and is used as a standard therapy for patients with advanced pancreatic cancer. It is one of the more effective drugs for sensitizing cells to radiation therapy, but most pancreatic cancers do not respond to gemcitabine alone[1–4]. The radiosensitizing properties of gemcitabine have been demonstrated both *in vivo* and *in vitro*[5–8], although the detailed interaction of gemcitabine has not been elucidated. In preliminary experiments with human lung carcinoma cells, van Putten *et al*. showed that gemcitabine treatment can inhibit the rate and extent of DNA double-strand break（DSB）repair[9]. In contrast, Lawtence *et al*. reported no detectable effect of gemcitabine on DNA DSB repair[10].

Radiation is used to kill cancer cells mainly by inducing DNA DSBs. The key DNA damage response protein, p53-binding protein 1（53BP1）, acts by binding to chromatin at the site of DSBs. 53BP1（also called TP53BP1）is a chromatin-associated factor that promotes immunoglobulin class switching. Cells have two different pathways for achieving DNA DSB repair: non-homologous end joining（NHEJ）and homologous recombination（HR）[11–14].

To evaluate the effect of gemcitabine, we used a wild-type Chinese hamster ovary（CHO）cell line. Immunofluorescence（IF）staining method was used to observe and detect DNA DSBs. In this study, we aimed to examine the effect of gemcitabine on DNA DSBs and elucidate its possible role in the process.

2. Materials and methods

2.1. Cell culture

Two Chinese hamster cell lines, namely, Chinese hamster ovary cells（CHO）and Ku80-deficient CHO cells（xrs5）, were grown on alpha-MEM medium（Life Technologies Japan, Tokyo, Japan）. All of the media were supplemented with 10% bovine calf serum（FBS; Hyclone, South Logan, UT, USA）in a 5% CO_2 humidified incubator in plastic flasks（Becton Dickinson, Billerica, MA, USA）at 37°C. In a subculturing process, the number of cells was

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measured to determine the cell proliferation ratio.

2.2. Cell treatments

Exponentially growing cells were treated with 5 μM gemcitabine (Tokyo Chemical Industry, Japan) for 24 h. A pre-incubation time of 24 h was adopted to avoid cell cycle stage dependence. After gemcitabine treatment, cells were trypsinized followed by neutralization of the trypsin with medium. Cell suspensions were diluted in fresh complete medium to a density of approximately 10^6 cells/ml.

2.3. Nucleus staining and Foci formation

To study the effects of gemcitabine, the shape and size of the cell nucleus were confirmed by IF staining. As an indicator of single cells, 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes), which strongly binds to DNA, was used for fluorescence microscopy. To visualize the DNA DSBs, the 53BP1 protein that accumulated at the site of DSBs was stained by IF method.

CHO cells grown on coverslips were incubated with or without 5 μM gemcitabine for 24 h at 37°C. At different intervals after treatment, the cells were fixed using 3.6% formaldehyde solution and permeabilized with 0.5% Triton-X100 in cytoskeleton (CSK) buffer. Subsequently, the cells were incubated with a rabbit polyclonal antibody against 53BP1 (Bethyl Laboratories, Montgomery, TX, USA) at a concentration of 0.2 μg/100 μL dissolved in TBS-DT (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, 125 g/mL ampicillin, 5% skim milk) for 2 h. After washing with PBS, samples were incubated with 2 μg/mL secondary antibody conjugated with Alexa Fluor®594 (Molecular Probes, Life Technologies Japan, Tokyo, Japan) for 1 h, and then with 2 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 30 min. To analyze the samples, coverslips were mounted onto slide glasses with 10% glycerol in PBS. Image analysis was performed on overlay projections using a fluorescence microscope (IX81; Olympus, Tokyo, Japan) with a mounted digital camera (DP72; Olympus). To determine a cell proliferation, the number of nuclei was counted on the monitor at a constant magnification of fluorescence microscope. To determine a cell enlargement, meanwhile, the size of nuclei was analyzed by counting the number of pixels which occupied nucleus on digital images. Each examination was repeated at least 20 times at different sites to determine the average values and standard deviations (SDs).

2.4. Statistical analysis

Data were analyzed using Student's *t*-test and Mann－Whitney test. A p-value of < 0.05 was considered statistically significant. SD values are presented in the figures if the assay could be repeated at least three times. Statistical analyses were performed with SPSS (version 19.0; Tokyo, Japan).

3. Results

3.1. Inhibitory effect of gemcitabine on cell proliferation

We investigated the inhibitory effect of gemcitabine on the proliferation of CHO and xrs5 cells at a concentration of 5.0 μM for 24 h treatment. **Figure 1** shows an image of DAPI-stained cells with or without 24 h of treatment with gemcitabine. In both CHO and xrs5 cells, it was confirmed that gemcitabine treatment reduced cell numbers.

To ascertain the lethal effect of gemcitabine, the number of nuclei was counted on a fixed area of monitor at a constant magnification. **Table 1** shows the results of average cell number which was obtained at least 20 times at different images. Gemcitabine treatment reduced the cell number to 79.7% in CHO cells and 80.6% in xrs5 cells compared to untreated cells. A significant reduction was confirmed in both CHO and xrs5 cells by the gemcitabine treatment (both $p < 0.001$) (**Fig. 2**).

3.2. Cell enlargement caused by gemcitabine

Next, we observed changes in the size of CHO and xrs5 cells by the use of gemcitabine at a concentration of 5.0 μM for 24 h treatment. Cell size was determined by the number of pixels in DAPI-stained cells occupying on a digital screen. The results showed that gemcitabine treatment significantly increased the

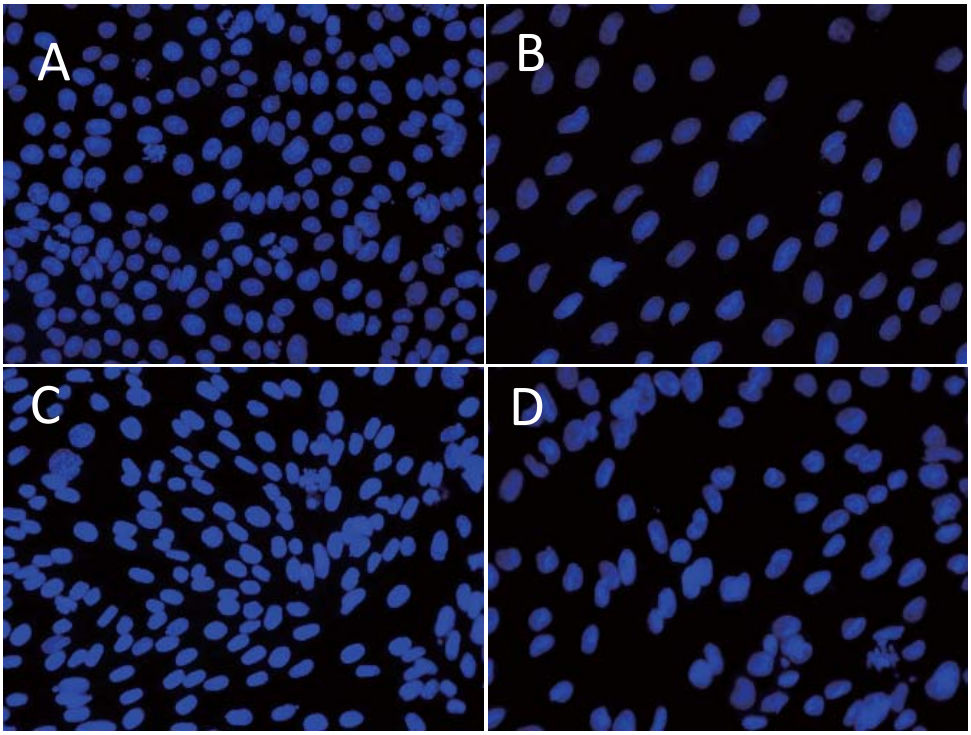

Figure 1 −Images of each cell nucleus in Chinese hamster ovary（CHO）and xrs5 cells with or without gemcitabine treatment for 24 h. The image shows cell nuclei stained with 2μM DAPI for 1 h.（A）CHO cells without gemcitabine treatment.（B）CHO cells treated with 5μM gemcitabine for 24 h.（C）xrs5 cells without gemcitabine treatment.（D）xrs5 cells treated with 5μM gemcitabine for 24 h.

number of pixels representing nuclei from 2944.4 ± 247.9 to 4204.1 ± 711.3（1.43-fold increase）in CHO cells, and from 2917.9 ± 244.8 to 4024.2 ± 653.6（1.38-fold increase）in xrs5 cells（Table 2）. **Figure 3** shows that gemcitabine treatment significantly increased the size of both CHO and xrs5 cells（both $p < 0.001$）, while no differences were observed between the CHO and xrs5 cell types（**Fig. 3**）. These data demonstrate that gemcitabine induces cell enlargement, while the extent of the enlargement does not depend on the cell type.

Table 1. Cell number on monitor

	Ave.	STDEV	*p* -value
CHO	60.85	8.62	< 0.001
CHO-Gem.	48.48	6.21	
xrs5	67.54	4.25	< 0.001
xrs5-Gem.	54.44	7.37	

3.3. DNA double strand breaks caused by gemcitabine treatment

The 53BP1 foci formation upon the use of gemcitabine was confirmed in the cells. CHO and xrs5 cells were incubated with or without 5 μM

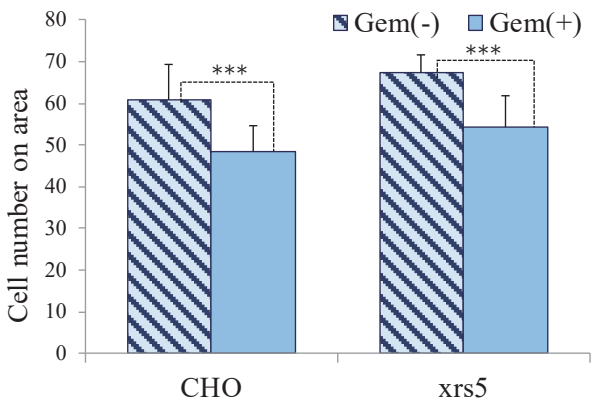

Figure 2 −Cell lethal effect of treatment with 5μM gemcitabine for 24 h. Susceptibility of Chinese hamster ovary（CHO）and xrs5 cells to mortality when left untreated（bar with diagonal stripes）or treated with gemcitabine（blue filled bar）. The vertical axis represents the number of cells confirmed on a digital screen. For each cell type, there was a significant difference between the untreated and gemcitabine-treated groups（$p < 0.001$）.

Table 2. Pixel value per cell

	Ave.	STDEV	*p* -value
CHO	2944.35	247.87	< 0.001
CHO-Gem.	4204.10	711.29	
xrs5	2917.85	244.84	< 0.001
xrs5-Gem.	4024.15	653.61	

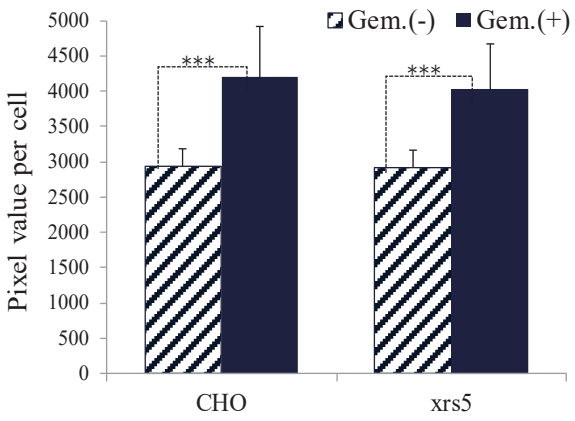

Figure 3 −Tendency for cell nucleus hypertrophy in Chinese hamster ovary (CHO) and xrs5 cells after 24 h of treatment with gemcitabine. The size of the cell nucleus is shown for CHO and xres5 cells without gemcitabine treatment (bar with diagonal stripes) or treated with 5μM gemcitabine for 24 h (dark blue filled bar). The vertical axis represents the number of pixels in the image when the nuclei were stained with DAPI. Data represent the average of values upon evaluation of 20 nuclei in each group. For each cell type, there was a significant difference between the untreated and gemcitabine-treated groups ($p < 0.05$).

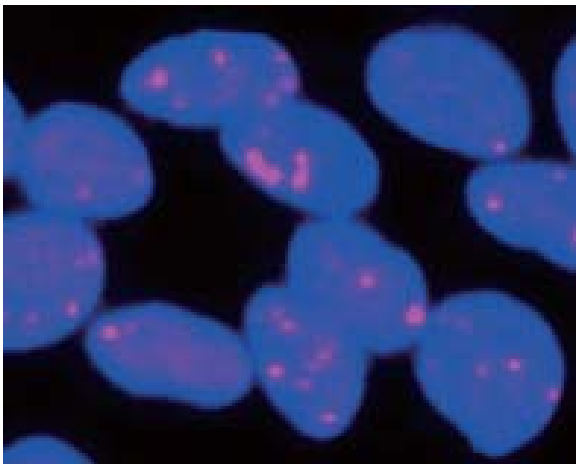

Figure 4 −P53-binding protein 1 (53BP1) foci formation in Chinese hamster ovary (CHO) cells treated with 5μM gemcitabine for 24 h. Immunofluorescence (IF) staining for 53BP1 was performed on the cells and the nuclei were stained with DAPI. Blue represents DAPI-stained cell nuclei and red represents 53BP1.

gemcitabine for 24 h before fixation. Subsequently, cells were incubated with a rabbit polyclonal antibody against 53BP1, Alexa-conjugated goat anti-rabbit immunoglobulin, and DAPI. **Figure 4** shows overlay projections of the 53BP1 and DAPI by immunofluorescence staining (IF). Blue represents DAPI-stained cell nuclei and red represents 53BP1, which is known to accumulate at the sites of DNA DSBs (**Fig. 5**).

The 53BP1 foci formation due to gemcitabine treatment in CHO and xrs5 cells is shown in **Figure 6**. Gemcitabine treatment significantly increased the number of 53BP1 foci in nuclei in both CHO and xrs5 cells (both $p < 0.001$). A small number of 53BP1 foci were also observed in gemcitabine-untreated cells. This indicates that the DNA DSB could occur by spontaneous generation. By the treatment of gemcitabine, the number of foci was increased 20.1-fold in CHO cells and 13.2-fold in xrs5 cells. It is confirmed that gemcitabine induces DNA DSBs alone, and the number of DNA DSBs was significantly higher in xrs5 cells than in CHO cells ($p < 0.001$).

3.4. Time dependent change of 53BP1 foci formation induced by gemcitabine

The 53BP1 foci formation induced by gemcitabine was observed with more detailed time sections. **Figure 7** presents the number of 53BP1 foci at 0.25 h (white bars), 2 h (shaded bars), and 24 h (dark blue bars) after the 24 h of gemcitabine treatment. Each number

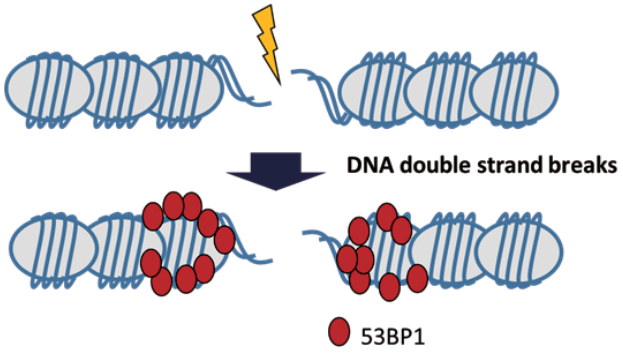

Figure 5 −Accumulation of p53-binding protein 1 (53BP1) at DNA double-strand breaks (DSBs). Accumulation of 53BP1 is an indicator of residual DNA DSBs.

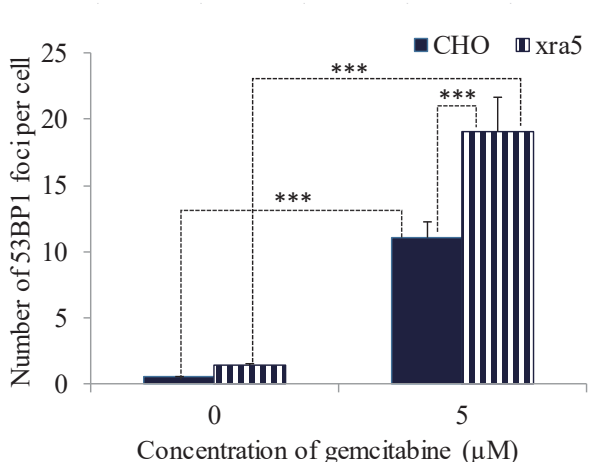

Figure 6 −Foci formation of p53-binding protein 1（53BP1）in Chinese hamster ovary（CHO）and xrs5 cells with or without gemcitabine treatment. Treatment with 5μM gemcitabine for 24 h markedly increased the number of 53BPI foci in both CHO（dark blue filled bar）and xrs5 cells（bar with vertical stripes）（both $p < 0.001$）. The number of 53BPI foci was significantly higher in xrs5 cells than in CHO cells（$p < 0.001$）.

represents the average number of 53BP1 foci in the nucleus for 20 cells. It was observed that the number of DNA DSBs showed no change over time in CHO cells with gemcitabine treatment, while an increasing trend of DNA DSBs induced by gemcitabine treatment was observed after 24 h in xrs5 cells. When compared with the findings at 0.25 h, the number of foci after 24 h increased 1.02-fold in CHO cells and 1.21-fold in xrs5 cells.

4. Discussion

In this study, we demonstrate that gemcitabine exerted cell enlargement as well as cell killing effects. Gemcitabine（dFdC）is a deoxycytidine analog that is well known for its antitumor activity. Intracellularly, dFdC is phosphorylated to its active metabolites by deoxycytidine kinase to dFdCMP, dFdCDP, and dFdCTP. dFdCTP is incorporated into DNA and as such can obstruct DNA replication and repair[15−17]. DNA DSBs, in which DNA is completely broken, are a serious form of DNA damage. Radiation is the most well-known cause of DNA damage, but other causes include carcinogens in food, tobacco, environmental chemicals, and reactive oxygen species[18]. In the present study, we demonstrated the effects of gemcitabine treatment alone on cell lethal as well as cell enlargement. Furthermore, we observed a marked increase in DNA DSBs induced by gemcitabine treatment alone. This indicates that gemcitabine not only inhibits DNA repair but also induces cell death by causing DNA DSBs.

Cell enlargement has been reported to be caused by

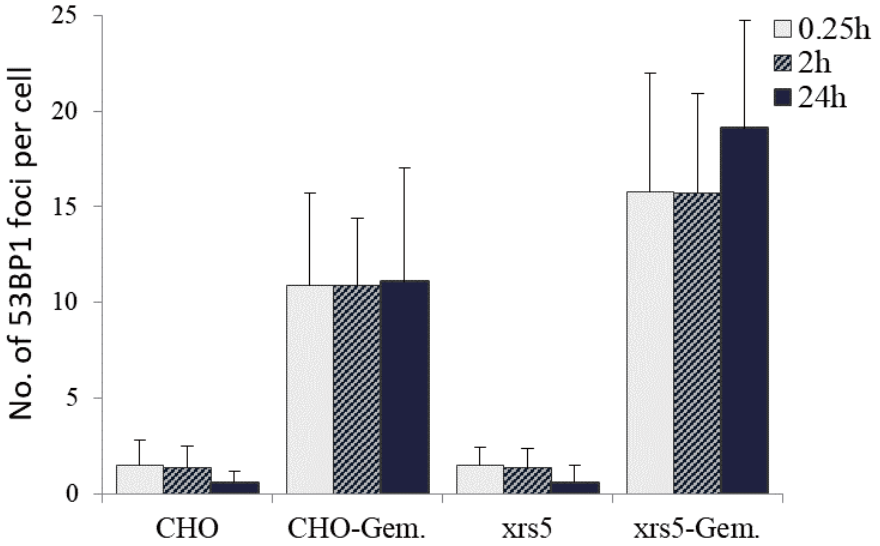

Figure 7 −Changes over time in p53-binding protein 1（53BP1）foci in Chinese hamster ovary（CHO）cells and xrs5 cells treated with 5μM gemcitabine for 24 h. The results represent the average number of 53BP1 foci at 0.25 h（white bars）, 2 h（bars with diagonal stripes）, and 24 h（dark blue filled bars）after the 24 h of treatment. Results represent the average number of 53BP1 foci in the nuclei of 100 cells. There was a significant difference in the number of foci between CHO and xrs5 cells in the gemcitabine-treated group（$p < 0.05$）.

the general accumulation of nuclear content, including bulk mRNA and proteins, accompanied by an increase in the size of the nucleus, which is dependent on new membrane synthesis[19]. Because gemcitabine is incorporated into DNA and can obstruct DNA replication, the cellular hypertrophy observed in this study may have been due to the accumulation of nucleic acids.

Upon comparing CHO and xrs5 cells, no differences were identified between these two cell types in terms of lethal effects of gemcitabine on the cells. However, in terms of DNA DSBs, the number of such breaks induced by gemcitabine was significantly higher in xrs5 cells than in CHO cells. Interestingly, a very small number of DNA DSBs were observed to develop spontaneously, which decreased over time, indicating that the DNA damage had been repaired. In contrast, the gemcitabine-induced DNA DSBs did not decrease in number over time. Even in xrs5 cells, an increase in their number was observed after 24 h.

Xrs5 is an X-ray-sensitive Chinese hamster ovary mutant cell line that is deficient in Ku80. Ku80 is a protein that, in humans, is encoded by the XRCC5 gene. Together, Ku70 and Ku80 make up the Ku heterodimer, which binds to DNA DSBs and is required for the non-homologous end-joining (NHEJ) pathway of DNA repair[20–21].

Homologous recombination (HR) and non-homologous end joining (NHEJ) are the two major pathways by which DSBs are repaired; their roles have been well characterized in somatic cells. HR is a high-fidelity process that occurs at the S/G2 phase of the cell cycle as it requires a template for repair[22]. In contrast, NHEJ ligates broken DNA ends and can introduce errors, but can occur at any stage of the cell cycle[11–14] (Fig. 8).

Because xrs5 cells are deficient in the Ku80 gene, DNA repair in them is dependent on the HR pathway. A possible explanation for the increased DNA DSBs after 24 h in xrs5 cells as observed in this study is the lack of activity of the HR repair pathway in the cells. Repair by HR requires a homologous chromosome, so the repair only works in the DNA synthetic phase prior to cell division. As the length of the cell cycle of CHO cells is 14 h, HR repair should be possible after 24 h.

Meanwhile, a recent study reported that, in xrs5 cells, DSB rejoining occurs in the G2 phase via the NHEJ pathway[23]. However, DSB ends can be rejoined correctly or incorrectly[24]. The increase in DNA DSBs after 24 h in xrs5 cells in this study may have involved such incorrect rejoining.

In any case, for repair of the increased DNA DSBs caused by gemcitabine in xrs5 cells, which cannot undergo NHEJ repair, pathways other than NHEJ may be required. Wachters *et al*. reported that they ruled out NHEJ as a target for gemcitabine because the radiosensitizing effect of gemcitabine was also observed in cells lacking functional DNA-PKcs or Ku80[25]. Yong *et al*. also reported that the radiosensitizing effect of gemcitabine is related to suppression of the HR pathway in rodent cell lines[26].

Our research group previously confirmed RAD51 foci formation in CHO cells that had been pretreated with gemcitabine. RAD51 is a 339-amino-acid protein that plays a major role in the HR of DNA during DSB

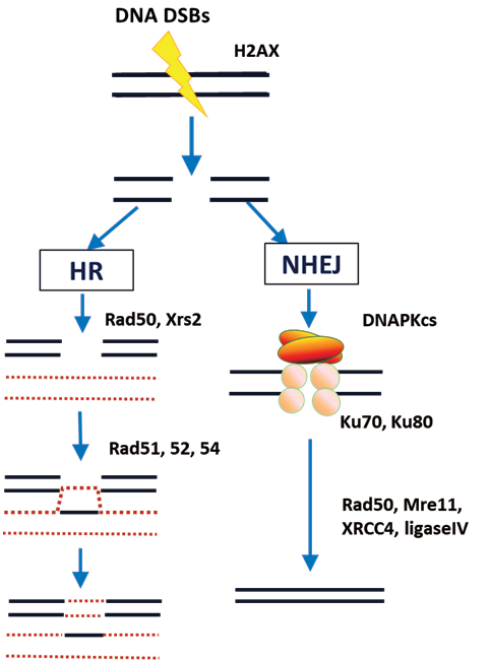

Figure 8 −DNA double-strand break repair pathway. Homologous recombination (HR) and non-homologous end joining (NHEJ) are the two major pathways by which DSBs are repaired.

repair[27]. Upon treatment with gemcitabine, there were very few foci of RAD51 clustered at HR repair sites compared with the number of DNA DSBs. Meanwhile, the number of RAD51 foci after irradiation along with gemcitabine pretreatment greatly increased and showed an increasing trend over time. Compared with the results obtained in this study, the repair pathway by which DNA DSBs induced by gemcitabine may differ from the pathway caused by the additive effects of irradiation.

The detailed pathway by which gemcitabine-induced DNA DSBs are repaired has not been elucidated. Our findings in this study－that the cell lethal effects of gemcitabine involve cell enlargement, and that repair of DNA DSBs induced by gemcitabine involves repair pathways other than the NHEJ pathway－should help elucidate more mechanistic and molecular details of the specific repair pathway associated with gemcitabine. Further investigation is required to elucidate in more detail the mechanism behind the involvement of HR and NHEJ in gemcitabine.

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