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**Title**

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**Author(s)**

Kinashi, Yuko; Okumura, Kakuji; Kubota, Yoshihisa; Kitajima, Erika; Okayasu, Ryuichi; Ono, Koji; Takahashi, Sentaro

**Citation**


**Issue Date**

2014-06

**URL**

http://hdl.handle.net/2433/188913

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**Type**

Journal Article

**Textversion**

author
Dose-rate effect was observed in T98G glioma cells following BNCT

Yuko KINASHI1)*, Kakuji OKUMURA1)*, Yoshihisa KUBOTA2), Erika KITAJIMA3), Ryuichi OKAYASU2), Koji ONO1) and Sentaro TAKAHASHI1)

1) Kyoto University Research Reactor Institute,

2) National Institute of Radiological Sciences,

3) Osaka High-technology College

Reprint requests to Yuko Kinashi, M.D., Ph.D.

Research Reactor Institute, Kyoto University,

Kumatori-cho, Sennann-gun, Osaka 590-0494, Japan

Tel.: 81-724-51-2437

Fax.: 81-724-51-2623

E-mail: kinashi@rrri.kyoto-u.ac.jp

* Authors with equal contributions

Keywords: dose-rate effect, BNCT, DNA-DSBs, glioma
ABSTRACT

Background: It is generally said that low LET radiation produce high dose-rate effect, on the other hand, no significant dose rate effect is observed in high LET radiation. Although high LET radiations are produced in BNCT, little is known about dose-rate effect of BNCT.

Materials and Methods: T98G cells, which were tumor cells, were irradiated by neutron mixed beam with BPA. As normal tissue derived cells, Chinese hamster ovary (CHO-K1) cells and DNA double strand breaks (DNA-DSBs) repair deficient cells, xrs5 cells were irradiated by the neutrons (not including BPA). To DNA-DSBs analysis, T98G cells were stained immunochemically with 53BP1 antibody. The number of DNA-DSBs was determined by counting 53BP1 foci.

Results: There was no dose-rate effect in xrs5 cells. D₀ difference between 4cGy/min and 20cGy/min irradiation were 0.5 and 5.9 at the neutron and gamma-ray irradiation for CHO-K1, and 0.3 at the neutron for T98G cells. D₀ difference between 20cGy/min and 80cGy/min irradiation for T98G cells were 1.2 and 0.6 at neutron irradiation plus BPA and gamma-ray. The differences between neutron irradiations at the dose rate in T98G cells were supported by not only the cell viability but also 53BP1 foci assay at 24 hours following irradiation to monitor DNA-DSBs.

Conclusion: Dose-rate effect of BNCT when T98G cells include 20ppm BPA was greater than
that of gamma-ray irradiation. Moreover, Dose-rate effect of the neutron beam when CHO-K1 cells did not include BPA was less than that of gamma-ray irradiation. These present results may suggest the importance of dose-rate effect for more efficient BNCT and the side effect reduction.

INTRODUCTION

Boron neutron capture therapy (BNCT) is a treatment that can selectively destroy tumor tissue by $^{10}$B (n,α) $^7$Li reaction without significant damage to normal tissue. In principle, $^{10}$B compounds are selectively delivered to tumor cells and exposed to low-energy thermal neutrons. The reaction with thermal neutron produces an alpha particle and $^7$Li nucleus that have high linear energy transfer (LET) values and enriched biological effect. Although it depends on the kinds of $^{10}$B compounds, DNA damage or decrease in cell viability produced by BNCT is greater than neutron alone or gamma-ray (Kinashi et al., 2011, Dagrosa et al., 2011).

It is generally accepted that for cell viability, low LET radiation such as X-ray and gamma-ray produce high dose-rate effect, on the other hand, no significant dose rate effect is observed in high LET radiation such as neutrons, alpha particle, and heavy ion (Metting et al., 1985, Ainsworth et al., 1976, Heiber et al., 1987, Yang et al., 1986, Furusawa et al., 2006, Fairchild et al., 1985). However, there is no data about dose-rate effect for BNCT. It is also
difficult to estimate dose-rate effect of BNCT from the past studies because the neutron beam used for BNCT is the mixed beam that contains thermal, epithermal and fast neutrons, and gamma-ray. A better understanding of dose-rate effect for BNCT will help to improve the efficiency of this therapy as well as to reduce the side effect in normal tissue.

DNA double strand breaks (DNA-DSBs) are potentially lethal lesions and important biological effect of ionizing radiation. DNA-DSBs can be repaired by DNA repair proteins, such as gamma-H2AX, DNA-PKcs, and 53BP1. Therefore, immunochemically stained foci for the proteins have been used for a sensitive and efficient marker for DNA-DSBs. Several studies using this method showed that the number of foci may reflect not only the dose but also the types of radiation. Okayasu et al. reported that the disappearance of phosphorylated DNA-PKcs foci in 180BR cells irradiated heavy ions were slow (Okayasu et al., 2006).

We investigated dose-rate effect of the neutron mixed radiation beam used for BNCT in Kyoto University Research Reactor (KUR) at cell survival fraction and DNA-DSBs. We report here that dose-rate effect of BNCT is observed in T98G cells that are human tumor cell line at above-mentioned both biological points.

MATERIALS AND METHODS

Cell culture
The Chinese hamster ovary-derived, wild-type CHO-K1 cells and the human glioblastoma cell lines, T98G were purchased from Riken BRC Cell Bank. The radiosensitive xrs5 mutant cells (Ku80-deficient CHO cell line) kindly supplied by Prof. P Jeggo (Jeggo et al., 1983, Kemp et al., 1984). CHO-K1 and xrs5 cells were cultured in Memo medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Biowest). T98G cells were grown in RPMI 1640 (Invitrogen) supplemented 10% heat-inactivated FBS (Biowest). These cells were maintained at 37°C in a humidified atmosphere with 5.0% CO₂.

10B compound and Irradiation experiments

For irradiation, the cells were trypsinized, suspended in the above mentioned medium, and then aliquoted to each teflon tube. A stock solution of 10B-para-boronphenylalanine (BPA) was used for BNCT experiment to T98G cells. T98G cell suspensions were incubated with BPA at 20ppm concentration 1 hour before neutron irradiation. The Heavy Water Facility of the Kyoto University Research Reactor (KUR) was used for neutron mixed beam irradiation (approximately 4cGy/min and 20cGy/min). Neutron fluencies were measured by radioactivation of gold foil and gamma-ray doses by TLD. The physical dose percentages to total dose of thermal (<0.5eV), epithermal (0.5eV-10keV), fast (>10keV) neutrons, and gamma-ray were approximately 30%, 3%, 22% and 45% respectively, when the reactor was operated at 4cGy/min or 20cGy/min (Fig. 1). Gamma-ray irradiation was carried out with a 60Co
gamma-ray irradiator at dose rate of about 4cGy/min, 20cGy/min, 80cGy/min and 2Gy/min.

Cell survival assay

Survival fraction of CHO-K1, xrs5 and T98G cells were determined by conventional colony formation assay. After irradiation, the cell suspension solutions were properly diluted, and the known number of cells were seeded in cell culture dishes and incubated for 7-9 days (CHO-K1 and xrs5) and 2 weeks (T98G). The cells were fixed with 70% ethanol and stained with crystal violet solution (Wako) for counting colonies.

Immunofluorescent staining

Irradiated T98G cells were seeded onto 22 mm × 22 mm cover slips in 6-well microplates, incubated for 30min, 1, 3, 6 and 24 hours, and washed with cold PBS (Invitrogen). The cells were fixed with 3.6% formalin/PBS for 15 min, permeabilized with 0.5% TritonX-100/PBS on ice for 15 min and washed thoroughly with PBS. The cover slips were incubated for 2 hours at 37°C with primary antibody, rabbit monoclonal anti-53BP1 antibody (Bethyl Laboratories) in TBS-DT solution (20 mM Tris-HCl, 137 mM NaCl, containing 0.1% Tween-20, 5% skim milk). After being washed three times with cold PBS, the cells were incubated for 1 hour at 37°C with secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen) in TBS-DT solution. After washing three times with PBS, The cover slips were mounted on slide glasses with DAPI (4’-6-diamidino-2-phenylindole) in 10%-glycerol/PBS to
counter-stain the nuclei.

Image acquisition and Analysis

Images were acquired with a fluorescence microscope (KEYENCE, BZ-9000). ImageJ (National Institutes of Health) were used for image processing and automated foci counting.

Some foci images were scored by eye.

RESULTS

Dose-rate effect was observed in CHO-K1 cells at cell survival assay

Fig. 2 shows cell survival fraction of CHO-K1 (referred as K1 in Fig. 2) and xrs5 cells irradiated with the neutron mixed beam. A scatter plot is used because the information of the dose rate was not available in real time so that the total doses were not always same in the mixed beam irradiation. Each point shows the survival fraction, and black and dot lines are used to fit log-linear model with Microsoft Excel software. As expected, xrs5 cells were more sensitive to the mixed beam than CHO-K1 cells. There was no significant difference for the cell survival at xrs5 cells between 4cGy/min and 20cGy/min irradiation (Fig. 2 and Table 1). On the other hand, dose-rate effect between 4cGy/min and 20cGy/min was observed in CHO-K1 cells irradiated with especially gamma-ray (Fig. 2B). The D0 differences in CHO-K1 cells were 0.5 and 5.9 for neutron and gamma-ray irradiation (Table 1). The surviving fraction for CHO-K1
cells after 20cGy/min gamma-ray irradiation was significantly lower than that after 4cGy/min irradiation.

Dose-rate effect of BNCT was greater than that of gamma-ray at T98G cell survival assay

Fig. 3 shows cell survival fraction of T98G cells irradiated with the neutron mixed beam alone (referred as no BPA in Fig. 3A) or the mix beam plus 20ppm BPA (referred as plus BPA in Fig. 3B). When T98G cells did not include BPA, D₀ between 4cGy/min and 20cGy/min irradiation did not show a big difference (D₀ difference was 0.3). 20cGy/min irradiation plus BPA produced a decrease in cell survival fraction, compared to 4cGy/min plus BPA (Fig. 3B) and the D₀ difference was 1.2 (Table 2). We also researched dose-rate effect of gamma-ray in T98G cells because it was thought that gamma-ray in the neutron beam significantly contribute to dose-rate effect. Assuming that T98G cells include 20ppm BPA, the dose-rate of 4cGy/min and 20cGy/min neutron irradiation is converted to 20cGy/min and 80cGy/min, due to ¹⁰B (n,α) reaction. In Fig. 4, the difference of D₀ between 20cGy/min and 80cGy/min in plus BPA group was larger than that in gamma-ray group.

Induction of 53BP1 foci in T98G cells

Dose-rate effect was greater in T98G cells incubated with BPA (Table 2 and Fig. 4). So, we researched DNA-DSBs, because it is said that high LET radiation induces more DNA-DSBs than low LET radiation. The results of 53BP1 foci in T98G cells 30min after neutron irradiation
are shown in Fig. 5. Although induction of 53BP1 foci increased with dose, the number of 53BP1 foci following 20cGy/min irradiation seemed to be larger than 4cGy/min irradiation, regardless of existence of BPA.

*Loss of 53BP1 foci in T98G cells*

Fig. 6 shows time dependence of 53BP1 foci after irradiation. The number of 53BP1 foci in not BPA including cells rapidly decrease to about zero, compared to the cells incubated BPA. When T98G cells include BPA, a lot of 53BP1 foci had remained even at 24 hours after neutron irradiation. Moreover, the difference between the cells plus BPA irradiated with 4cGy/min and 20cGy/min was significant at 24 hours post irradiation.

**DISCUSSION**

In this report, we showed that dose-rate effect was observed at cell survival rate in T98G cells following BNCT, and the effect was greater even if it was compared with gamma-ray irradiation at the same dose-rate (Fig. 4). Little dose-rate effect of gamma-ray or the neutron mixed beam (not irradiated with BPA) was observed in xrs5 cells. On the other hand, there was the dose-rate effect in CHO-K1 cells. This means that dose-rate effect is not observed in DNA damage repair deficient cells, such as xrs5 cells. DNA-DSBs were repaired, when T98G cells were irradiated with the neutron beams or X-ray (Fig. 6) (Short et al., 2007).
Therefore for T98G cells, the dose-rate effect would be observed.

We treated CHO-K1 and xrs5 cells derived from normal tissues, and T98G cells that are tumor cells to investigate the dose-rate effect of BNCT. In BNCT, highly $^{10}$B distributed tumor cells are exposed to thermal neutrons. The alpha particles and $^7$Li nuclei produced by $^{10}$B (n,α)$^7$Li reaction have short paths (~10μm), same as a cell size (Liu et al., 2009). Therefore, it is thought that only tumor cells are affected by BNCT and normal cells are irradiated with the neutron mixed beam. These present results may suggest that dose-rate effect of the neutron mixed beam for BNCT would contribute to both higher cytotoxicity reaction for tumor cells through BNCT and relative decline of biological effect to normal cells.

The kinetics of the number of 53BP1 foci for DNA-DSBs detection was also further evidence to the dose-rate effect of the neutron mixed beam and BNCT. High-LET radiation induces DNA-DSBs that are not repaired or more difficult to be repaired compared with low-LET radiation (Okayasu et al., 2006, Hill et al., 1999, Terato et al., 2008). Although there did not seem to be the significant difference about induction of DNA-DSBs, we showed that the DNA-DSBs induced by the neutron mixed beam alone was fast or easy to be repaired compared to the mixed beam plus BPA. Moreover, we thought that the significant difference between 4cGy/min and 20cGy/min about the number of 53BP1 foci 24 hours after BNCT irradiation might reflect the difference of the survival rate produced by dose-rate effect in the case of...
including BPA (Fig. 3B and Fig. 6). We demonstrated the relationship between DNA-DSBs and the lethal potential, provided that kinetics of 53BP1 foci correlated with DNA-DSBs repair.

There is the hypothesis that residual foci are unable to pass mitosis (Marková et al., 2007). Although this means that few foci are observed in the cell exceeding the duration of the cell cycle after irradiation, recent studies show that the residual 53BP1/gamma-H2AX foci may be available for biological dosimetry. Primary induced foci and residual foci may be different types of foci (Marková et al., 2011). It is reported that the doubling time of T98G cells are 22-26 hours (Choi et al., 1997, Nakada et al., 2005). We also calculated the doubling time to be approximately 24 hours from their growth curve analysis. So, we concluded that the difference of 53BP1 foci between 4cGy/min and 20cGy/min at 24 hours after BNCT irradiation was the variance of the degree of DNA-DSBs repair.

In this study, using cell survival assay, we found that greater dose-rate effect were observed in T98G cells underwent BNCT than gamma-ray, and that the effect were less in CHO-K1 cells irradiated with the neutron beam alone, compared with gamma-ray. Time dependence of 53BP1 foci was not direct evidence of dose-rate effect of BNCT because the converted absorbed dose was not same between 4cGy/min and 20c/min irradiation. However, the difference of 53BP1 foci reflected the difference of the survival rate results. Although we have thought that dose-rate effect of BNCT was not that of alpha particles or 7Li nuclei
produced by $^{10}\text{B} \,(n,\alpha)\,^7\text{Li}$ reaction, we are interested in this result.
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FIGURE LEGENDS

Fig. 1. The physical doses percentage of thermal, epithermal, fast neutrons, and gamma-ray in the neutron mixed beams, when samples were irradiated at KUR operated at 4cGy/min or 20cGy/min.

Fig. 2. Dose-rate effect on survival fraction for CHO-K1 and xrs5 cells irradiated with neutron mixed beam (n) (A) or gamma-ray (γ) (B).

Fig. 3 Dose-rate effect at survival fraction for T98G cells irradiated with neutron mixed beam in case of not including BPA (A) or including BPA (B). In Fig. 3B, the horizontal axis is the absorbed dose converted assuming that the concentration of BPA in T98G cells is 20ppm.

Fig. 4 Dose-rate dependence of D₀ for T98G cells. The dose rate of plus BPA + neutron irradiation group is converted assuming that the concentration of BPA in T98G cells is 20ppm.

Fig. 5. The number of 53BP1 foci induced in T98G cells at 30 min after the neutron mixed beam irradiation in case of not including BPA (A) or including BPA (B). The data are compiled from two independent experiments. The error bars indicate the standard errors of the mean.
Fig. 6. Time dependent loss of 53BP1 foci in T98G cells following approximately 50 min neutron mixed beam irradiation at 4cGy/min and 10 min at 20cGy/min. Note that the absorbed dose converted assuming that the concentration of BPA in T98G cells is 20 ppm is different between 4cGy/min and 20cGy/min irradiation. The converted dose was approximately 10 Gy in 4cGy/min neutron irradiation plus BPA, and 8 Gy in 20cGy/min plus BPA. The data are compiled from two independent experiments at each point. The error bars indicate the standard errors of the mean.
Table 1. $D_0$ for CHO-K1 and xrs5 cells irradiated with neutron mixed beam or gamma-ray

<table>
<thead>
<tr>
<th></th>
<th>CHO-K1</th>
<th>xrs5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutron mixed beam 4cGy/min</td>
<td>2.2</td>
<td>0.40</td>
</tr>
<tr>
<td>Neutron mixed beam 20cGy/min</td>
<td>1.7</td>
<td>0.35</td>
</tr>
<tr>
<td>Gamma-ray 4cGy/min</td>
<td>7.4</td>
<td>0.53</td>
</tr>
<tr>
<td>Gamma-ray 20cGy/min</td>
<td>1.5</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Table 2. $D_0$ for T98G cells irradiated with neutron mixed beam in case of including BPA or not.

<table>
<thead>
<tr>
<th></th>
<th>no BPA</th>
<th>plus BPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutron mixed beam 4cGy/min</td>
<td>1.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Neutron mixed beam 20cGy/min</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Figure(s)

Fig. 1

- Thermal neutrons (-0.5eV)
- Epithermal neutrons (0.5eV–10keV)
- Fast neutrons (10keV–)
- Gamma-ray

Percentage (%)

4 cGy/min

20 cGy/min
Figure(s)

Fig. 2
Fig. 3

[A] Figure(s)

- Survival Fraction vs. Dose (Gy)
  - Data points for two conditions:
    - no BPA + 4cGy/min
    - no BPA + 20cGy/min
  - Linear regression lines:
    - R² = 0.9399
    - R² = 0.8774

[B] Survival Fraction vs. Conversion Dose (Gy)

- Data points for two conditions:
  - plus BPA + 4cGy/min (conversion=20cGy/min)
  - plus BPA + 20cGy/min (conversion=80cGy/min)
- Linear regression line:
  - R² = 0.9738
  - R² = 0.9518
Fig. 4

The figure shows a bar graph representing different D0 (Gy) values under various dose rates. The dose rates are 4 Gy/min, 20 Gy/min, 80 Gy/min, and 2 Gy/min. The graph compares the effects of gamma-rays and the combination of BPA and neutrons (converted dose) on D0 values. The graph indicates higher D0 values for the gamma-ray exposure compared to the combined BPA and neutron exposure.
Fig. 5

(A) Graph showing the number of 53BP1 foci per cell against dose (Gy) for different conditions: no BPA + 4cGy/min, no BPA + 20cGy/min.

(B) Graph showing the number of 53BP1 foci per cell against conversion dose (Gy) for different conditions: plus BPA + 4cGy/min (conversion=20cGy/min), plus BPA + 20cGy/min (conversion=80cGy/min).
Fig. 6

- **4 cGy/min 0 ppm**
- **plus BPA + 4 cGy/min (conversion=20 cGy/min)**
- **20 cGy/min 0 ppm**
- **plus BPA + 20 cGy/min (conversion=80 cGy/min)**

Number of 53BP1 foci (cell⁻¹)

Time after irradiation (hour)

Fig. 6