Radiobiological Characterization of Proton Beam at the National Cancer Center in Korea

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Estimation of the relative biological effectiveness (RBE) of the proton beam at the National Cancer Center Proton Therapy Center in Korea (NCCPTC) is required clinically for the treatment of cancer. The proton beam was fixed at 190 MeV with 6 cm a spread out Bragg peaks (SOBP) for which corresponds to most frequent clinical condition. The RBE was estimated from the survival of human salivary gland (HSG) cells using the traditional colonogenic and MTT assays. The HSG cells were also irradiated in a cell-stack chamber and monitored for survival to identify whether the characteristic depth-dependent survival pattern was observed. The RBE of the NCCPTC was estimated to be 1.024 ± 0.007 and 1.049 ± 0.028 at the middle of SOBP using colonogenic and MTT assays, respectively. Further analysis of the biological response of proton exposure revealed no difference compared to conventional X-ray treatment in western blot, and FACS analysis. The proton beam of the NCCPTC also exhibited the characteristic depth-dependent survival pattern. The estimated RBE value of NCCPTC was slightly smaller than generic RBE value of 1.1 for protons of the majority of centers. Due to the recommendation of a generic RBE of 1.1 for protons, a representative RBE value of 1.1 was assigned for clinical application for proton beams at the NCCPTC.

INTRODUCTION

The National Cancer Center Proton Therapy Center (NCCPTC) is the first proton therapy facility in Korea. Proton beams have an important feature arising from the physical aspects of their dose distribution which enables proton beams can provide highly localized and uniform doses of radiation to tumors while sparing the surrounding normal tissues compared to conventional modalities using photons and electrons. Probably the most important of these biological phenomena is a markedly increased efficiency of cell killing. This is why proton beam therapy is considered as a promising new treatment for malignant tumors and the number of proton beam therapy facilities in the world has increased over the last ten years.

However, when using different types of radiation, equal physical doses of radiation do not produce equal biological effects because of the differences in their energy-deposition patterns. The concept of relative biological effectiveness (RBE) has been introduced to account for this feature. Conventionally, the RBE is the ratio of the dose of high-energy photons such as 60Co γ-rays or linear accelerator X-rays relative to any other particle to produce the same biological response. RBE is a simple concept but its clinical application is complex because value of RBE is varied depending on the several factors including particle type, energy, dose, dose per fraction, number of fractions, cell or tissue type, and varies between early and late reactions following irradiation. Thus, RBE must be defined before the proton beam at the facility can be used clinically.1-3) After applying the RBE value, it is to ensure that radiation oncologists can take benefits from the large pool of clinical results obtained with photon beams. Proton therapy is based on the use of a single RBE value (equals 1.1 at almost all institutions), which is applied to all proton beam treatments independent of dose/fraction, position in the SOBP, initial beam energy or the particular tissue.

To define the RBE of the NCCPTC, we have investigated radiation dose-survival curves of the cultured human salivary gland (HSG) cells using 190 MeV protons produced by...
the NCCPTC’s cyclotron particle accelerator and X-rays from a 6 MV linear accelerator. The HSG cells were derived from the salivary gland tumors and previously used for an in vitro RBE study in Japan. Application of this cell line enabled direct comparison between the NCCPTC and other proton therapy centers that had also used HSG cells. In addition, we evaluated the characteristic depth-dose profile of the proton beam and also compared several biological characteristics induced by radiation from proton beams and X-rays.

MATERIALS AND METHODS

Proton beams and X-rays

The fixed-energy cyclotron of the NCCPTC (Ion Beam Applications SA, Louvain-la-Neuve, Belgium) produces proton beams at 230 MeV. The beam energy is then varied by the energy selection system (ESS) in the range of 70–230 MeV to control the depth-dose. The energy variability is attained using a graphite degrader of variable thickness, which is followed by a collimator and slit system to define the emittance and energy of the beam. To define the RBE of the proton beams, the beam energy of proton was fixed at 190 MeV with 21 cm range (it results from the energy) and 10 cm diameter snout. The Bragg peak of the proton beams was spread out over 6 cm width; the dose rate in the middle of the spread out Bragg peak (SOBP) was approximately 2.2 Gy/min. Referential X-ray was obtained by a 6 MV conventional linear accelerator (Clinac 2100CD; Varian Medical Systems, Palo Alto, CA, USA). A radiation dose was delivered with a 100 cm source surface distance (SSD), a 10 × 10 cm collimated field size and a dose rate of 2.2 Gy/min. The photon and proton beam were calibrated using the International Atomic Energy Agency protocol TRS-398.

Cell culture and calculation of RBE

The HSG cells were obtained from the Human Science Research Resources Bank (Osaka, Japan), and maintained in DMEM with 10% FBS, 100 units/ml penicillin, 10 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were seeded to 106 cells/25 cm2 flask, and irradiated in a 10 cm diameter snout. The irradiated cells were immediately harvested in 0.2% Triton X-100 in PBS, and incubated overnight with an anti-γ-H2AX antibody (Jackson Immunoresearch, West Grove, PA, USA) diluted 1:500 in 3% goat serum and 3% bovine serum albumin in PBS. The antibody complexes were detected by fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) with 4’,6’ diamidino-2-phenylindole (DAPI) for nuclear staining. All images were obtained by Zeiss Axio Image M1 fluorescence microscopy with an AxioCam HRC CCS camera (Zeiss, Berlin, Germany). The extent of γ-H2AX foci formation was esti-
mated by the percentage of nuclei with $\gamma$-H2AX foci out of a total of 500 nuclei in ten randomly chosen microscopic fields. No fluorescence was detected if the primary antibody was omitted.

**Western blot cell cycle analysis**

Western blot analysis was performed as described in a previous report. The following primary antibodies were used: pCHK2-T68, pRb-S807/811 (Cell Signaling Technologies, Danvers, MA, USA), Rb, and $\alpha$-Tubulin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

Flow cytometry was performed as previously described. Trypsin-harvested HSG cells were resuspended, and fixed in 70% ethanol. The resulting cells were washed with PBS, resuspended in a solution containing RNase A and propidium iodide, and the cellular fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The DNA content and the cell cycle data profiles were analyzed by CellQuest (BD Biosciences, San Jose, CA, USA).

**Statistical analyses**

The data points of each dose-effect relation have been fitted by polynomial fit of Origin (version 6.0; Microcal Software, Inc., Northampton, MA, USA). Statistical significant differences and 95% confidence intervals were calculated by T test (http://www.physics.csbsju.edu/stats/t-test.html). $P \leq 0.01$ was considered statistically significant.

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**Fig. 1.** Relative biological response of HSG cells at the plateau, and mid-SOBP positions of the proton beam, compared to X-rays. (A) Cell survival curves after irradiation with 190 MeV proton beams with a 6 cm SOBP, and 6 MV X-rays. Each curve represents the average of three independent triplicate experiments. Every point represents the mean ± SD. (B) Western blot and (C) flow cytometric analysis of HSG cells after 7 Gy irradiation and 24 hr incubation. X and Y axes represent the contents of DNA and numbers of distributed cells, respectively. CTL: control (non-irradiated) cells.
RESULTS

The HSG cells were irradiated at two different locations, one was in the middle of the SOBP (mid-SOBP) (18 cm depth from the surface), and the other was at the plateau region (3 cm depth from the surface). Along the beam line, the physical dose in the plateau was 75.1% of that in the mid-SOBP. When the HSG cells were irradiated, the duration of irradiation was adjusted to produce the same dose in both positions. The cell survival results from three independent experiments are summarized in Fig. 1A and Table 1. The mean D10 (10% survival dose) for the proton beam was 4.70 ± 0.21 Gy at the mid-SOBP location, and 4.73 ± 0.26 Gy at the plateau location, while the mean D10 for the X-ray was 4.81 ± 0.23 Gy. The RBEs calculated from the above results were 1.024 ± 0.007 at the mid-SOBP location and 1.021 ± 0.064 at the plateau.

Next, we tested whether biological influence and response mechanism were altered by the type of radiation or by the depth in the proton beam. We first analyzed the pattern of proteins responsible for DNA damage repair and cell cycle regulation from the cells exposed to X-ray and the proton beam, both at the plateau and mid-SOBP locations. As shown in Fig. 1B, the different modes of irradiation did not appear to induce different expression patterns of the 68th (threonine) residue phospho-CHK2, the 807/811th (serine) residues of phospho-Rb and Rb. We also checked the immediate response patterns of these proteins at 30 min after irradiation and found similar patterns in different types of irradiation (data not shown). The distributions of cell cycles in above conditions were analyzed by flow cytometry but no notable differences in cell cycle arrest were found between different types of irradiation (Fig. 1C). These results suggested that biological influence of proton beams shows similar pattern to conventional X-ray.

The most of RBE estimation of in vitro study is estimated from the colony formation assay which has many limitation for broad application and need a lot of efforts and time. Thus, we tested whether the MTT assay is suitable to measure biological responsiveness of proton beam. The estimated RBE from the MTT assay at the mid-SOBP was 1.049 ± 0.028, which was a 2.44% deviation from the RBE estimation by the colonogenic assay (Fig. 2). The rapid and convenient features of this colorimetric assay also lead us to consider as alternative way to check the biological effectiveness on proton assessment.

To see whether proton beam of NCCPTC possesses the characteristic depth-dependent survival pattern, we measured survival of the HSG cell along the beam tract using cell-stack chamber. As shown in Fig. 3A, the cell survival pattern of NCCPTC displays a similar shape as the physical dose pattern and a SOBP of the expected size (150–210 mm) with dramatic distal fall-off. This feature will be a reason of sparing property of the frontal normal tissues in proton treatment. Then, we estimate the direct DNA damage in the distal fall-off region by measuring the foci formation of γ-H2AX which is a very sensitive protein sensor of DNA damage.9,10) As expected, most of the cells located in the SOBP displayed obvious γ-H2AX foci (Fig. 3B and 3C). The strongest signal appeared in mid-SOBP (86.8%) followed by the plateau region (80.8%). Importantly, the number of damaged cells in

| Table 1. Summary of RBE experiments |
|------------------------------------|
| D10 of Survival (Gy) | X-ray | Mid-SOBP | Plateau | RBE |
|----------------------|-------|---------|---------|-----|
| 1                    | 5.094 | 4.951   | 4.677   | 1.029 | 1.089 |
| 2                    | 5.008 | 4.871   | 5.203   | 1.028 | 0.962 |
| 3                    | 4.354 | 4.283   | 4.299   | 1.016 | 1.013 |
| MEAN                 | 4.81 ± 0.23 | 4.70 ± 0.21 | 4.73 ± 0.26 | 1.024 ± 0.007 | 1.021 ± 0.064 |

Fig. 2. The relative effectiveness of irradiation measured by the MTT assay in the mid-SOBP location of the proton beam compared to X-ray. Every point represents the mean ± SD.
the distal location (2 cm away from the end of the SOBP) was similar to those of unirradiated control (8.1% versus 6.4%). This finding suggests that the absence of a cytotoxic effect of the proton beam at the distal region is due to no DNA damage occurring in this region.

**DISCUSSION AND CONCLUSION**

Currently, the NCCPTC is the only radiotherapy center to treat patients using proton beams in Korea. In order to optimize the proton treatment, we estimated the RBE for clinical application. In measuring the RBE using cultured cells, we employed the HSG cell line for preclinical radiobiological assessment. The HSG cells were also used for RBE estimation in Japan, which enabled direct comparison with the other proton therapy centers using HSG cells in their RBE assessment. The Hyogo Ion Beam Medical Center (HIBMC) is one of the facilities that used HSG cells in RBE assessment, revealing the RBE at D10 from 1.01 to 1.05 depending on the depth of the proton beam. When we compared the results at the same conditions including depth and size of the SOBP, RBEs in the mid-SOBP appeared from the HIBMC and the NCCPTC, were similar (1.02 and 1.024, respectively). Moreover, the overall RBE of all five proton therapy facilities currently working in Japan were estimated 1.034, which is also comparable to the RBE of the NCCPTC (personal communication).

Currently, the colony formation assay, which measures the ability to form a colony from a single cell, is frequently used for in vitro RBE estimation. However, many cell lines are not adequate for this type of study due to aggregation during plating leading to colony formation from more than one cell and failure of colony formation resulting in a low proliferation rate. Therefore, the colony formation assay prevents the application of various cell lines from several tumors due to these limitations. Moreover, extremely low cell density and isolated culture conditions are far from the real environment of tumors. The MTT assay is a widely accepted standard assay for measuring cellular proliferation and was used to determine cytotoxicity of potential medicinal agents and other toxic materials. The mitochondrial reductase enzyme turns yellow MTT to purple formazan by reduction which happens only in active enzymes of living cells. Therefore conversion is directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death to cells can be deduced, through the production of a dose-response curve. Therefore, the RBE based on the MTT assay can be achieved in typical culture conditions with the presence of communication between the neighboring cells while the RBE from the colony formation assay was measured based on the survival of a single cell in isolated conditions. The resulting RBE deduced from the MTT assay at the same location was found to be 1.049 ± 0.028, which shows only 2.44% deviation from the RBE derived from the colonogenic assay. Since proliferating cells are metabolically more active than non-proliferating resting cells, it is suitable not only for the determination of viability and proliferation of cells but also for the determination of cellular activity. The rapid and convenient features of this colorimetric assay make it an

Fig. 3. Cell survivals and DNA damages after cell-stack irradiations of proton beam at NCCPTC. (A) Cell survivals after cell-stack irradiation by 190 MeV proton beams, with 6 cm SOBP. The physical dose of 3.5 Gy at the center of SOBP were administered in two independent triplicate experiments. Each experiment was marked by open and closed circles. The points represent actual measurement of cell survival by the colonogenic assay. (B) Stainings of γ-H2AX foci (red) for the measurement of DNA damage in several locations along the tract of the proton beam. The stainings of nuclei with DAPI (blue) are also shown in the lower right boxes. SOBP + 2 cm represents the location of 2 cm distal after the SOBP end (23 cm). (C) Foci formations were counted from in the indicated locations. Every value represents the mean ± SD.
attractive alternative method for assessing biological effectiveness, especially when using a cell line not suitable for the colonic assay. The most important feature of the proton beam is its characteristic depth-dose profile, which shows that the dose-deposition increases slowly with depth up to a maximum reached in the target volume, and then disappears suddenly down-stream the target volume. This characteristic of the proton beam provides highly localized doses to tumors while sparing the surrounding normal tissues. To determine whether the proton beam of the NCCPTC possesses these properties, we estimated the extent of survival depending on the depth of the proton beam using a cell-stack chamber. Our results showed the presence of the SOBP with the expected position and size. The lethality of these areas were 41.0 ± 2.9% in plateau area (10–107 mm, N = 14, 95% confidence interval: 37.7–44.3) and 63.3 ± 7.1% in the SOBP (152–208 mm, N = 30, 95% confidence interval: 61.0–65.5), respectively. When we compared the biological lethality of these areas, the difference was statistically significant (P ≤ 0.001). And it is interesting that no survival determination in the distal fall-off comes from whether no biological damage or DNA damage but recovered by repair process. To examine this question, we measured the extents of γ-H2AX foci at similar depths even though difference in administrated physical doses. This is because the γ-H2AX foci are too sensitive to even 0.1 Gy of X-ray irradiation. However, the populations damaged in the distal region (2 cm away from the end of SOBP) (8.1%) showed similar results to those of unirradiated control cells (6.4%) with such moderate sensitivity. This finding suggested the absence of toxicity at the distal region was due to no DNA damage occurring in this region.

Proton therapy is granted as rapidly developing cancer treatment but required extensive assessment for optimal adjustment of clinical therapeutic comparing conventional radiation therapy. The RBE of the proton facility should be estimated for generating prescriptions and the measured RBE of the NCCPTC was found to be 1.024 (95% confidence interval: 0.951–1.097). It is true that RBE values for protons are varied depending on the depth, dose level, and dose rate, but individual RBE settings by depth are not common. Instead, a representative clinical RBE value of 1.1 was assigned for all proton beams at the NCCPTC after the thorough discussion by radiation oncologists and researchers. And further studies will be performed for estimation of the biological effectiveness in a SOBP of NCCPTC proton beams from regenerating jejunal crypts of mice.

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