資料

Examining the effect of gemcitabine on DNA double-strand breaks using an immunofluorescence method in mammalian cells

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Abstract : Gemcitabine（dFdC）is a deoxycytidine analogue that is well known for its anti-tumor activity, especially in pancreatic cancer. It is an effective drug for sensitizing cells to radiation, but the exact interaction of dFdC and radiation has not been elucidated. In this study, we evaluated the effect of gemcitabine on radiosensitization in mammalian cells using immunofluorescence（IF）to examine DNA double-strand breaks（DSBs）induced in cell nuclei. To detect the site of DNA DSBs, IF staining was performed to observe phosphorylated p53-binding protein 1（53BP1）-positive foci over time. Cells were treated with or without 5 μM gemcitabine for 24 hours prior to treatment with or without post-γ-ray irradiation. Gemcitabine could induce DNA DSBs without irradiation, which were not repaired over time. After irradiation, significantly higher numbers of 53BP1 foci were observed in cells pre-treated with gemcitabine than in untreated cells. Higher numbers of 53BP1 foci were confirmed to remain over time in the cells treated with both gemcitabine and irradiation. Overall, our data suggest that the enhanced cell killing effect observed with gemcitabine treatment is dependent on the inhibition of cellular DNA DSB repair mechanisms.

Key words: Radiosensitization, gemcitabine, 53BP1, DNA double-strand break, immunofluorescence

1. Introduction

Gemcitabine（4-amino-1-[3,3-difluoro-4-hydroxyl-5-（hydro-xymethyl）Tetrahydrofuran-2-yl]-1H − pyrimidin-2-one; dFdC）is a deoxycytidine analogue that is well known for its anti-tumor activity and is used as a standard therapy for patients with advanced pancreatic cancer. It is one of the most 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 with radiation 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 on DNA DSB repair by gemcitabine[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 a DSB. 53BP1（also called TP53BP1）is a chromatin-associated factor that promotes immunoglobulin class switching. Cells have DNA DSB repair pathways, known as non-homologous end joining（NHEJ）and homologous recombination（HR）[11–14].

To evaluate the radiosensitization effect of gemcitabine, we used a wild-type Chinese hamster ovary（CHO）cell line. Immunofluorescence（IF）staining 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 radiosensitization process.

2. Materials and methods

2.1. Cell culture procedures

CHO cells were cultured in alpha-MEM medium（Life Technologies Japan, Tokyo, Japan）, supplemented with 10% fetal bovine 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.

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2.2. Cell treatments

Exponentially growing cells were treated with 5 μM gemcitabine (Tokyo Chemical Industry, Japan) for 24 hours. A pre-incubation time of 24 hours was adapted to avoid cell cycle stage dependency. After gemcitabine treatment, cells were detached using trypsin and resuspended in medium. Then, the cell suspensions were diluted in fresh complete medium to a density of approximately 10^6 cells/mL.

Irradiation of cultured cells was performed using a Gammacell instrument. A Gammacell is an irradiator mounted with Cs-137, which is used widely for biological studies of radiosensitization effects. To examine the effects of radiosensitization, cells were irradiated with constant exposure dose of gamma rays from ^{137}Cs.

2.3. 53BP1 foci formation

To visualize the DNA DSBs, the 53BP1 protein that accumulated at the site of DSBs was stained using IF. CHO cells grown on coverslips were incubated with or without 5 μM gemcitabine for 24 hours at 37° C. After incubation, cells were irradiated with 1 Gy followed by IF staining. At different time intervals after irradiation, cells were fixed using a 3.6% formaldehyde solution and permeabilized with 0.5% Triton-X100 in cytoskeleton (CSK) buffer. Subsequently, 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 hours. 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 hour, then with 2 μg/mL 4′, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 30 minutes. 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). Nuclei size was analyzed by the value of pixels that occupied the nucleus on digital images using the fluorescence microscope. The number of nuclei was counted on the monitor. Each value was obtained repeatedly at least 20 times to determine average values and standard deviations (SDs).

2.4. Statistical analysis

Data were analyzed using the 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. 53BP1 foci formation using IF

3.1.1. 53BP1 foci formation in control CHO cells

The 53BP1 foci formation was confirmed in control CHO cells that were untreated with gemcitabine or irradiation. CHO cells were incubated without gemcitabine before fixation. Subsequently, cells were incubated with a rabbit polyclonal antibody against 53BP1, Alexa-conjugated goat anti-rabbit immunoglobulin, and DAPI. Figure 1 shows overlay

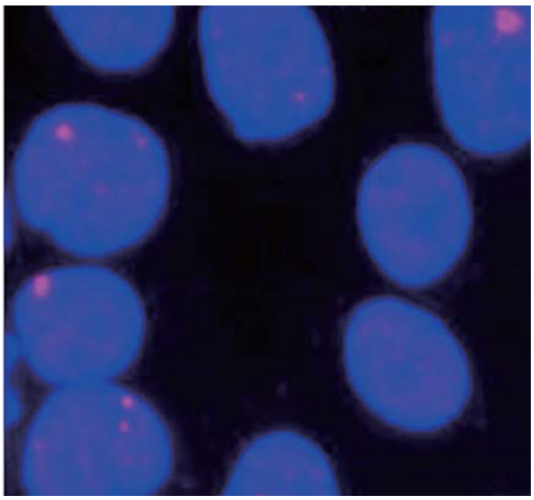

Figure 1. P53-binding protein 1 (53BP1) foci formation in control Chinese hamster ovary (CHO) cells. CHO cells were not treated with gemcitabine. Immunofluorescence (IF) staining for 53BP1 was performed on the cells and the nuclei were stained with DAPI. IF images show few 53BP1 foci in the CHO cell nuclei.

projections of the 53BP1 and DAPI IF signal. The images indicate scattered 53BP1 foci in the nuclei of CHO cells that were stained with DAPI. Despite no treatment with gemcitabine, some 53BP1 foci were confirmed in the control CHO cells.

3.1.2. 53BP1 foci formation is induced by irradiation

Next, we confirmed that further 53BP1 foci formation could be induced by irradiation. CHO cells were incubated without gemcitabine, exposed to 1 Gy γ-rays, then fixed after 15 minutes (Fig. 2A) or 2 hours (Fig. 2B). The images represent a typical pattern of 53BP1 in the nuclei of CHO cells.

3.1.3. 53BP1 foci formation is induced by both gemcitabine and irradiation

53BP1 foci formation was induced by both gemcitabine and irradiation, as shown in Figure 3. CHO cells were incubated with 5 μM gemcitabine for 24 hours, then exposed to 1 Gyγ-rays before fixation. The images represent extensive 53BP1 foci in the nuclei of CHO cells after 15 minutes (Fig. 3A) and 2 hours (Fig. 3B).

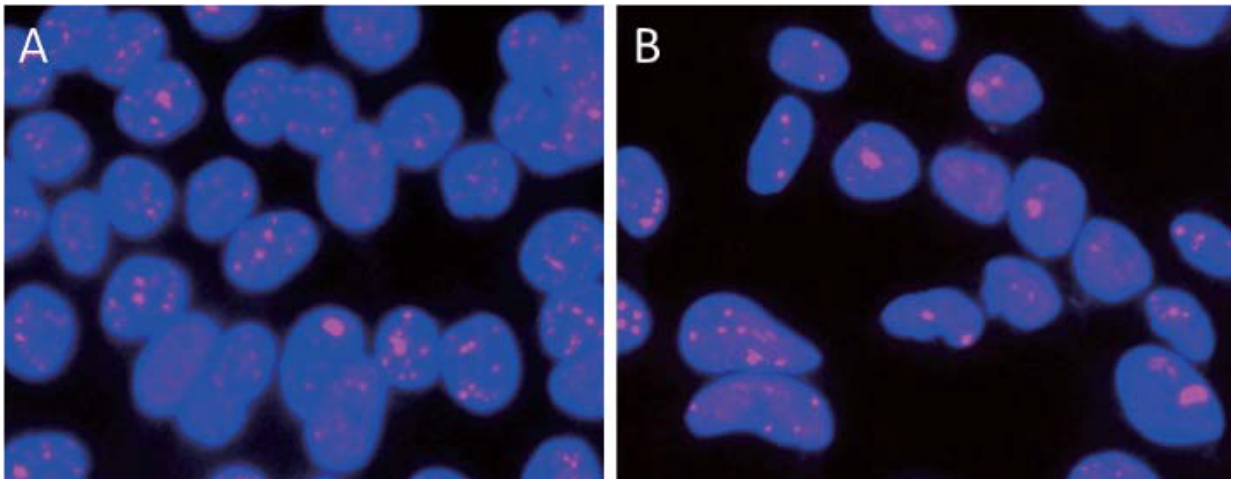

Figure 2. P53-binding protein 1 (53BP1) foci formation in Chinese hamster ovary (CHO) cells irradiated with 1 Gy γ-rays. CHO cells were incubated without gemcitabine treatment for 24 hours before immediate fixation. Immunofluorescence (IF) staining for 53BP1 was performed on the cells and the nuclei were stained with DAPI. IF images show 53BP1 foci in the nuclei of CHO cells (A) after 15 minutes and (B) after 2 hours.

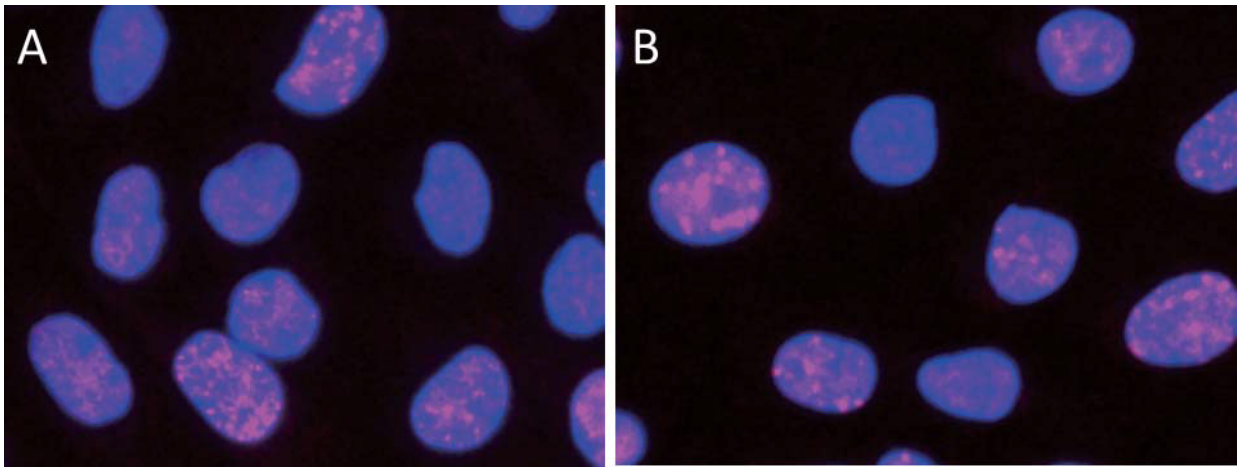

Figure 3. P53-binding protein 1 (53BP1) foci formation in Chinese hamster ovary (CHO) cells treated with gemcitabine followed by irradiation with 1 Gy γ-rays. CHO cells were incubated with 5 μM gemcitabine for 24 hours, then exposed to 1 Gy γ-rays. Immunofluorescence (IF) staining for 53BP1 was performed on the cells and the nuclei were stained with DAPI. IF images show high numbers of 53BP1 foci in the nuclei of CHO cells irradiated (A) after 15 minutes and (B) after 2 hours.

3.2. Change of 53BP1 foci number over time

3.2.1. 53BP1 foci number in control cells

We analyzed the number of 53BP1 foci in the control CHO cells that were not treated with gemcitabine or irradiation. Figure 4A shows the changes in 53BP1 foci number over time. At all timepoints examined, small numbers of 53BP1 foci were confirmed. The average number of foci was 1.5 (± 1.3) after 15 minutes, then slightly reduced to 0.6 (± 0.6) after 24 hours.

3.2.2. 53BP1 foci number induced by gemcitabine

The changes in 53BP1 foci number induced by gemcitabine are shown in Figure 4B. Cells were treated for 24 hours with 5 μM gemcitabine, then fixed and stained. Different from the control cells, gemcitabine treatment resulted in an increased number of 53BP1 foci. The average foci number was 10.9 (± 8.7) after 15 minutes and 11.1 (± 6.8) after 24 hours. No decrease in foci number was observed even after 24 hours.

3.2.3. 53BP1 foci number induced by irradiation

The effect of irradiation only was also examined. Cells were exposed to a dose of 1 Gy γ-rays without incubation with gemcitabine, then fixed and stained. 53BP1 foci formation increased compared with the induction seen with gemcitabine alone. However, the foci number reduced rapidly over time (Fig. 4C). The average foci number was 17.0 (± 3.4) after 15 minutes, which reduced to 0.94 (± 0.6) after 24 hours. The foci number confirmed at 24 hours was almost the same as that observed in the control cells with no irradiation.

3.2.4. 53BP1 foci number induced by both gemcitabine and irradiation

Figure 4D shows the changes in 53BP1 foci formation induced by both gemcitabine and irradiation. Cells were incubated with 5 μM gemcitabine for 24 hours, exposed to 1 Gy γ-rays, then fixed and stained. The average number of 53BP1 foci observed here was the highest among all experiments. The average foci number was 25.7 (± 13.8) after 15 minutes, which reduced to 14.7 (± 7.6) after 24 hours. Additionally, the decline rate appeared to be lower than that observed in previous experiments because 54% of the foci number seen at 15 minutes remained after 24 hours.

3.3. The synergistic effect between gemcitabine and irradiation

Tables 1−3 show the effect of gemcitabine with or without irradiation after 15 minutes, 2 hours, and 24 hours. At all timepoints, significant differences were confirmed between the use of gemcitabine and/or irradiation. However, in terms of changes in foci number over time, the effect of gemcitabine and irradiation showed quite a different pattern (Fig. 5). With gemcitabine treatment alone, the number of 53BP1 foci increased 7.3-fold compared with that in control cells ($p < 0.001$). The number of foci was similar after 15 minutes and 2 hours. Additionally, 1 Gy irradiation increased the 53BP1 foci number 1.5-fold compared with that in gemcitabine-treated cells. However, the 53BP1 foci number rapidly reduced after 24 hours to almost the same level observed in control cells. In cells treated with both gemcitabine and irradiation, the foci number after 15 minutes was more than 2.4-fold higher than with gemcitabine alone and more than 1.5-fold higher than with irradiation alone. Furthermore, 57.2 % of the foci number at 15 minutes remained at the 24-hour timepoint.

4. Discussion

In this study, we examined the number of DNA DSBs caused by gemcitabine or irradiation using IF staining of the 53BP1 protein. 53BP1 is phosphorylated and associates with chromatin at the site of DSBs. 53BP1 localizes rapidly to discreet foci within the nucleus of cells exposed to DNA DSB-inducing agents, allowing IF staining of 53BP1 to represent sites of DSBs[15] (Fig. 6).

In control CHO cells with no gemcitabine or irradiation treatment, very few 53BP1 foci were observed. DNA DSBs can be produced from various

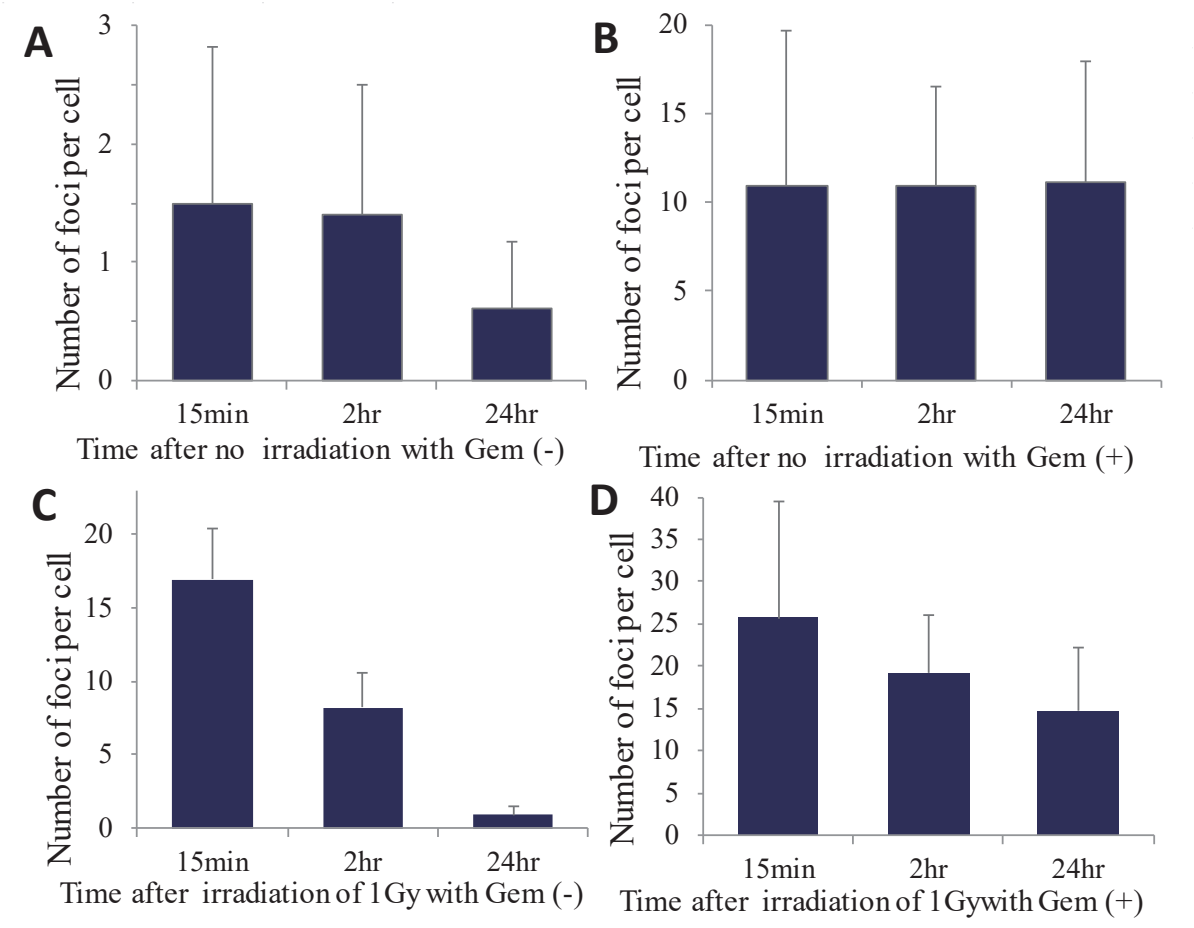

Figure 4. Change of p53-binding protein 1 (53BP1) foci number in Chinese hamster ovary (CHO) cells with or without gemcitabine prior to treatment with or without post-γ irradiation. Immunofluorescence (IF) staining for 53BP1 was performed on the cells and the nuclei were stained with DAPI. (A) CHO cells were incubated for 24 hours with no gemcitabine or irradiation treatment before immediate fixation. The average number of foci were 1.5 (± 1.3) after 15 minutes, which slightly reduced to 0.6 (± 0.6) after 24 hours. (B) Change of 53BP1 foci number induced by gemcitabine. CHO cells were incubated for 24 hours with 5 μM gemcitabine before immediate fixation. The average foci number was 10.9 (± 8.7) after 15 minutes and 11.1 (± 6.8) after 24 hours. (C) Change of 53BP1 foci number induced by irradiation. CHO cells were exposed to a dose of 1 Gy γ-rays without pre-incubation with gemcitabine. The average foci number was 17.0 (± 3.4) after 15 minutes, which rapidly reduced to 0.94 (± 0.6) after 24 hours. Change of 53BP1 foci number induced by gemcitabine and irradiation together. CHO cells were incubated with 5 μM gemcitabine for 24 hours, then exposed to 1 Gy γ-rays. The average foci number was 25.7 (± 13.8) after 15 minutes, which reduced to 14.7 (± 7.6) after 24 hours.

Table 1. P53-binding protein 1 (53BP1) foci formation induced by gemcitabine with or without irradiation after 15 minutes

	0Gy		1Gy		*p* -value
	Ave.	SD	Ave.	SD	
Gem (-)	1.5	1.3	17.0	3.5	$p < 0.001$
Gem (+)	10.9	8.9	25.7	14.2	$p < 0.001$
p -value	$p < 0.001$		$p < 0.001$		

Table 2. P53-binding protein 1 (53BP1) foci formation induced by gemcitabine with or without irradiation after 2 hours

	0Gy		1Gy		*p*-value
	Ave.	SD	Ave.	SD	
Gem (-)	1.4	1.1	8.3	2.4	$p < 0.001$
Gem (+)	10.9	5.7	19.2	7.1	$p < 0.001$
p-value	$p < 0.001$		$p < 0.001$		

Table 3. P53-binding protein 1 (53BP1) foci formation induced by gemcitabine with or without irradiation after

	0Gy		1Gy		*p*-value
	Ave.	SD	Ave.	SD	
Gem (-)	0.6	0.6	0.9	0.6	$p < 0.001$
Gem (+)	11.1	7.0	14.7	7.7	$p < 0.001$
p-value	$p < 0.001$		$p < 0.001$		

sources, such as ionizing radiation, or from errors occurring during normal DNA replication or recombination[15]. Our data obtained here support these results.

Next, we confirmed that the number of 53BP1 foci significantly increased following treatment with 5 μM gemcitabine for 24 hours. In addition, the foci number did not appear to change over time, even after 24 hours. Furthermore, the number of 53BP1 foci induced by irradiation was larger than that induced by gemcitabine, and this number rapidly decreased after 24 hours. Our data suggest that the DNA DSB-related pathways differ between gemcitabine and irradiation. Gemcitabine is phosphorylated intracellularly to its active metabolites (gemcitabineMP, gemcitabineDP, and gemcitabineTP) by deoxycytidine kinase. Because gemcitabineTP is incorporated into DNA and obstructs DNA replication and repair[16–18], the DNA

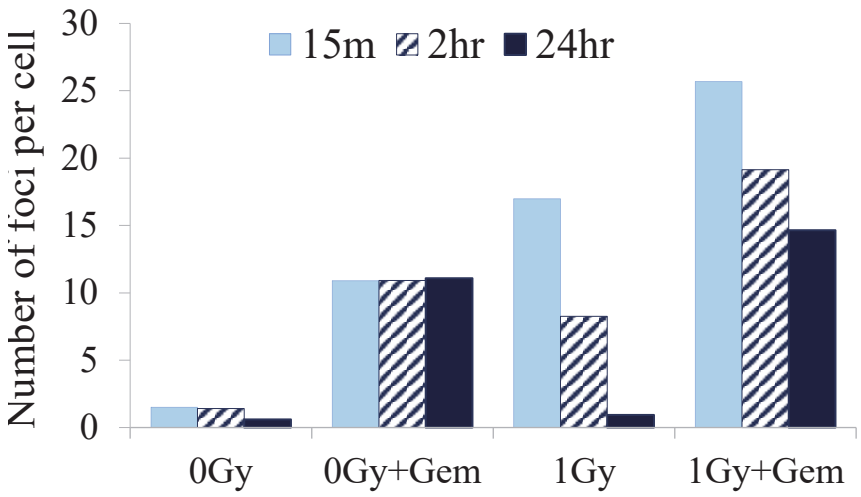

Figure 5 – Change of p53-binding protein 1 (53BP1) foci number with or without gemcitabine and/or irradiation treatment. In each treatment condition, shown as '0 Gy', '0 Gy + Gem.', '1 Gy', and '1 Gy + Gem.', the 53BP1 foci number was compared over time.

DSBs induced by gemcitabine likely cannot be repaired. In contrast, it is well known that radiosensitivity in mammalian cells is correlated with irradiation dose. Ionizing radiation induces an array of lesions in DNA, including base damage, single-strand breaks, and DSBs. DNA DSBs are generally thought to be the most relevant lesion in radiation-induced killing of cells. Unless the lethal dose of irradiation is used, DNA DSBs can be repaired using the HR or NHEJ pathway[11–14]. NHEJ ligates two broken ends, whereas HR refers to the use of the sequence homologous to the DSB site, resulting in gene conversion. Different cell conditions can affect whether NHEJ or HR is initiated for DSB repair[19]. In mammals, NHEJ is reportedly the most prominent cellular DNA repair pathway of radiation-induced DNA DSBs[9]. Because a 1 Gy dose is not high enough to kill cells, the results of this study suggest that most of the DNA DSBs induced by 1 Gy irradiation could be repaired after 24 hours.

Our data also showed that the 53BP1 foci formation induced by gemcitabine and irradiation together induced the largest number of DNA DSBs compared with gemcitabine or irradiation alone, suggesting a synergistic effect between them. In addition, the foci number remained high after 24 hours compared with the other conditions. The peculiarity that gemcitabine has no effect on DNA DSB repair[10] could enhance the synergistic effect between gemcitabine and irradiation.

In a previous study, we reported that the radiosensitizing effect of gemcitabine was not observed in Ku80-deficient CHO cells (xrs5)[20]. Ku80 is a protein encoded by the XRCC5 gene. Ku70 and Ku80 make up the Ku heterodimer, which binds to DNA DSB ends and is required for the NHEJ DNA repair pathway. Because xrs5 cells are deficient in NHEJ repair[21–22], a strong radiosensitizing effect was confirmed in xrs5 cells compared with normal CHO cells. Conversely, we did not confirm a synergistic effect with gemcitabine in the xrs5 cells. Cell survival rates were assessed using colony formation assays, which demonstrated that there was no difference in survival between cells treated with irradiation alone and those treated with irradiation and gemcitabine.

In lower eukaryotes such as yeast, DSBs are repaired by RAD52-dependent HR. RAD52 binds to DNA ends, thereby protecting them from exonuclease activity and activating end-to-end interaction and HR[23]. In vertebrates, however, DSBs are primarily repaired by Ku-dependent NHEJ. In mammals, NHEJ is the most prominent cellular DNA repair pathway of radiation-induced DNA DSBs[9]. In fact, the strong radiosensitizing effect confirmed in xrs5 cells in our previous study showed that NHEJ is the principal repair pathway of radiation-induced DNA DSBs. In addition, the lack of synergistic effects with irradiation and gemcitabine in xrs5 cells suggests that the effects of gemcitabine-induced radiosensitization on the NHEJ repair pathway are involved.

Additionally, our research group confirmed RAD51 foci formation in mammalian cells that were irradiated and pre-treated with gemcitabine. RAD51 is a 339-amino acid protein that plays a major role in HR of DNA during DSB repair[24]. The detailed repair pathway of gemcitabine-induced radiosensitization has not been elucidated. Our finding in this study − that the DNA DSBs induced by gemcitabine likely cannot be repaired − will help elucidate more mechanistic and molecular details of the specific repair pathway associated with gemcitabine-induced radiosensitization.

Further investigation is required to elucidate a more detailed mechanism for the involvement of HR and NHEJ in gemcitabine-induced radiosensitivity.

In conclusion, this study shows that gemcitabine treatment can increase the number of DNA DSBs that are not repaired in CHO cells. The effect of gemcitabine-induced radiosensitization can inhibit the repair of DNA DSBs induced by irradiation. These results indicate that gemcitabine induces DNA DSBs alone and can synergize with irradiation to obstruct cell-mediated repair of the DSBs.

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