ている。
このことから、治療成績に2.5か月の生存期間延長が期待できると仮定し、コントロールとなる手術＋X線分割外照射＋化学療法（テモゾロミド）に対する手術＋BNCT＋X線分割外照射＋化学療法（テモゾロミド）のハザード比はおおむね0.4程度と概算できる。このことからは治療成績を解析し得る治療実施症例は約20例ほどになるが、さらに条件を厳しく見積もり、登録目標症例数が45例に設定されている。今回の再解析では、生存期間中央値が18か月を達成した段階で有意な差を持って本研究で提唱した新規プロトコール“BNCT＋X線分割外照射＋化学療法”の有効性を示し得る。BNCTが原子炉中性子源に頼らざるを得ない現状では、目標症例数への予定期間内での到達困難も予想されたが、今回の解析結果から改めて計画の妥当性が見いだせた。

2） 本研究では今回臨床試験で得られた薬物動態を解析し、照射中のホウ素濃度は非常に良好に保たれていることが示された。また、本研究ではこれまでの大阪医科大プロトコールを踏襲し、ホウ素化合物としてＢＳＨとＢＰＡを併用しているが、２剤併用の際に問題となる個々の薬物由来の腫瘍・組織内硼素濃度の推定に関してはおおむね良好に算出できていたといえる。すなわち、ＢＰＡ 400 mg/kg/2hr + 100 mg/kg/hの単剤プロトコールから得られたBPA由来血中ホウ素濃度は、照射中にほぼ減衰することなく一定値を示し、その値は以前に実施されてきたＢＰＡ 250 mg/kgの治療プロトコールで得られるより高い値をとることが示されている。

ＢＳＨを併用することで、照射後のホウ素濃度は照射前より緩やかに減衰しているが、ＢＳＨ由来のホウ素に関してはＢＰＡ投薬までの採血で得られる値（血中ホウ素濃度）から算出可能であり、照射中の各々の由来ホウ素濃度が算出可能となっており、これらは事後評価の値と良好に相関し、照射前の採血のみでＢＮＣＴにより付与される腫瘍・正常組織の線量がシミュレーション可能であった。

3） 本試験で新たに用いた3層分割X線分割外照射法（8, 16, 24Gyのgradient）は、BNCTから寄与される比較的浅い部位での高線量を考慮した照射法であり、従来の20～30Gyの均一なＸ線分割外照射の追加で得られた良好な抗腫瘍効果を維持しつつ、長期生存で危惧された浅部での照射線量を低減できている。

また個々の患者毎で異なる腫瘍深度を鑑みても、あらゆる状況で正常脳の線量低減と深部腫瘍の線量向上が可能であり、プロトコール規定によって、いずれの施設・症例においても均質な治療計画が実施できると考えられた。

Ｅ．結論

1） 本研究で提唱した初発膠芽腫に対する新規放射線化学療法による有効治療法確立は、実施計画に無理がなく、十分にその有効性を示し得る計画立案である。

2）これまでに実施してきた同プロトコール（ＢＰＡ、ＢＳＨの併用投与法とBPA照射中静注）と本研究での薬物動態はほぼ一致し、照射中の血中濃度を各薬物由来の濃度として算出する手法の妥当性が見いだせた。

3） 本研究で用いた8-, 16-, 24Gyの3層分割XRTでは、従来のBNCT併用XRTと同等の腫瘍線量を保ち、かつ正常脳の最大線量を減じた治療法となっていることが示された。

Ｆ．健康危険情報

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Ｇ．研究発表

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(発表誌名巻号・頁・発行年等も記入)

H. 知的財産権の出願・登録状況
(予定を含む。)

1. 特許取得
なし
2. 実用新案登録
なし
3. その他
特記事項なし

厚生労働科学研究費補助金（医療技術実用化総合研究事業）
（分担）（総合）研究報告書

初発膠芽腫に対する新規放射線化学療法による有効治療法確立のための臨床研究

研究分担者　道上宏之　岡山大学・助教

研究要旨
「新規ホウ素製剤の開発によるBNCTの発展を目指した研究」
現在の臨床研究において,BNCTによる脳腫瘍患者の生存期間の延長が期待される。しかし、腫瘍組織でのホウ素濃度の上昇は認められるが、腫瘍細胞内部までの取り込みが認められないことが多い。ホウ素中性子捕捉反応の効果を十分に生かすために、細胞内導入型のホウ素製剤の開発を目指す。

A．研究目的

現在、悪性神経膠腫に対する治療法は、手術療法・放射線療法・化学療法による集学的治療法であるが、その予後は極めて悪い。その理由として、血液脳関門に囲まれた脳という特殊な環境に発生した腫瘍であるため、他の癌腫で使用可能な分子標的薬等の多くの抗がん作用を有する薬剤の使用が非常に困難である。その中で、大阪医科大学脳神経外科を中心とするBPAとBSHの2種類のホウ素製剤を併用した中性子捕捉療法が、非常に高い効果をあげている。BNCTはホウ素の取り込んだ細胞に対して中性子線の照射を行うと、核反応を引き起こし、細胞障害を引き起こす。しかしながら、細胞内にホウ素を取り込んでいない腫瘍細胞に対しては効果が非常に少ない。この弱点を克服し、さらなるホウ素中性子捕捉療法を発展させるためには、新規のさらなる効果をもたらすホウ素製剤の開発が急務である。今回は、初発膠芽腫患者に対するBNCTの臨床研究を進めるのと同時に、その臨床研究から出た知見を研究へと盛り込み、新規ホウ素製剤開発の基礎研究も行う。

B．研究方法

膠芽腫に対する臨床研究において使用されるホウ素製剤は、BPA(ホウ素フェニルアラニン）とBSH(Sodium mercapto undecahydrododecaborato)の2種類である。BPAは、ホウ素一個に対してアミノ酸が結合した化合物であり、アミノ酸の取り込みの高い増殖スピードの速い腫瘍細胞に多く取り込まれる。一方BSHは、ホウ素12個からなるホウ素原子がカゴ型に配列した化合であり、多くのホウ素を効率よく運ぶ長所はあるものの、細胞内へ導入されないためBNCTの効果が低いといわれている。今回我々は、ペプチドを用いた世界で初めてのホウ素ペプチド製剤の開発に成功した。ホウ素の細胞内局在を変化させ、中性子を照射した時の核へのダメージをシミレーションすることにより、細胞内にホウ素を導入することが重要であるかの検討を行った。本研究では、BSH-peptideと呼ばれる新規ホウ素製剤の開発に成功し、報告を行う。

（倫理面への配慮）

動物実験棟に関しては、岡山大学動物実験の規約に従い、委員会の承認を得て行う。

C．研究結果

ホウ素化合物の細胞内局在による中性子照射後の核へのダメージのシミュレーションは、京都大学原子炉実験所にて共同研究にて行われた。細胞内導入効果のない細胞外膜に付着しただけのホウ素は、核へと局在するホウ素と比較して効果が60～100倍程度違うことが判明した。

細胞膜通過ペプチド（11R）は分解によりアミノ酸へと変化するため、非常に安全な新規ホウ素製剤であることが確認された。また、BSHを多数搭載したmulti-BSH-peptideを作製することに成功し、ペプチドによるホウ素製剤開発の分野の開発に成功した。腫瘍細胞に対しての投与で、投与2時間目よりホウ素の細胞内導入及び核への局在を確認した。さらに、担癌モデル動物を用いて、腫瘍のある腫瘍部位へ特異的にBSH-peptideが局在することを証明した。このホウ素製剤は、細胞内及び核へと導入されていることを動物実験レベルにて確認した。中性子照射により、従来のBSHと比較して1/100以下の低濃度にて効果を呈することを証明した。

D．考察

腫瘍部におけるホウ素濃度が高い方が中性子捕捉療法により効果をもたらすことは周知の事実であるが、シミュレーションにより効果を定量したのは世界で初めてである。今回作製したBSH-peptideは、「細胞透過型ホウ素ペプチド」（2011年10月19日出願）特願2011-230059として、承認された。また、BSH-peptideに関する論文がアクセプトされた。（Biomaterials. 2014 Mar;35(10):3396-405)

E．結論

ペプチドを用いた新規ホウ素製剤の開発に成功し、特許取得、今後の発展が期待される。

F．健康危険情報

研究代表者による総合研究報告書参照

G．研究発表

1．論文発表

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H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得

1)「細胞導入ペプチドと皮膚導入促進剤とを組み合わせた皮膚導入システム及び美白剤」（2011 年 8 月 31 日）（特願 2011-189687）

2)「細胞透過型ホウ素ペプチド」（2011 年 10 月 19 日出願）（特願 2011-230059）

3)「ペプチドタグによるタンパク質の細胞内運搬」（2011 年 10 月 25 日）特願 2011-233812

4)「アクチン重合定量測定法を利用した抗浸潤薬新規スクリーニング法」（2012 年 6 月 19 日出願）（特願 2012-137489）

5)「抗がん剤」（フルボキサミンを配合することを特徴とする抗脳腫瘍治療薬剤　第二医薬用途）（出願日 2012 年 11 月 30 日）（特願 2012-263317）

2. 実用新案登録
なし

3. その他
特記事項なし

研究成果の刊行に関する一覧表

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RADIATION ONCOLOGY

CASE REPORT Open Access

Boron neutron capture therapy with bevacizumab may prolong the survival of recurrent malignant glioma patients: four cases

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Abstract

Background and importance: Recurrent malignant gliomas (RMGs) are very difficult to control, and no standard treatments have been established for them. We performed boron neutron capture therapy (BNCT) for patients with RMG. BNCT enables high-dose particle radiation to be applied selectively to tumor cells. However, RMG cases generally receive nearly 60 Gy X-ray irradiation prior to re-irradiation by BNCT. Therefore, even with tumor-selective particle radiation BNCT, radiation necrosis in the brain and symptomatic pseudoprogression may develop. In four of our recent patients with RMG after BNCT, we applied the anti-VEGF antibody bevacizumab to treat two pathological entities. This approach appeared to prolong survival. Here we present the case reports of these four consecutive patients with RMG and discuss the novel use of bevacizumab in this context.

Clinical presentation: Four patients with RMGs were treated with BNCT at our institutes. Upon the referral for BNCT, they were assessed as belonging to the recursive partitioning analysis (RPA) class 3 (n = 3 patients) or RPA class 4 (n = 1 patient) (the RPA classification for RMG was advocated by Carson et al. in 2007). The estimated median survival times for RPA classes 3 and 4 were 3.8 and 10.8 months, respectively, after some treatment at the recurrence. We applied BNCT for these four patients and administered bevacizumab when the lesions were considered radiation necrosis or symptomatic pseudoprogression. The class 3 patients survived after the BNCT for 14, 16.5 and > 23 months, and the class 4 patient survived > 26 months, with favorable improvements in clinical symptoms.

Conclusion: BNCT with the addition of bevacizumab for radiation necrosis or symptomatic pseudoprogression improved the clinical symptoms and prolonged the survival in RMG patients.

Keywords: Bevacizumab, Boron neutron capture therapy, Recurrent malignant glioma

Background

The prognosis of recurrent malignant gliomas (RMGs) is poor, and no standard treatment has been established [1]. Since 2002 at our institute, we have been applying a form of tumor-selective particle radiation, boron neutron capture therapy (BNCT), for RMGs and observed favorable survival outcomes [2,3]. BNCT is a biochemically targeted radiotherapy based on the nuclear capture and fission reactions that occur when non-radioactive boron-10, which is a constituent of natural elemental boron, is irradiated with low-energy thermal neutrons to yield high-linear-energy transfer alpha particles and recoiling lithium-7 nuclei. These particles are released within a very short range such as 9 μm, and therefore the cytotoxic effects are confined within boron-10-containing cells [4].

Boron-10-containing compounds can be accumulated selectively in tumor cells by several mechanisms. For example, boronophenylalanine (BPA) is selectively and preferentially accumulated in tumor cells via the augmented metabolism of amino acids compared to normal cells. Even with this novel and selective particle radiation technique, radiation damage — chiefly radiation necrosis (RN) and symptomatic pseudoprogression (psPD) — often occurs [5,6]. The radiation damage is especially likely in RMG cases, because full-dose X-ray treatment (XRT) is generally part of the treatment history in such cases.

Bevacizumab (BV), an anti-vascular endothelial growth factor (VEGF) antibody, has recently been used for the

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treatment of symptomatic RN [7,8]. Based on our analysis of human RN surgical specimens, we previously demonstrated that the edema in RN is caused by the overexpression of VEGF in reactive astrocytes [9]. Following this determination, we used BV in an attempt to control the symptomatic RN and the symptomatic psPD encountered after BNCT for RMGs [5,7]. Here we present a case series report of our last four consecutive cases of RMG treated with BNCT and BV, with >18-month observation periods. All four patients had RMGs after primary treatment with XRT and chemotherapy consisting chiefly of temozolomide (TMZ). The patients' profiles and survival data are listed in Table 1. Three of the patients were classified as recursive partitioning analysis (RPA) (advocated by Carson et al. in 2007 [1]) class 3 and one was classified as RPA class 4.

Case presentation

Case 1

A 44-year-old male's craniotomy showed anaplastic astrocytoma. He received standard chemoradiotherapy (XRT 60 Gy with TMZ). Unfortunately the lesion recurred with aggravation of aphasia and right hemiparesis, which forced him to retire from his job. The Karnofsky performance status (KPS) was 70%, and he was classified as RPA class 3. The patient was then referred to our institute for BNCT. Upon referral, MRI showed a slightly enhanced lesion with mild perifocal edema (Figure 1). A simultaneous fluorine-18-labeled BPA positron emission tomography (F-BPA-PET) image showed marked tracer uptake in the left parietofrontal region (Figure 1), with a 6.0 lesion/normal (L/N) brain ratio of the tracer, indicating that the lesion was a highly malignant tumor. BNCT was applied for this patient according to our recent protocol for RMGs and meningiomas [10]. Briefly, only BPA was administered over a 2-hr period (200 mg/kg/hr) just prior to and during the neutron irradiation (100 mg/kg/hr). The neutron irradiation time was decided based on a simulation not to exceed 12.0 Gy-Eq (Gray-equivalent) for the peak brain dose. The 10-B concentration in the blood during the neutron irradiation was 23.0 parts per million (ppm). By BNCT, the maximum brain dose, maximum tumor dose, and minimum tumor dose were estimated as 11.4, 118, and 36.1 Gy-Eq, respectively. Here, "Gy-Eq" corresponds to the biologically equivalent X-ray dose that would have equivalent effects on tumors and on the normal brain. The dose estimation was performed by the measurement of blood boron concentration and F-BPA-PET data prior to neutron irradiation as described elsewhere [2,6,10].

After the BNCT, an MRI showed gradual enlargement of both perifocal edema and contrast enhancement, whereas sequential F-BPA-PET showed a favorable decrease of tracer uptake (Figure 1, lower panel). F-BPA-PET was originally developed to estimate the absorbed dose in BNCT, as described above [2,11,12]. The background uptake of the tracer F-BPA is very low compared to that of fluorodeoxy-glucose and even compared to that of methionine as a tracer. Thereafter, RN and psPD have been differentially diagnosed from tumor progression by F-BPA-PET [6,13]. Ten months after the BNCT, the patient's KPS worsened to 60%, and so we administered BV 5 mg/kg biweekly, three times. Just prior to the BV administration, F-BPA-PET showed a more decreased L/N ratio, which indicated that the aggravation shown by MRI was RN and not a recurrence of the tumor. After the BV treatment, MRI showed improvement of the perilesional edema and a decrease in contrast enhancement. The BV treatment stabilized the patient's symptoms for 6 months but then his symptoms recurred, prompting us to perform a re-challenge with BV another three times. The patient is now stable and doing well, 23 months after the BNCT (Table 1).

Case 2

A 41-year-old man underwent surgery for his right parietal glioblastoma (GBM) with subtotal excision. Standard treatment with XRT and TMZ was performed, but the tumor recurred 5 months after the surgery. Upon referral for BNCT, the patient's KPS was assessed as 90% and he was classified as RPA class 4. MRI showed a definitively enhanced lesion with moderate perifocal edema (Figure 2). A simultaneous F-BPA-PET image showed marked tracer uptake in the right parietal region with a 3.8 L/N ratio of the tracer, indicating that the lesion was a recurrent malignant tumor and not psPD (Figure 2,

Table 1 The background of the four patients with recurrent malignant glioma (RMG)

Case No.	Age	Sex	Hist.	RPA class	Irradiated dose (Gy-Eq)			BV cycles	PsPD or RN	Survival (Months from BNCT)
					Brain (Max)	Tumor (Max)	Tumor (Mini)	(Months from BNCT)		
1	43	M	AA	3	11.4	118	36.1	3 (11 M)	RN	23 M, alive
2	41	M	GBM	4	12.1	88.5	36.6	4 (14 M)	RN	26 M, alive
3	60	M	AA	3	10.8	110	82.3	6 (4 M)	PsPD	16.5 M
4	34	F	AOA	3	11.5	71.6	30.1	6 (2 M)	PsPD	14 M

Hist, histology; RPA, recursive portioning analysis; BV, Bevacizumab; PsPD, pseudoprogression; RN, radiation necrosis; BNCT, boron neutron capture therapy.

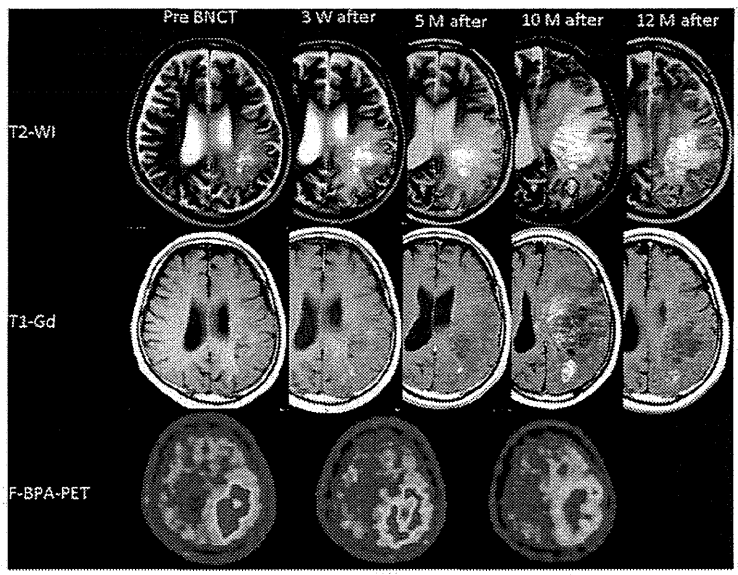

Figure 1 Sequential change of T2-weighted MRI (upper column), Gd-enhanced T1-weighted MRI (middle column) and F-BPA-PET (lower column) of Case 1, a 44-year-old male. The timing of the MRIs is depicted above the MRIs. F-BPA-PET images were taken just before the BNCT and at 1 month and 10 months after the BNCT. These PET images show the gradual decrease of the tracer uptake as a promising effect of the BNCT. BV was started 10 months after the BNCT, and the MRI showed marked improvement of both perifocal edema and contrast enhancements by BV treatment.

lower panel). He was treated with BNCT, with the same protocol as Case 1. The boron-10 concentration in the blood during the neutron irradiation was 30.2 ppm. By BNCT, the maximum brain dose, maximum tumor dose, and minimum tumor dose were estimated as 12.1, 88.5, and 36.6 Gy-Eq, respectively. One week after the BNCT, a contrast-enhanced T1-weighted MRI showed a marked shrinkage of the mass, and that at 3 months later showed slight enlargement of the enhanced lesion, which was presumed to be psPD. Periodic MRIs showed

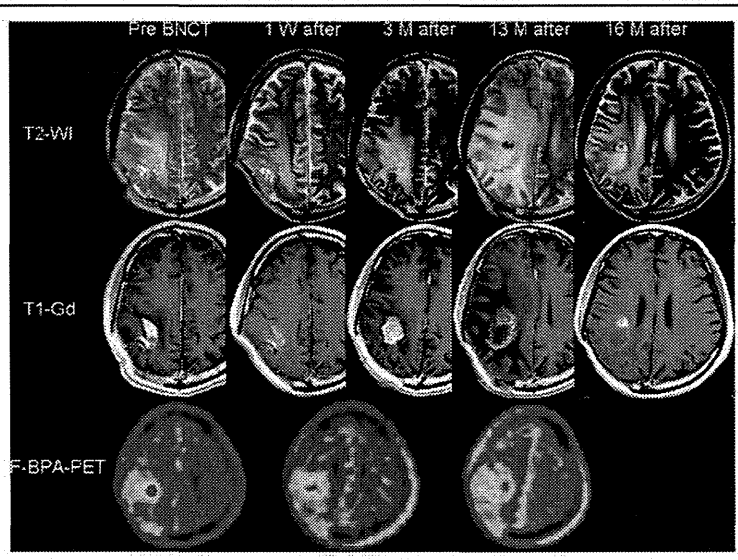

Figure 2 Sequential change of T2-weighted MRI (upper column), Gd-enhanced T1-weighted MRI (middle column) and F-BPA-PET (lower column) of Case 2, a 41-year-old man. The timing of the MRI is depicted above the MRI. F-BPA-PET images were taken just before the BNCT, 1 month after and 12 months after the BNCT. These PET images show the gradual decrease of the tracer uptake as a promising effect of BNCT. BV was started 13 months after the BNCT, and an MRI showed a marked positive effect of the BV treatment on the perifocal edema and contrast enhancements.

gradual enlargement of both the enhanced lesion and perifocal edema, whereas F-BPA-PET showed a gradual decrease of the tracer uptake. The final L/N ratio, 1 year after BNCT, was 2.3. This L/N ratio and the MRI 13 months after the BNCT suggested that the lesion was RN.

The patient was not able to continue his work as a cook, and we decided to begin intravenous BV treatment biweekly (5 mg/kg). After four treatments, MRI showed marked improvement in the perifocal edema and left hemiparesis. The patient is now doing well and has resumed his work as a cook, 26 months after the BNCT, without tumor progression or recurrence of the RN.

Case 3

A 56-year-old male experienced speech disturbance and mild right hemiparesis. First he received a craniotomy with a diagnosis of gemistocytic astrocytoma, followed by fractionated XRT (total 50 Gy) and repetitive chemotherapy with nitrosourea. Three years later, a recurrent lesion appeared with Gd enhancement on MRI. Re-craniotomy revealed GBM histologically. After surgery, the enhanced lesion gradually grew and the patient's sensory aphasia worsened despite the repeated administration of TMZ. He was referred to our institute for BNCT. Upon his referral, he was assessed as RPA class 3. The boron-10 concentration in the blood during the neutron irradiation was 30.0 ppm. Using BNCT, the maximum brain dose, maximum tumor dose, and minimum tumor dose were estimated as 10.8, 110, and 82.3 Gy-Eq, respectively, as shown in Table 1. His right hemiparesis and aphasia gradually worsened after the BNCT, even with an escalating dose of corticosteroids. Four months after the BNCT, a follow-up MRI and F-BPA-PET suggested that the lesion was symptomatic psPD, not tumor progression. The patient was successfully treated with BV, as we recently reported, along with the periodic changes of the neuroimages and the detailed clinical course [5]. We lost this patient to local tumor progression 16.5 months after the BNCT.

Case 4

A 27-year-old female manifested left hemiparesis. A right frontal enhanced mass was removed gross/totally, and the histological diagnosis was anaplastic oligo-astrocytoma. She received fractionated XRT (total 72 Gy) and repetitive chemotherapy with nitrosourea. The lesion recurred and re-craniotomy was performed 4 years later, with the same pathological diagnosis. This was followed by TMZ chemotherapy. Unfortunately, a recurrence was confirmed by MRI and she was referred to us for BNCT. The boron-10 concentration in the blood during the neutron irradiation was 21.4 ppm. By BNCT, the maximum brain dose, maximum tumor dose, and minimum tumor dose were 11.5, 71.6, and 30.1 Gy-Eq, respectively (Table 1). After the BNCT, her hemiparesis gradually became aggravated despite an increased dose of corticosteroids. MRI taken 2 months after the BNCT showed an enlarged enhanced lesion with increased perilesional edema. We judged this aggravation as symptomatic psPD. We started BV treatment for her. The patient was bedridden just prior to the BV treatment, but after two BV treatments her hemiparesis improved markedly and she could walk. Her neuroimages and clinical symptoms showed marked improvement, as we reported previously [5]. Unfortunately we lost her because of tumor extension to the opposite hemisphere 14 months after the BNCT.

The neuroimages, including F-BPA-PET scans of Cases 3 and 4, were published elsewhere [5] and thus are not included in this brief report.

Discussion

In comparison with many Phase I and II trials for RMG [1], BNCT showed a marked survival benefit for RMG in our previous study, in which BV was not used [3]. Briefly, BNCT resulted in median survival times (MSTs) (months and 95% confidence intervals) as follows: for all RPA classes (Classes 1–7), 10.8 (7.3–12.8) (n = 22), and in the poor-prognosis group (RPA class 3 + 7), 9.1(4.4–11.0) (n = 11). In a meta-analysis reported in the Journal of Clinical Oncology [1], the MSTs in all RPA classes and in the poor-prognosis group (RPA class 3 + 7) were 7.0 (6.2–8.0) (n = 310) and 4.4 (3.6–5.4) (n = 129), respectively. These data showed the superiority of BNCT for RMGs, especially in poor-prognosis groups. In comparison, our previous data showed MSTs of RPA class 3 and 4 as 7.3 and 12.0 months, respectively, although the number of the patients was quite limited: 4 cases in class 3 and 3 cases in class 4 [3].

In our recent patients undergoing BNCT for RMGs, we have begun to treat RN or symptomatic psPD aggressively by administering BV. We applied intravenous BV treatment for four recent RMG patients treated with BNCT at our institute and in whom we encountered RN or symptomatic psPD; these cases are reported here. Three of these four patients were classified as RPA class 3 and one as class 4 (Table 1). The estimated survival time of class 3 patients is 3.8 months and that of class 4 patients is 10.8 months [1]. Our three class 3 patients survived for 14, 16.5, and > 23 months, and the class 4 patient has survived for over 26 months.

At a glance, BNCT with BV seemed to prolong the survival of RMGs strikingly in comparison not only with Carson's data set but also with our previous BNCT data. Although of course no definitive conclusion can be drawn from such a small number of cases.

In our limited experience, there is no obvious histological difference between RN and psPD [6]. The center part of each pathology is characterized as histological necrosis, and

marked angiogenesis is observed in the boundary of the necrotic core and normal brain tissue [9]. Clinically, most psPD occurs at a relatively early stage after intensive treatments and is self-limiting without severe sequelae [14]. In most cases, psPD improves over time without intensive treatments. On the other hand, RN often shows severe symptoms and occurs at least a half year after radiotherapy. Thereafter, symptomatic psPD is especially difficult to distinguish from RN. In Table 1, we distinguish them only from the duration of the symptomatic onset after BNCT.

We have described herein the use of BV for RN or psPD after BNCT. BV was approved for the treatment of RMGs as an anticancer agent [15,16], and several trials of re-irradiation using XRT or hypo-fractionated stereotactic radiotherapy in combination with BV just before radiotherapy for RMGs have recently been conducted, with favorable preliminary safety and response results [17-19]. The authors of those reports described the role of BV not only as an anticancer agent but also for normalizing the perfusion pressure and oxygenation effects during irradiation. BV may also prevent RN and symptomatic psPD after re-irradiation.

We are now planning a prospective clinical trial of BNCT using BV immediately after neutron irradiation for RMG patients with poor prognosis (class 3 + 7). We are also conducting a clinical trial of BNCT for RMGs using a small accelerator in-hospital, instead of an atomic reactor. We hope to determine whether accelerator-based BNCT with BV could be used as a standard treatment for RMGs.

Conclusion

BNCT with the addition of BV for radiation necrosis or symptomatic pseudoprogression improved the clinical symptoms and might prolong the survival of RMG patients.

Consent

Written informed consent was obtained from the patient for the publication of this report and any accompanying images.

Abbreviations

BNCT: Boron neutron capture therapy; BPA: Boronophenylalanine; BV: Bevacizumab; GBM: Glioblastoma; Gy-Eq: Gray-equivalent; KPS: Karnofsky performance status; L/N: Lesion/normal; PET: Positron emission tomography; ppm: parts per million; psPD: pseudoprogression; RMG: Recurrent malignant gliomas; RN: Radiation necrosis; RPA: Recursive portioning analysis; TMZ: Temozolomide; XRT: X-ray treatment.

Competing interests

There is no conflict of interest to disclose for any of the authors.

Authors' contributions

S-IM conceived of the study and participated in the follow-up of patients. SK, RH, and MS applied BNCT in the atomic reactor. MF followed the patients with bevacizumab. TK selected the patients for BNCT. All authors read and approved the final manuscript.

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High linear-energy-transfer radiation can overcome radioresistance of glioma stem-like cells to low linear-energy-transfer radiation

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Ionizing radiation is applied as the standard treatment for glioblastoma multiforme (GBM). However, radiotherapy remains merely palliative, not curative, because of the existence of glioma stem cells (GSCs), which are regarded as highly radioresistant to low linear-energy-transfer (LET) photons. Here we analyzed whether or not high-LET particles can overcome the radioresistance of GSCs. Glioma stem-like cells (GSLCs) were induced from the GBM cell line A172 in stem cell culture medium. The phenotypes of GSLCs and wild-type cells were confirmed using stem cell markers. These cells were irradiated with ^{60}Co gamma rays or reactor neutron beams. Under neutron-beam irradiation, high-LET proton particles can be produced through elastic scattering or nitrogen capture reaction. Radiosensitivity was assessed by a colony-forming assay, and the DNA double-strand breaks (DSBs) were assessed by a histone gamma-H2AX focus detection assay. In stem cell culture medium, GSLCs could form neurosphere-like cells and express neural stem cell markers (Sox2 and Musashi) abundantly in comparison with their parental cells. GSLCs were significantly more radioresistant to gamma rays than their parental cells, but neutron beams overcame this resistance. There were significantly fewer gamma-H2AX foci in the A172 GSLCs 24 h after irradiation with gamma rays than in their parental cultured cells, while there was no apparent difference following neutron-beam irradiation. High-LET radiation can overcome the radioresistance of GSLCs by producing unrepairable DNA DSBs. High-LET radiation therapy might have the potential to overcome GBM's resistance to X-rays in a clinical setting.

Keywords: glioblastoma multiforme; glioma stem cells; linear energy transfer; neutron beams; gamma rays

INTRODUCTION

Radiation therapy with surgery and chemotherapy is the standard treatment for glioblastoma multiforme (GBM) [1]. However, the prognosis of patients with GBM has not improved in recent decades, and almost half of GBM patients do not survive the first year after diagnosis. Thus, another, more promising therapy for GBM is needed. Recently, some reports have shown the presence of glioma stem cells (GSCs) in malignant gliomas [2–4]. These cells are highly resistant to radiotherapy because of their enhanced checkpoint response to radiation [5]. Other studies have shown that GSCs express high levels of sirtuin family genes (especially the SirT1 gene) and that these upregulations are relevant to radiosensitivity because they modulate apoptotic activity in response to irradiation to GSCs [6]. As a result, GSCs are now known to play important roles in tumor progression and relapse after radiotherapy and chemotherapy, and new therapeutic strategies targeting GSCs should be developed to treat patients with GBM. In the previous reports, radioresistance of GSCs was studied in a subpopulation with a specific phenotype. In these studies, it was difficult to use appropriate control cells for the GSCs. Therefore, we induced glioma stem-like cells (GSLCs) in which the phenotypes of GSCs

were enriched, and used the wild-type GBM cells as controls in this study.

On the other hand, we have applied boron neutron capture therapy (BNCT) for malignant brain tumors, including GBM [7–9]. This is a unique tumor-selective particle radiotherapy using neutron irradiation, especially thermal neutron irradiation. Boron-10 (^{10}B) releases alpha (^{4}He) and ^{7}Li particles through ^{10}B (n,α)^{7}Li reaction. The key players in the anti-tumor effects of BNCT are these high linear-energy-transfer (LET) particles. With BNCT, good results have already been achieved for patients with newly diagnosed GBM and recurrent malignant glioma [9, 10], although the numbers of such cases in clinical trials have been limited.

So far, the radioresistance of GSCs has been examined mainly in terms of low-LET radiation such as X-rays or gamma rays. Therefore, we hypothesized that high-LET radiation could overcome the radioresistance of GSCs. In fact, a previous study showed that high-LET radiation was more effective than low-LET radiation for promoting DNA damage [11]. Here, we employed a reactor neutron-beam irradiation system that produces high-LET proton particles through elastic scattering and nitrogen capture reaction. We analyzed the usefulness of high-LET radiation for overcoming the radioresistance to low-LET radiation in GSCs using GSLCs, as well as the ability of these cells to recover from radiation-induced DNA damage by a gamma-H2AX assay.

MATERIALS AND METHODS

Cell culture

The human GBM cell line A172 was purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) with penicillin and streptomycin at 37°C in an atmosphere of 5% CO_2. GSLCs were induced from A172 cells in serum-free medium (SFM) as described previously [12]. The SFM was composed of DMEM/F12 (Sigma-Aldrich, St Louis, MO), 20 ng/ml basic fibroblast growth factor (Peprotech, Rocky Hill, NJ), 20 ng/ml epidermal growth factor (Peprotech), 2 μg/ml heparin (Sigma-Aldrich), and B27 supplement (50×; Life Technology/Invitrogen).

Western blot analysis

Cells were cultured for 7 d in each culture medium. Protein samples were prepared with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Immune complexes were formed by incubation with the stem cell markers CD133 (Cell Signal Technology, Danvers, MA), Sox2 (Cell Signal Technology), and Musashi (Cell Signal Technology) overnight at 4°C. As a control for the housekeeping gene products, Ku70 (Thermo Scientific, Waltham, MA) was employed. Blots were washed and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive protein bands were detected by using an enhanced chemoluminescence Advance Western Blotting Detection Kit (GE Health Care, Buckinghamshire, UK), and Image Reader LAS-1000 Pro ver. 2.5 (Fuji Photo Film, Tokyo, Japan).

Fluorescence-activated cell sorting analysis

Cells were cultured for 7–14 d in each culture medium. Cells were collected and incubated with anti-CD133 antibody (Bioss, Woburn, MA) for 1 h at 37°C. After washing, the cells were incubated with Alexa Fluor 647-labeled anti-rabbit secondary antibody for 30 min at 37°C, then analyzed by fluorescence-activated cell sorting (FACS) using a BD FACS Aria Cell Sorter (BD Bioscience, San Jose, CA).

Gamma-ray and neutron-beam irradiation

Two sets of A172 cells, one cultured with serum-containing medium (DMEM + 10% FBS) and the other cultured with SFM, were trypsinized, and single-cell suspensions were placed into a Teflon tube and irradiated at room temperature by neutron beams or gamma rays.

At the Heavy Water Column of the Kyoto University Research Reactor (KUR), neutron-beam irradiation was performed at a power of 1 MW. The neutron fluence was measured from the radioactivation of gold foil. Contaminating gamma rays, including secondary gamma rays, were measured with thermoluminescence dosimeter (TLD) powder. The TLD used was beryllium oxide (BeO) enclosed in a quartz glass capsule. BeO itself is sensitive to thermal neutrons [13]. The average neutron fluxes were 1.0×10^{9} n/cm^2/s for the thermal neutron range (less than 0.6 keV), 1.6×10^{8} n/cm^2/s for the epithermal neutron range (0.6–10 keV), and 9.4×10^{6} n/cm^2/s for the fast neutron range (more than 10 keV). The total absorbed doses resulting from fast, epithermal, and thermal neutron-beam irradiation were calculated as the sum of the absorbed doses attributed primarily to ^{1}H(n,n)^{1}H, ^{14}N(n,p)^{14}C, and contaminating gamma rays. The dose-converting coefficients and details of the calculation method have been described previously [14, 15].

Gamma-ray irradiation was applied using a ^{60}Co gamma-ray irradiator at a dose rate of 1.3 Gy/min.

Colony-forming assay

Cell survival was defined using a colony-forming assay. The irradiated cells were seeded into 100 mm dishes at various densities depending on the physical dose that cells received, and cultured in a serum-containing medium. After 13–15 d, the colonies were stained with methylene blue. A cell cluster containing at least 50 cells was considered a single colony. The surviving fraction was calculated as the number of colonies of treated cells divided by that for the control cells. The D_{10} values were derived by linear quadratic model analysis