Abstract. Background/Aim: The tumor suppressor gene p53 is mutated in glioblastoma. We studied the relationship between the p53 gene and the biological effects of boron neutron capture therapy (BNCT). Materials and Methods: The human glioblastoma cells; A172, expressing wild-type p53, and T98G, with mutant p53, were irradiated by the Kyoto University Research Reactor (KUR). The biological effects after neutron irradiation were evaluated by the cell killing effect, 53BP1 foci assay and apoptosis induction. Results: The survival-fraction data revealed that A172 was more radiosensitive than T98G, but the difference was reduced when boronophenylalanine (BPA) was present. Both cell lines exhibited similar numbers of foci, suggesting that the initial levels of DNA damage did not depend on p53 function. Detection of apoptosis revealed a lower rate of apoptosis in the T98G. Conclusion: BNCT causes cell death in glioblastoma cells, regardless of p53 mutation status. In T98G cells, cell killing and apoptosis occurred effectively following BNCT.

At the Kyoto University Research Reactor Institute, more than 450 cancer patients have been treated with boron neutron capture therapy (BNCT). BNCT takes advantage of the T(n,α)7Li reaction resulting from low-energy thermal neutron irradiation, and can selectively cause cancer cell death due to the alpha particles and 7Li recoil nuclei produced by the reaction. Because both types of particles have high biological effects and projected ranges of less than 10 μm, BNCT destroys tumor tissues without significant damage to normal tissues.

Research on cell death is important from the standpoint of the biological effects of radiotherapy, and the p53 tumor suppressor gene plays an important role in the cell death program. The most commonly mutated tumor suppressor gene, p53 regulates genes that control both cell-cycle checkpoints and programmed cell death via apoptosis. Activation of p53 after irradiation can lead either to inhibition of proliferation or cell death (1-3). The efficacy of many anticancer drugs is reduced in p53-mutant cells (4), although one previous study reported that cells expressing mutant p53 were more likely to induce apoptosis than wild-type cells (5).

Few groups have studied the role of p53 in BNCT in oral squamous carcinoma cells (6, 7). They reported that cancer cells with mutant p53 were more resistant than cells with wild-type p53. Since the 1980s, treatment of brain tumors has been the primary application of BNCT in Japan (8, 9). More than 50% of patients with malignant brain cancer have tumors with mutant p53 (10, 11). The purpose of this study was to study the relationship between the p53 tumor-suppressor gene and the biological effects of BNCT in two types of human glioblastoma cells: A172, expressing the wild-type (wt) p53 gene, and T98G, expressing a mutant (mu) p53 gene.

Materials and Methods

Cell culture. Two types of human glioblastoma cell lines, A172 and T98G, were purchased from RIKEN BRC Cell Bank (Tsukuba, Japan). A172 are wild-type for p53, whereas T98G cells are p53-mutant (12). Cells were cultured in MEM-α medium (Invitrogen, Carlsbad, California, USA) supplemented with 10% heat-inactivated FBS (Bio-west, Nuaillé, France) and maintained at 37˚C in a humidified atmosphere with 5% CO₂.

Neutron Irradiation. After trypsinization, cells were suspended in the medium described above and aliquoted into Teflon tubes for irradiation. To investigate the biological effects of BNCT, some samples were incubated in medium containing 20 μl/ml of BPA (boron phenylalanine) for 1 h before irradiation. Cells were irradiated using the neutron beam at the Heavy Water Facility of the Kyoto University Research Reactor (KUR) operated at 1 MW power output. The thermal neutron fluences were measured by gold-foil activation analysis. The gamma-ray dose, including secondary gamma rays, was measured using a thermoluminescence dosimeter. Cell survival assay. The survival rates of the irradiated cells were determined using conventional colony assays. After irradiation, the cell suspensions were diluted, and known quantities of cells were seeded in cell-culture dishes. Cells were then incubated for 15-20 days. In order to visualize and count colonies.

Correspondence to: Yuko Kinashi, MD, Ph.D., Research Reactor Institute, Kyoto University, Kumatori-cho, Sennann-gun, Osaka, Japan. Tel: +81 724512347, Fax: +81 724512623, e-mail: kinashi@rri.kyoto-u.ac.jp

Key Words: Boron neutron capture therapy, neutron, p53, 53BP1, apoptosis.
Immunofluorescence staining. DNA double-strand breaks (DSBs) in glioblastoma cells following neutron irradiation were detected using the 53BP1 (TP53: tumor suppressor gene 53-binding protein) foci assay. Immunofluorescence staining was performed as described previously (13). Briefly, irradiated cells were incubated 1 or 3 h, and washed with cold PBS (Invitrogen). After fixation with 3.6% formalin (Wako Pure Chemical Industries Ltd, Osaka, Japan) cells were washed with PBS and permeabilized with 0.5% Triton X-100 (Calbiochem (Merk Millipore), Tokyo, Japan) in PBS on ice for 5 min. After these steps, cells were washed with PBS and incubated for 2 h at 37˚C with 1 μl/ml anti-53BP1 primary antibody (Bethyl Laboratories Inc, Montgomery, Texas, USA) in 5% skim milk in 1× TBS buffer, pH 7.4 (Nippon Gene). After three washes with PBS, the cells were incubated for 1 hour at 37˚C with 1 μl/ml Alexa Fluor 594–conjugated goat anti–rabbit IgG secondary antibody (Invitrogen) in the aforementioned TBS-DT solution. The cells were fixed and stained by 10% glycerol (Wako) with 1% DAPI (4’6-diamidino-2-phenylindole, Chemical-Dojin CO., Ltd, Kumamoto, Japan). Immunofluorescence images were acquired using a fluorescence microscope (KEYENCE, BZ-9000), and then analyzed using the BZ-9000 optional software and Image-J (National Institutes of Health).

Apoptosis detection. We applied the TUNEL method (14) to apoptosis detection. Irradiated cells were were detected using the IN Situ Cell Death Detection Kit, TMR red (Roche Diagnostics, Tokyo, Japan) 5 or 24 h after irradiation. Using this kit, DNA strand breaks in apoptotic cells were labeled by the TUNEL method. After labeling, the cells were mounted on glass slides in 10% glycerol (Wako) with 1% DAPI in PBS to stain nuclear DNA.

Cells were observed under a fluorescence microscope, and then analyzed using the BZ-9000 optional software and Photoshop Elements 11 (Adobe).

Results

Figure 1 shows survival fractions of A172 (wt p53) cells and T98G (mu p53) cells following neutron irradiation used for BNCT. In the absence of BPA, T98G cells were more resistant than A172. On the other hand, in the presence of BPA, the survival-fraction curves of both cell types were similar. Thus, the difference in radiation sensitivity observed in the absence of BPA cells was reduced in the presence of this compound. These results can be confirmed by comparison of D10, calculated from the Figure 1 survival curves (Table I). In absence of BPA, A172 cells required 1.7 Gy radiation dose for 90% cell death, but T98G required 5.3 Gy, a nearly 3-fold increase. On the other hand, in the presence of BPA, the D10 of A172 was 0.8 Gy. Table I shows RBE (Relative Biological Effectiveness) values calculated from the D10 dose of A172 and T98G cells following mixed-neutron beam irradiation. The RBE of A172 was about 2-fold larger than that of T98G in the absence of BPA, but slightly smaller than that of T98G in the presence of BPA.

Figure 2 shows that the number of 53BP1 foci increased as a function of the neutron-irradiation dose. There was little difference in the number of foci in A172 and T98G after
exposure to 1.0 Gy. Although a previous study reported that the number of foci after irradiation changed in a time-dependent manner (15), we observed little difference in the number of foci between 1 and 3 h (Figure 2). This result indicates that foci formation was nearly completed 1 h after neutron irradiation, and that DNA damage was not repaired within 3 hours. Foci number did not differ significantly in the presence or absence of BPA. Furthermore, there was little difference in the number of foci between the two types of glioma cells with different p53 status.

Figure 3 shows images that were acquired and analyzed by fluorescence microscopy and Photoshop Elements 11 following TUNEL detection. The images in Figure 3 depict cells 5 h after 1.015 Gy irradiation with the mixed-neutron beam. T98G induced apoptosis only in the presence of BPA, whereas A172 cells induced apoptosis whether or not BPA was present. The rate of induction of apoptosis following neutron irradiation, determined by counting apoptotic cells (Figure 4), differed significantly between A172 cells and T98G cells. Apoptosis was less frequent in T98G than in A172 at each dose point, but the frequency rose in both cell types following addition of BPA.

**Discussion**

In the present study, we investigated the p53-related biological effects of BNCT in two types of human glioblastoma cells, A172 (wt p53) and T98G (mu p53) status. The biological effects of A172 and T98G cells were compared in three experiments: colony formations to determine survival fractions, 53BP1 foci assay to detect DNA damage, and TUNEL staining to detect apoptosis.

![Figure 2. Number of 53BP1 foci 1 and 3 hours after the irradiation. The right graph shows the number of foci 1 hour after irradiation, and the left graph shows the number of foci 3 hours after irradiation. Each data point represents the average number of foci in more than 50 cells.](image)

Table I. Relative biological effectiveness calculated by D10 dose* of the irradiated A172 and T98G cells by neutrons for BNCT.

<table>
<thead>
<tr>
<th></th>
<th>A172</th>
<th>A172+BPA</th>
<th>T98G</th>
<th>T98G+BPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{10}$(Gy) of BNCT</td>
<td>1.7</td>
<td>0.82</td>
<td>5.2</td>
<td>1.1</td>
</tr>
<tr>
<td>$D_{10}$(Gy) of gamma-ray**</td>
<td>4.8</td>
<td>7.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBE</td>
<td>2.8</td>
<td>5.9</td>
<td>1.3</td>
<td>6.4</td>
</tr>
</tbody>
</table>

*Each $D_{10}$ was calculated from survival curve which shows on Figure 1. **Co60 gamma-ray system.

The survival rates revealed that neutron irradiation killed cells more effectively than gamma rays. The RBE values were 3.0 for A172 and 1.3 for T98G in the absence of BPA, and 5.9 for A172 and 6.4 for T98G in the presence of 20 ppm BPA. Another group reported that the RBE of carbon ions ranges between 3.3 and 3.9 in glioblastoma cells (16). Relative to the RBEs of carbon radiotherapy, the most popular form of particle radiotherapy for glioblastoma, the cell-killing effects of thermal neutrons were equal or lower, but the effects were much higher when BPA was present. In other words, BPA is an essential component of BNCT.

T98G (mu p53) was more radioresistant than A172 (wt p53), but the difference in radiosensitivity decreased when BPA was present. In the absence of BPA, the RBE of A172 was 2.3-fold larger than that of T98G, whereas in the presence of 20 ppm BPA, the RBE of A172 was slightly

171
smaller than that of T98G. An earlier study revealed some difference in sensitivity to carbon ion or alpha particles between A172 and T98G (17). Our results suggest that BNCT is an effective treatment for glioblastoma that can reduce differences in radiosensitivity due to the functional status of p53.

Observation of DNA double-strand breaks (DSBs), as determined by 53BP1 foci, revealed no significant change between 1 and 3 h after irradiation. Because another group reported that the number of 53BP1 foci per cell decreased with time after a peak at 30 min after X-ray irradiation (18), our result suggests that repair of DNA damage after BNCT is slower than that after X-ray irradiation. Consistent with this, another study showed that DNA DSBs induced by high LET (linear energy transfer) radiotherapy such as BNCT was not repaired for a long time in comparison to damage induced by carbon-ion beams and X-rays (19). The persistence of DNA damage may be one factor contributing to the high cell-killing effect of BNCT.

Apoptosis induction clearly differed between T98G and A172. It is generally accepted that p53 mutations increase resistance to programmed cell death in response to ionizing irradiation (20, 21). Our results showed that T98G (mu p53) was more resistant than A172 (wt p53) to apoptosis induced by neutron irradiation. When neutron irradiation was combined with BPA (i.e. BNCT), apoptosis was also induced in T98G (mu p53). High-dose irradiation with LET heavy ions induces p53-independent apoptosis (22). The energy of $^4$He and $^7$Li ions produced by the $^{10}$B(n,α)$^7$Li reaction is increased to 1.47 MeV (alpha particle) and 0.84 MeV ($^7$Li particle) in the presence of boron; the ranges of LET for alpha particles and $^7$Li particles are 50-231 keV/μm and 65-266 keV/μm, respectively. Mutations in the p53 tumor-suppressor gene are common genetic alterations in primary
malignant human brain tumors (23, 24). In the T98G cells (mu p53), cell killing and apoptosis occurred effectively following BNCT, possibly due to p53-independent apoptosis or another mechanism. The results of this study demonstrate that BNCT caused cell death in glioblastoma cells, regardless of mutant p53 status.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan No. 26461884; and by a research grant from Kansai Genshi-ryoku Kondankai, Osaka Japan and KUR research program for scientific basis of nuclear safety.