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ORIGINAL ARTICLES

Radiosensitivity estimation of human brain tumors using primary culture technique and the cytokinesis-block micronucleus assay

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Abstract: Clinical relevance of *ex vivo* radiosensitivity estimation, using primary culture technique and cytokinesis-block micronucleus (CBMN) assay, was investigated in 7 patients with primary brain tumors. The tumors consisted of 4 astrocytomas, 1 oligodendroglioma, 1 glioblastoma multiforme and 1 teratoma. They underwent partial resection of the tumor and 6 of the 7 patients received postoperative radiotherapy. CBMN assay was performed using primary cultured tumor cells that originated from the resected tumor specimens, and the parameters, the average of number of micromuclei per binucleated cells (MN/BNC) induced by 2 Gy irradiation after subtraction of that for control (MN2) and the MN/BNC increasing rate induced by irradiation (MNIR) were obtained. In the 6 patients who received postoperative radiotherapy, tumor response was evaluated by serial MRI or CT scan. Although MN2 and MNIR showed some differences among various histological types, those for oligodendroglioma and glioblastoma multiforme were suggestive of being radiosensitive and radioresistant, respectively, when considering those for fibroblast from human normal tissues. Furthermore, it seemed that the values of MN2 and MNIR of the tumors were related to the tumor responses by subsequent postoperative radiotherapy. These results suggest that our method could be useful for predicting clinical outcome of treatment to human brain tumors.

Key words: human brain tumor, radiosensitivity, cytokinesis-block micronucleus assay, primary culture, postoperative radiotherapy, tumor response

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1. Introduction

For personalizing and optimizing radiotherapy, as well as technical issues like improving dose distribution recently advanced, it is important to estimate various radiobiological properties of tumor such as the intrinsic radiosensitivity, proliferative activity, oxygenation status, and omics-based molecular characteristics if possible[1-7]. For this purpose, we introduced the cytokinesis-block micronucleus (CBMN) assay[8], known as one of the available tools to assess an individual radiosensitivity of human tumors[4,5,9,10]. However, so as to obtain tumor cells from fresh surgical specimen, rapid and overwhelming growth of contaminated normal tissue cells as well as bacterial contamination are unavoidable technical problems when using tumor materials accompanied with necrosis, inflammation, infection, etc. Furthermore, regarding postoperatively irradiated human tumors, it is often difficult to directly confirm the estimated radiosensitivity *in vivo* or tumor response on images by computed tomography (CT), magnetic resonance imaging (MRI), and/or radionuclide imaging. From the viewpoints, we considered primary brain tumors to be among the most appropriate materials. It is because they develop intracranially and without bacterial infection and the total removal is usually difficult, meaning that the radioresponse of the residual tumor is observable. In the present study, we examined the radiosensitivity of human brain tumors using the primary culture technique and the CBMN assay, compared it with tumor response by postoperative radiotherapy, and investigated the clinical relevance in the management of the tumors.

2. Materials and Methods

Patients and Treatment

Seven patients presenting with primary brain tumor underwent partial resection of tumor, and 6 of the 7 subsequently received postoperative radiotherapy between May 1997 and May 1998 at Hirosaki University Hospital. They consisted of 5 males and 2 females. The age ranged from 6 to 61 years with a median of 39. No previous chemotherapy was performed before surgery. Histologically, the tumors consisted of 4 astrocytomas, 1 oligodendroglioma, 1 glioblastoma multiforme and 1 teratoma. Postoperative radiotherapy was carried out by a linear accelerator (NEC, Tokyo) and 10 MV X-ray was employed. Radiotherapy was given using rotational irradiation, rotational conformation irradiation or three-dimensional conformal irradiation. Conventional dose fractionation with a fraction size of 1.8-2.0 Gy was employed and the total target dose to the planning target volume ranged from 50 to 60 Gy. Informed consent to treatment for entry into the research was obtained from each of the patients or from the parents for the one being a minor, according to Helsinki Declaration[11]. There was no ethical committee in Hirosaki University Hospital at that time.

Assay Procedure

All of the specimens were obtained at partial resection of tumor. Fresh tumor specimens were minced with scissors on dish and treated at 37°C for 2-3 h with a cocktail of 1 mg/ml collagenase/dispase (Boehringer, Germany) dissolved in phosphate-buffered saline (PBS). Then the tumor cell suspension was gently treated by pipetting and filtered through a fine wire mesh. After removal of the collagenase/dispase solution by centrifugation and rinsing with 0.9% NaCl, the tumor cells were plated into a chamber slide of which the bottom was slide glass (Iwaki). The number of chamber slides ranged from 10 to 15, depending on the tumor tissue volume obtained. The culture medium was Eagle's minimal essential medium (Gibco), supplemented with 20% fetal calf serum and 0.2 mg/ml gentamicin sulfate. The incubation was performed at 37°C in a humidified atmosphere of 5% CO_2 in air, and the culture medium was exchanged when considered necessary. The incubation was continued until sufficient growth of tumor cells, which were morphologically discriminated from

contaminated normal tissue cells, by an inverted phase-contrast microscope[12]. When insufficient growth of tumor cells and/or overwhelming growth of contaminated normal tissue cells such as fibroblast were shown, the culture was considered unsuccessful and discontinued.

Then irradiations were carried out by the linear accelerator (10 MV X-ray) with the assistance of water phantoms located around and above the chamber slides. The doses of 1, 2, and 4 Gy were given at room temperature. Control cells received sham irradiation. Immediately after irradiation, Cytochalasin B (CB, Sigma, Germany) was added. CB was dissolved (1 mg/ml) in dimethyl sulfoxide and the stock solution of CB was diluted with culture medium to a final concentration of 1.5 μg/ml. The details of cytokinesis-block were described elsewhere.[9] Newly-induced micronuclei by cytotoxic agents can be detected in a dividing fraction of cells when adding CB. Cultures were terminated depending on the number of binucleated tumor cells. The appropriate period for fixing the irradiated cells was around 6-7 days and the culture medium with the added CB was exchanged when necessary.

After the cells were rinsed with 0.9% NaCl, they were fixed for 20 min with methanol at room temperature. Then the media chamber was removed. The cells on the glass slide were air-dried overnight and stained with 4, 6-diamidino-2-phenylindole (DAPI; 100μg/ml in Tris buffer, pH 7.0; Serva, Germany). The MN in the binucleated tumor cells were scored at a magnification of 400× using a microscope equipped with fluorescence and phase contrast (Olympus). At least 100 binucleated tumor cells, more than 200 whenever possible, were assessed to count MN.

The average number of MN per single binucleated cell (MN/BNC) was calculated mostly from duplicated samples, but occasionally from only a single sample due to unsuccessful culture. From the dose response curve as to MN/BNC as a function of radiation dose, two parameters, MN/BNC induced by 2Gy irradiation after subtraction of that for control (MN2) and the MN/BNC increasing rate induced by irradiation (MNIR, Gy^{-1}), were obtained (Fig. 1). As a control for radiosensitivity, the same procedures were performed for the fibroblasts derived from the macroscopically normal tissues in the resected specimens of 3 patients.

Evaluation of Tumor Response

For the 6 patients who received postoperative radiotherapy after partial resection, evaluation of tumor response, according to the criteria in the guidelines to evaluate the response to treatment in solid tumors (RECIST)[13], was performed by serial MRI and/or CT scan.

3. Results

Assay Data

The assay data for the fibroblasts (*n=3*) and the tumors (*n=7*) are shown in Table 1. The dose response curve of MN/BNC as a function of the radiation dose is shown in Fig. 1. The average values of MN2 and MNIR for fibroblast were 0.20 and 0.09 Gy^{-1}, respectively. As to the tumors, the MN2 ranged from 0.03 to 0.53, and the MNIR from 0.02 to 0.31 Gy^{-1}. It appeared that some differences in the values existed among various histological types. For oligodendroglioma (*n=1*), the values of MN2 and MNIR were high, 0.53 and 0.31 Gy^{-1}, respectively, suggesting radiosensitive. On the other hand, for glioblastoma multiforme (*n=1*), those of MN2 and MNIR were very low, 0.06 and 0.02 Gy^{-1}, respectively, suggesting radioresistant. Astrocytoma (*n=4*) showed intermediate values between the two. The MN2 ranged from 0.08 to 0.17, and the MNIR from 0.02 to 0.19.

Table 1. Patients, radiosensitivity parameters and tumor responses

No.	Gender	Age	Histology	Tumor Location	MN2	MNIR (Gy^{-1})	Radiotherapy (TTD/fr)	Tumor Response[12]
			Fibroblast ($n=3$)		0.20	0.09		
1.	Male	6	Teratoma	Pineal region	0.34	0.13	50.4Gy/28fr	CR
2.	Male	61	Oligodendroglioma	Cerebrum	0.53	0.31	54Gy/30fr	PR
3.	Male	39	Astrocytoma	Cerebrum	0.16	0.09	60Gy/30fr	SD
4.	Male	16	Astrocytoma	Cerebellum	0.1	0.11	–	–
5.	Male	39	Astrocytoma	Cerebrum	0.08	0.05	60Gy/30fr	SD
6.	Female	19	Astrocytoma	Cerebellum	0.17	0.02	50Gy/25fr	SD
7.	Male	54	Glioblastoma	Cerebrum	0.06	0.02	60Gy/30fr	SD

MN2: The average number of micromucleus per binucleated cells (MN/BNC) induced by 2Gy irradiation after subtraction of that for control. MNIR: The MN/BNC increasing rate induced by irradiation (Gy^{-1}).
TTD/fr: Total target dose/ fraction number of radiotherapy. Glioblastoma: Glioblastoma multiforme. CR: Complete response. PR: Partial response. SD: Stable disease.

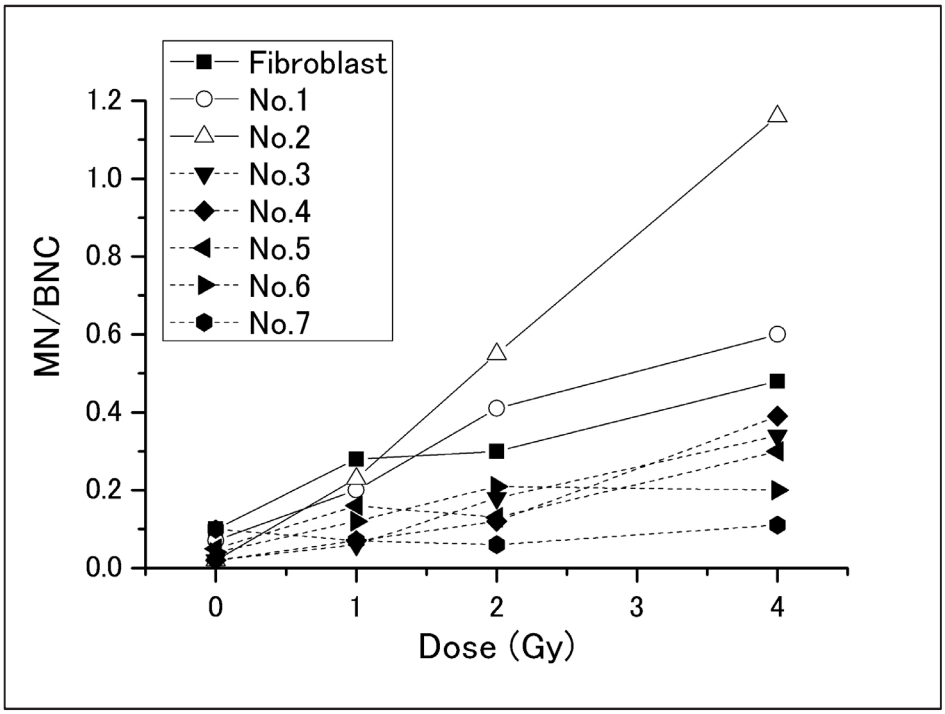

Fig. 1
Dose response curves of human fibroblast and brain tumors.
MN/BNC: The average number of micronuclei per binucleated cells.

Relationship between the parameters and tumor response

Tumor responses in the 6 patients, who received postoperative radiotherapy after partial resection, were evaluated for a period of 3 to 6 months after completing radiotherapy (Table 1). Both of the parameters, MN2 and MNIR, related well to tumor response. When we set the boundary value of MN2 as 0.20, the average one for fibroblast, it could clearly discriminate the tumor response into two groups. The tumors with an MN2 below 0.20

showed stable disease, while those with an MN2 above 0.02 showed complete or partial response.

4. Discussion

Various attempts for predicting tumor response to radiotherapy have been tried for personalizing and optimizing anti-tumor therapy, although clinical decision-making in radiotherapy is still based chiefly on classical clinico-pathological assessment. In particular, the assays to determine intrinsic radiosensitivity, oxygenation status and proliferative activity have been intensively investigated[7]. Our method, employing primary culture of tumor and the CBMN assay, is among the clinically-applicable assays to determine intrinsic radiosensitivity *ex vivo*. For fibroblast, the average value 0.20 of MN2 (n=3) was in line with the similar parameter mean induced MN frequencies after irradiation of 3.5 Gy[14], suggesting the validity of our method. Applying a similar method for various types of human solid tumors, Shibamoto *et al.* reported that the average number of micronuclei per binucleate cell at 2 Gy irradiation (after subtraction of the value at 0 Gy) ranged from 0.052 to 0.35 and tumors which produced more micronuclei after irradiation showed a better response to radiotherapy[5]. West *et al.* also demonstrated that for tumor survival fraction at 2 Gy (SF2), the other way to determine intrinsic radiosensitivity of tumor *ex vivo* using primary culture, significantly correlated with outcome of radiotherapy for cervical cancer[1].

Though the number of patients was small, our results were similar with those in the previous reports. Interestingly, a patient presenting with oligodendroglioma (No. 2), known to be often responsive to chemoradiation, showed high MN2 and MNIR and a clinically good tumor response. In contrast, a patient presenting with glioblastoma multiforme (No. 7), well known for its radioresistance, showed low MN2 and MNIR and a clinically poor tumor response. It was suggested that our method could be useful in predicting outcome of subsequent postoperative radiotherapy to primary brain tumors. Of the two parameters, MN2 appeared more predictive, which might reflect tumor response of radiotherapy in conventional dose fractionation scheme. In addition, newly introduced parameter MNIR appeared promising, too, which might reflect dose response relationship of the tumors, although dose range was limited up to 4 Gy.

However, it is also true that employing primary culture technique itself constitutes a barrier for routine clinical application of our method. The processes reaching *ex vivo* determination of intrinsic radiosensitivity are time-consuming and the yield of clonogenic culture of tumor cells often appears insufficient. In actuality, obtaining the radiosensitivity parameters in sufficient time before starting postoperative radiotherapy was difficult, and there were a large proportion of unsuccessful primary cultures (data not shown). Seven cases with successful primary culture might be quite fortunate ones from the standpoint of the growth balance between tumor cells and contaminated normal tissue cells, because the growths of the tumor cells were slow on the whole. These suggest that our method might not be so practical for clinical use. Of course, radiotherapy is flexible and changeable. So, for some patients, important and useful information regarding intrinsic radiosensitivity could play a certain role in changing treatment strategy even during the period of radiotherapy. However, in general, decision-making to treat patients presenting with tumor should be done prior to starting therapeutic procedures.

Therefore, recently, we are paying special attention to omics-based molecular tumor biology, because there have already been surprising developments of companion diagnostics and/or molecular signatures to guide therapeutic decision-making for some types of malignancies[7]. Systems-biology approaches should be noted, too, which include

modelling the radiosensitivity gene network and that for the hub-based gene network determining cellular radiophenotype[15, 16]. Further, precision oncology approaches will be crucial also for radiotherapy[17-19]. These approaches will eventually become more and more rapid and practical enough for clinical use, employing fresh tumor samples, with the advancement of computerization and the accumulation of related data. By combining these new analytic methods with ours of classical type, if possible, the clinical evaluation of tumor response will be of interest and importance to understand new aspects concerning radiosensitivity of human tumor.

Conflict of Interest

The authors declare that they have no conflict of interest.

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