The effect of combining temozolomide treatment with bevacizumab for intramedullary spinal cord glioma

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The effect of combining temozolomide treatment with bevacizumab for intramedullary spinal cord glioma

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ABSTRACT

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Study Design: The current standard therapies for the management of intramedullary malignant gliomas include surgery, chemotherapy, and radiotherapy. Concurrent or adjuvant temozolomide (TMZ) has been considered an emerging new treatment for intramedullary malignant gliomas. Although TMZ does not cure spinal cord tumor patients, it significantly improves patient survival and quality of life.

Objective: In this study, we hypothesized that treatment with bevacizumab accelerates the therapeutic effect of TMZ on intramedullary gliomas in an animal model.

Methods: C6 glioma cells were injected into the T5 level of the spinal cord, and TMZ and bevacizumab were administered 5 days after C6 inoculation. Tumor size was analyzed using histology and magnetic resonance imaging (MRI) at 13 days after tumor inoculation.

Results: Histological analyses and MRI findings showed that combined treatment with TMZ and bevacizumab reduced tumor mass. Neurologic outcomes demonstrated that combined therapy improved hind limb function more than TMZ alone or control group.

Conclusion: This study shows that bevacizumab could be useful in combination with TMZ to increase the therapeutic benefits of TMZ for intramedullary spinal cord tumors.

Key words : bevacizumab, temozolomide, intramedullary spinal cord tumor

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I. INTRODUCTION

Intramedullary spinal cord tumors (IMSCTs) are relatively rare neoplasms of the central nervous system (CNS), comprising 4 to 10 % of CNS tumors.[1] Surgical therapy is the current accepted treatment for malignant tumor.[2,3] The prognosis for patients with malignant lesions, however, remains discouraging, due to a high recurrence rate and the rapid and progressive infiltration of tumors into the spinal cord parenchyma, which underlies clinical morbidities associated with malignant IMSCTs. Recently, clinical advances in multimodality treatments using numerous chemotherapeutic agents after surgical resection, and radiotherapy have led to an improvement in expected survival of IMSCT from 9 to 20 months.[4,5] However, limitations such as poor CNS drug penetration and dose-limiting toxicities have restricted their use.

Temozolomide (TMZ), an alkylating agent, is available-orally and is currently used to treat patients with high-grade gliomas, having received approval by the Food and Drug Administration as an effective anti-glioma chemotherapeutic.[6] This agent exerts its anti-tumor effects through several

distinct mechanisms of action. Conventional chemotherapeutic agents for CNS tumors have poor efficacy, due to low penetration of the blood-brain barrier.[7,8] By contrast, TMZ is a small drug that infiltrates the blood-brain barrier because of its lipophilic properties.[9] TMZ changes to its active metabolite 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC) at physiological pH levels, and the cytotoxicity of MTIC induces alkylation of DNA at the O6 and N7 positions of guanine in tumor cells.[10] Combination therapy with TMZ improves patient survival and glioblastoma sensitivity compared radiotherapy alone.[11,12]

Recently, many studies have investigated combination therapy using chemotherapy and anti-angiogenic agents.[13-15] Anti-angiogenic therapy offers several potential advantages over conventional cytotoxic chemotherapy. Unlike antitumor agents, anti-angiogenic drugs target vascular endothelial cells, which are easily accessible and genetically stable.[16] In particular, bevacizumab is an anti-angiogenic agent and a monoclonal antibody that targets vascular endothelial growth factor (VEGF). The use of this antibody has been found to improve the survival and inhibit angiogenesis.[17]

In this study, we developed a novel therapy for IMSCTs, using bevacizumab and TMZ for IMSCTs. We investigated whether the combined therapy of bevacizumab and TMZ has greater the anticancer effects on IMSCTs than the conventional single therapy of TMZ.

II. MATERIALS AND METHODS

1. Cell culture and establishment of stable C6 cell lines

C6 (glioma) cells were purchased from American Type Culture Collection (Rockville, MD). C6 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and, 1% penicillin/streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. To establish stable Dr-red expressing C6 cell lines, 1μg of the pBudCE4.1-DsRed plasmid was lipofected into into 1×10^4 normal C6 cells according to a previously described protocol.[18] After 24 hr, different C6 cells were transferred into 100-mm dishes. The transfected cells were selected using the 200μg/ml Zekocin (Invitrogen, Carlsbad, CA) until formation of visible colonies.

2. The anticancer efficacy of the TMZ or bevacizumab *in vitro*

The anticancer efficacy of TMZ was determined by measuring Ds-Red expression and mitochondrial metabolic activity of C6 cells cultured in the presence of various concentration of TMZ or bevacizumab. The mitochondrial metabolic activity of the cells was determined using the MTT assay. C6 cells were plated in 96-well plates at 2×10^4 cells/well, and cultured for 24 hr. Various concentration of TMZ (Sigma, St. Louis, MO) or bevacizumab (Roche Korea Co, Seoul, Korea) were added to each well of the C6 cell culture. After incubation for 48 hr at 37°C, cultured cells were rinsed with phosphate buffered saline (PBS), and 200μl MTT (3-(4,5-dimethylthiazol-2-

yl)- 2,5-diphenyltetrazolium bromide, 2mg/ml in PBS, Sigma,) was added. After incubation for 4 hr at 37℃, the MTT solution was removed. The resulting insoluble particles were dissolved in 100μl dimethyl sulfoxide hybrid-max (Sigma, St. Louis, Mo) for 30 min, and the absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay plate reader.

3. The anticancer efficacy of TMZ or bevacizumab in a spinal cord tumor model

The Animal Care and Use Committee of the Medical Research Institute of Yonsei University College of Medicine approved all of the protocols. All experiments were performed according to international guidelines on the ethical use of animals, and the number of animals used was minimized. Spinal cord tumors were was induced in adult male Sprague Dawley rats (250-300g; Orient Bio, Kyungki-do, Korea). After anesthesia (Zoletil, 50mg/ml), laminectomy was performed at the T5 level and C6 glioma cells (1.0×10^5 cells in 5μl PBS) were injected into the T5 position using a Hamilton syringe (Hamilton, Bonaduz, Switzerland). Five days after tumor inoculation, the rats were randomly assigned to one of four experimental groups (n=5 for each group). The control group received an injection of medium. The TMZ group received oral administration of 50mg/kg TMZ once a day for 7 days. The bevacizumab group received an intraperitoneal injection of 7mg/kg bevacizumab every other day for 7 days. The combined treatment group was received intraperitoneally 7mg/kg bevacizumab and oral administration of

50mg/kg TMZ.

4. Histological analysis and Functional tests

Rat hind limb strength was assessed following the Basso, Beattie, and Bresnahan (BBB) scale.[19] Briefly, rats were placed in an open field testing area, allowed to adapt, and then were observed for 5 min. The BBB scores of each rat were recorded preoperatively to ensure a baseline locomotor rating of 21. After the operation, the BBB scores of all treatment groups were recorded daily for 11 days. After the final injection, rats were sacrificed and then perfused with saline containing 4% paraformaldehyde (Merck, Germany). For histological analyses, the tumor regions were dissected and sectioned with a thickness of 10μm. The sections were processed for standard hematoxyline and eosin (H&E) staining. Apoptotic activity was evaluated by the terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method using ApopTag Plus Fluorescein In situ Apoptosis Detection kit (Chemicon International. Temecula, CA). The tumor mass was measured by magnetic resonance scanning.

5. Statistical Analysis

Data are presented as mean ± standard deviation (SD). The statistical significance was analyzed between groups by ANOVA (one-way analysis of variance). All statistical analyses were performed using the Medcalc Program (Medcalc Software). P values of less than 0.05 were considered as significant ($p<0.05$).

III. RESULTS

1. Effect of bevacizumab and TMZ in C6 glioma cells *in vitro*

We established stable C6 cell lines (DsRed-C6 cells) (Figure 1.). For determination of the effectiveness of TMZ or bevacizumab in glioma, we evaluated the effects of various concentrations of TMZ or bevacizumab on the cytotoxicity of C6 cells using the MTT assay. Significant inhibition of cell proliferation was observed in DsRed-C6 cells treated with 100μM of TMZ (Figure 2). By contrast, the cell proliferation rate of DsRed-C6 cells was not changed in the treatment group receiving 10μM TMZ (Figure 2.B, C). RT-PCR analysis of the C6 glioma cells showed mRNA expression of VEGF (Figure 3.A). Treatment of DsRed-C6 cells with bevacizumab did not change cell numbers or MTT assay data (Figure 3.C, D). To evaluate of the apoptosis effect of TMZ and bevacizumab on C6 cells, a TUNEL assay was performed 48 hr after treatment TMZ and bevacizumab (Figure 4). The number of the TUNEL-positive cells in TMZ treated group was significantly higher than that in the control or bevacizumab groups (Figure 4.B, C). The mRNA level of p53, an apoptosis-related gene, was higher in the control or bevacizumab groups (Figure 4.A)

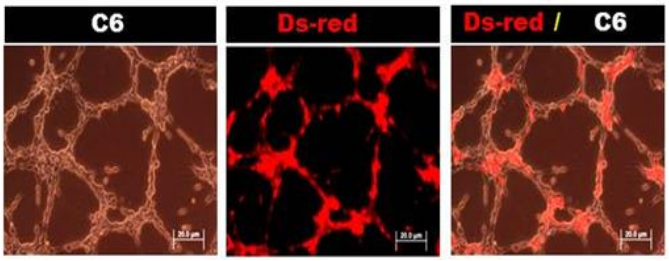

Figure 1. Characterization of C6 glioma. Morphology of stable DsRed-expressing C6 cells. Scale bars indicate 20 μm.

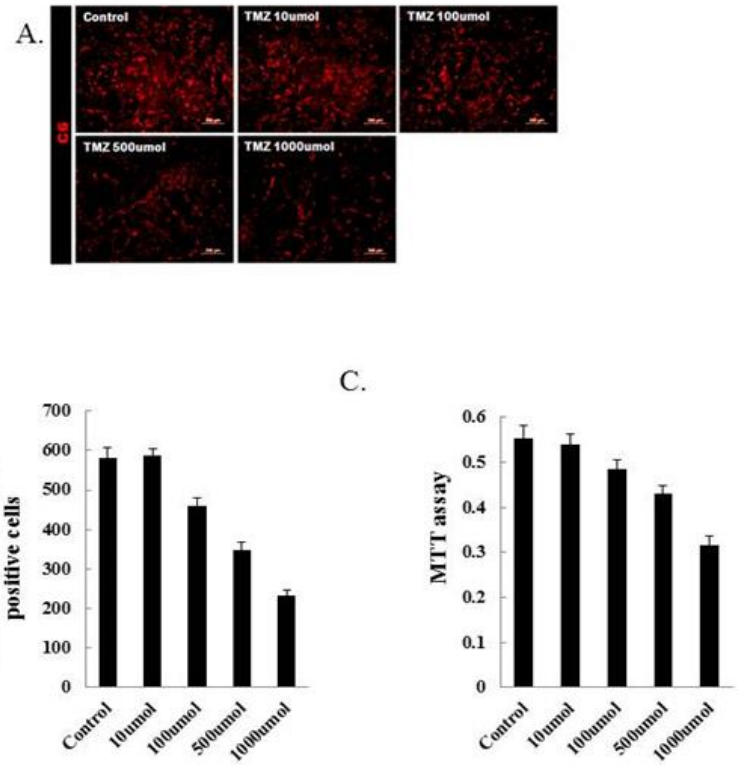

Figure 2. Characterization of C6 glioma by TMZ. (A) Micrographs of DsRed-expressing C6 cells treated with different doses of TMZ. C6 cells (red) were incubated with increasing doses of TMZ for 2 days. Cell survival was measured by counting DsRed -positive C6 cells (B) and MTT assay (C). Scale bars indicate 200 μm.

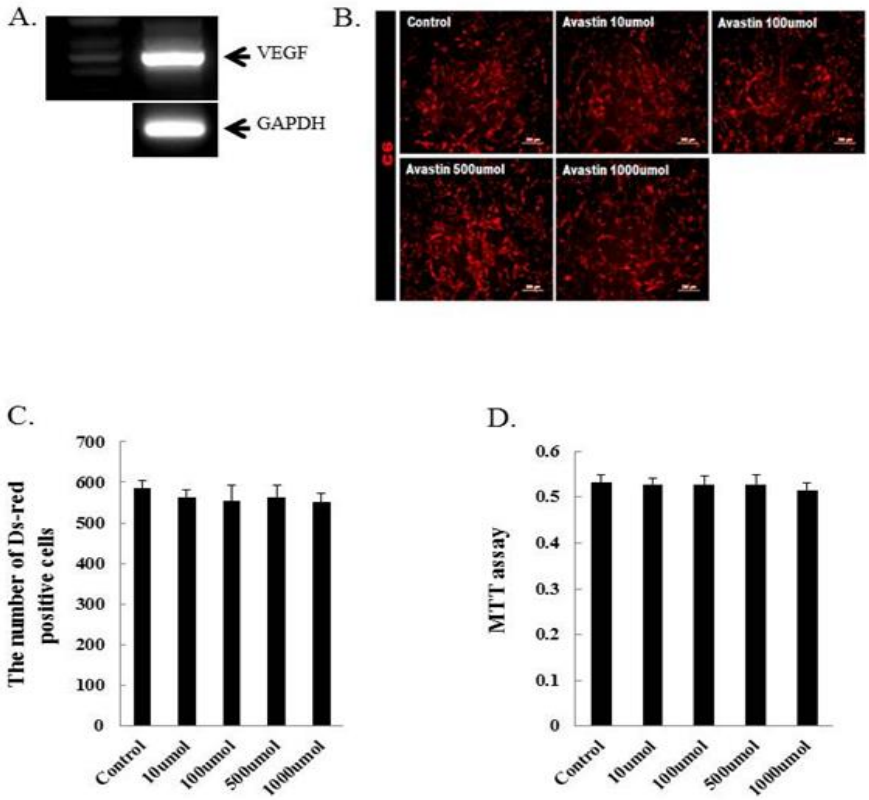

Figure 3. Characterization of C6 glioma by Avastin. (A) RT-PCR analysis of VEGF gene expression in C6 gliomas (DsRed). (B) Photomicrographs show DsRed-expressing C6 glioma treated with different doses of bevacizumab. Cell survival was measured by counting DsRed-positive cells (C) and MTT assay (D). Scale bars indicate 200 μm.

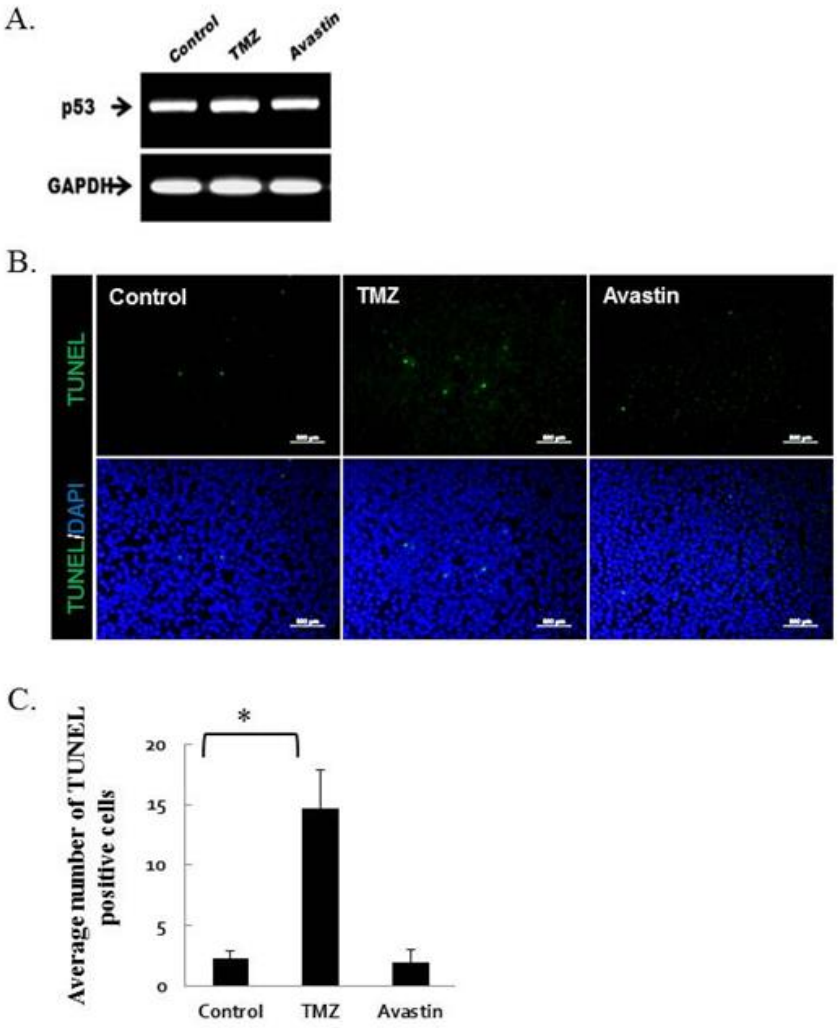

Figure 4. Apoptotic effect of TMZ and bevacizumab in C6 glioma. (A) mRNA expression of apoptosis-related genes. Increased mRNA expression of apoptosis-related gene p53 was associated with TMZ-induced apoptosis. (B) TUNEL staining in C6 glioma cells after TMZ or bevacizumab treatment. Nuclei were stained with DAPI. Apoptosis-positive neuclei were labeled TUNEL staining. (C) TUNEL-positive cells in C6 glioma after TMZ or bevacizumab. * $p<0.05$.

2. Effect of TMZ and bevacizumab on apoptosis

Apoptosis in the IMSCT area was analyzed by TUNEL assay (Figure 5). The TUNEL-positive cell was higher with combined therapy using bevacizumab and TMZ than with in the bevacizumab or TMZ therapy alone (Figure 5.A). High numbers of TUNEL-positive cells were observed in the combined therapy group, and apoptotic cells were increased in the bevacizumab or TMZ group compared with the control group. The average number of TUNEL-positive cells in the tumor area was 26 ± 7 in the control group, 175 ± 31 in the bevacizumab group, 200 ± 25 in the TMZ group, and 312 ± 39 in the combined treatment group (Figure 5.B). Apoptosis in the IMSCTs was further determined by measuring an apoptosis-related gene using RT-PCR analysis (Figure 5.C). RT-PCR analysis showed that mRNA expression of p53, which is an apoptosis-promoting molecule, was more extensive in the combined bevacizumab and TMZ treatment group than in the other groups.

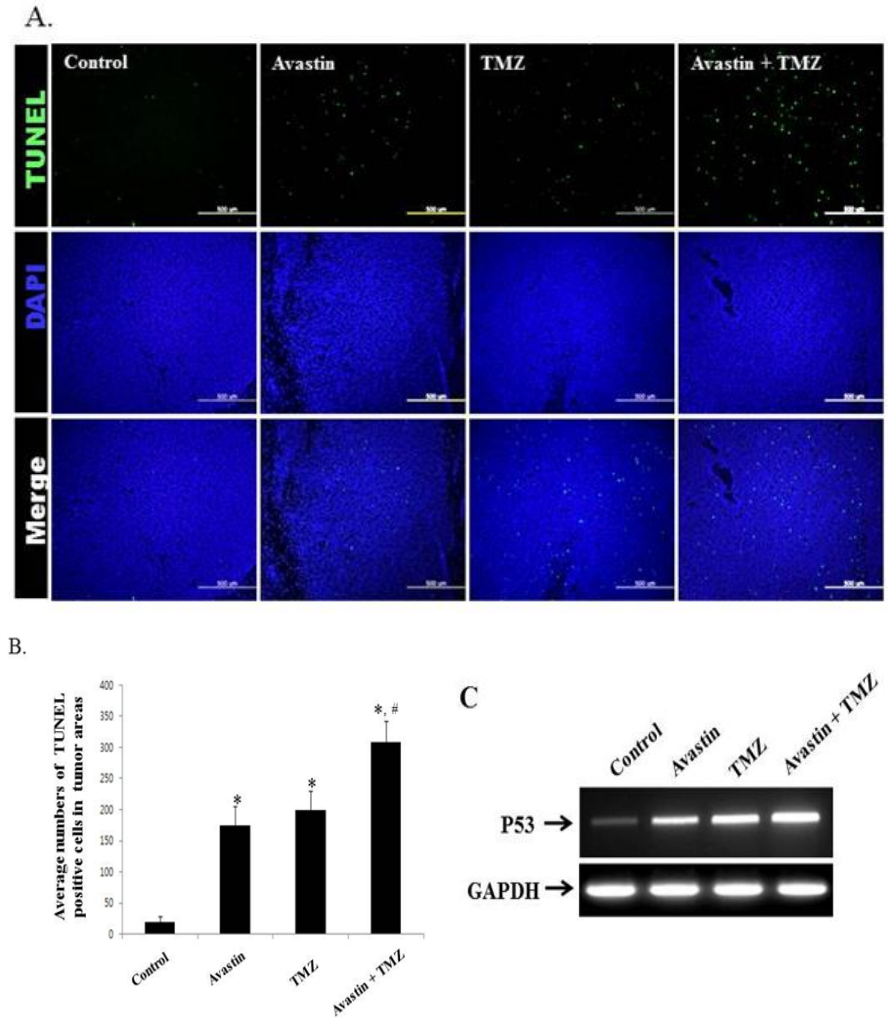

Figure 5. Apoptosis analysis of IMSCT area at 8 days after therapy. (A) TUNEL staining in IMSCTs. Apoptotic cells and cell nuclei were stained by TUNEL (green) and DAPI (blue), respectively. (B) Number of TUNEL-positive cells in the IMSCT area. *$p<0.01$ compared to control group; $^{\#}p<0.01$ compared to bevacizumab group and TMZ group. (C) RT-PCR analysis of an apoptosis-related gene. mRNA expression of an apoptosis-related gene was more extensive in the combined therapy group than in the TMZ group.

3. Effects of TMZ and bevacizumab on spinal cord tumors

Anticancer efficacy was evaluated by measuring tumor volume using histological analyses and MRI of the tumor area (Figure 6, 7). Histological analyses show the tumor area in the T5 region. The whole area of spinal cord was covered with tumor in the control group. Combined bevacizumab and TMZ treatmont was significantly reduced tumor area compared with the other groups (Figure 6). The tumor area in the group treated with bevacizumab or TMZ alone was smaller than that in the control group. The tumor area in the combined therapy group was decreased by approximately 60% relative to the control group. The tumor volume was evaluated to be approximately 52mm^2 ± 4.8 in the control group, approximately 34.8 ± 3.6 mm^2 in the bevacizumab group, approximately 31.7± 4.5 mm^2 in the TMA group, and approximately 18 ± 3.4 mm^2 in the combine therapy group (Figure 7).

Median hind-limb BBB scores remained at 16 for 4 days immediately after tumor injection for all four groups (Figure 8). The control group BBB score began to show a decline on day 6 and reached 2.5 on day 13. The BBB score in the bevacizumab or TMZ alone groups began to decline on day 7 and reached approximately~8 on day 13. However, the combined treatment group had significantly higher mean BBB score than bevacizumab or TMZ alone groups.

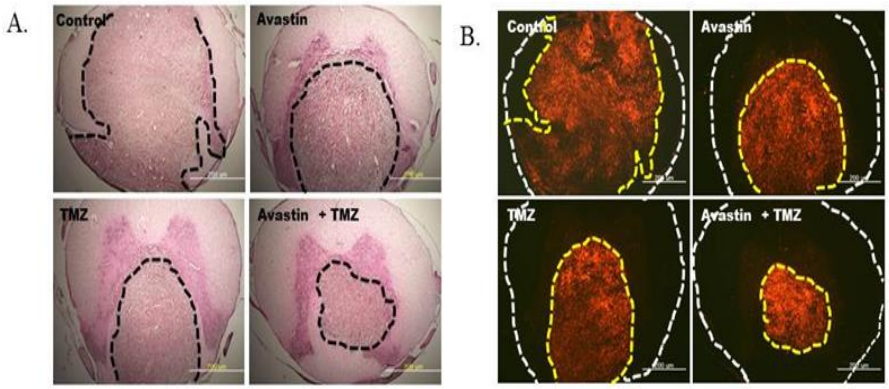

Figure 6. Histological analysis of IMSCTs. (A) H&E staining of IMSCT sections at 8 days treatment. The dotted lines indicate the IMSCT area. (B) Photomicrographs of DsRed-expressing C6 glioma (red) in the spinal cord at 8days. The white dotted lines indicatd the spinal cord, and the yellow dotted lines indicate the DsRed-expressing cells. The area of DsRed-expressing cells was smaller in the combined treatment group than in the other groups.

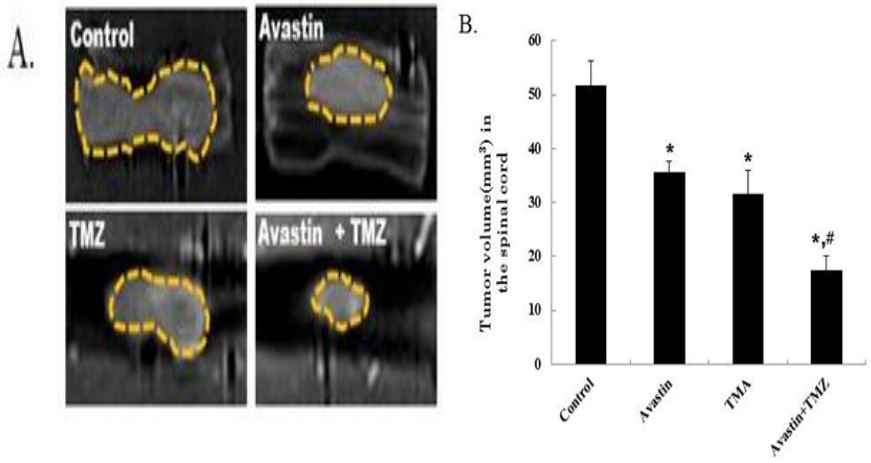

Figure 7. MRI analysis of IMSCTs. (A) MRI analysis of IMSCTs. (B) Tumor volume in control, bevacizumab, TMZ and combined bevacizumab and TMZ. Spinal cord tumor volumes were calculated based on MRI results. *$p<0.05$ compared to control group; $^{\#}p<0.05$ compared to bevacizumab or TMZ group.

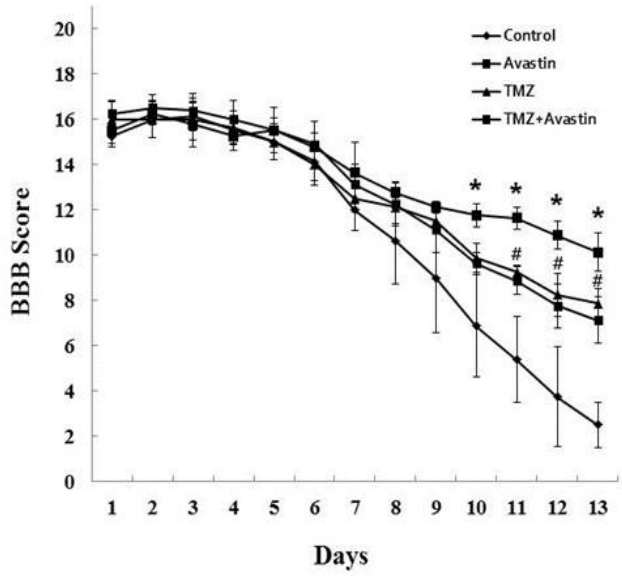

Figure 8. Functional test of IMSCT animals. Hind-limb function was examined every day for 13 days after inoculation using the Basso–Beattie–Bresnahan (BBB) score. *$p<0.01$ compared to control, bevacizumab and TMZ groups; $^{\#}p<0.01$ compared to control group.

IV. DISCUSSION

Despite aggressive treatments, there has been only slight improvement in the prognosis of intramedullary malignant glioma using current standard therapeutic approaches including operation, radiation therapy, chemotherapy, and immunotherapy. [20,21] This study showed that combined therapy with TMZ and bevacizumab increased apoptosis-positive C6 glioma cells and decreased growth of spinal cord tumors. Also, combined treatment with bevacizumab with TMZ reduced a rapid decline of hind-limb BBB scores.

Treatment with bevacizumab and TMZ inhibited the growth of IMSCTs compared to treatment with TMZ alone. Intramedullary malignant gliomas are non-metastasizing, locally infiltrating, and hypervascularized tumors.[22-24] Despite important advances in surgery, radiation therapy and chemotherapy, patients with malignant gliomas continue to have poor prognosis and survival rate.[25,26] TMZ is an imidazotetrazine derivative and the most commonly used chemotherapy agent for malignant gliomas. Many other studies have reported the ability of TMZ to increase survival of animals with tumors.[13] Its effect has also been demonstrated in several clinical trials.[9] TMZ reduced the volume of tumor mass, and this effect was accompanied by a reduction in tumor cell proliferation. Although TMZ improves tumor patient survival and quality of life, it does not cure spinal cord malignant glioma. The present study reveals that combined treatment with bevacizumab and TMZ decreased tumor growth in the spinal cord. Bevacizumab is a recombinant monoclonal antibody, targeting the VEGF, which prevents the ligand from binding to the VEGF

receptor, thereby inhibiting angiogenesis.[27,28]. The anti-cancer effects of bevacizumab have been shown in several studies, which describe a reduction the rate of tumor growth by inhibition of host angiogenesis.[27-30] The few clinical trials undertaken to treat pancreatic neuroendocrine tumors have employed combined bevacizumab and TMZ therapy.[14] In the present study, the combined administration of bevacizumab and TMZ inhibited tumor growth to a greater extent than treatment with bevacizumab or TMZ alone.

In this study, the combined treatment of bevacizumab and TMZ had a greater effect on the apoptosis of C6 glioma cells than TMZ therapy alone. Conventional chemotherapeutic agents for CNS tumors have had poor efficacy, because of difficult infiltrating the blood-brain barrier.[7,8] TMZ is a small, orally available drug that infiltrates the blood-brain barrier due to its lipophilic properties.[9] TMZ is a methylating and alkylating agent of DNA used in the therapy of malignant gliomas. Bevacizumab also reduces the rate of tumor growth by inhibition of host angiogenesis.[29] Bevacizumab is routinely used in combination with chemotherapy for the treatment of gliomas.[31,32] These combined anticancer therapies stimulate autophagy of tumor cells, which enhances apoptosis of cancer cells.[33-35] In the present study, the greater cell apoptosis after the combined treatments may be due to the inhibition of autophagy. TMZ therapy stimulates the generation of reactive oxygen species and extracellular signal-regulated kinase activation, which consequently leads to inhibition of autophagy in glioma cells.[36] Inhibition of autophagy by TMZ induces apoptosis of glioma cells and promotes the

anticancer effects of bevacizumab, such as inhibition of angiogenesis and induction of cell apoptosis.[30,37,38]

The present study demonstrates that a combination of bevacizumab and TMZ maintained better walking function of the hind limbs and reduced tumor mass than TMZ treatment alone. (Figure 6, 7, 8) These results may be due to inhibited host angiogenesis by bevacizumab, which suppresses the provision of nutrients and oxygen to the tumor.[39] In addition, anti-angiogenesis increases tumor hypoxia.[39,40] As a result, bevacizumab and TMZ combination therapy decreases tumor mass and maintains survival of neurons.

Further studies are necessary to assess the clinical utility of this approach. Combined bevacizumab and TMZ therapy needs to promote animal survival for the long-term study of spinal cord tumors. Another limitation of this study for clinical application is the use of C6 glioma cells, which are a non-human cell line. This may result in imperfect therapeutic effects of bevacizumab or TMZ on spinal cord tumor. Thus, the same experiments should be repeated using a human tumor cell line. Finally, as the present study used a small-animal model, the experiments should be repeated in a large-animal model.

V. CONCLUSION

The combined treatment of bevacizumab and TMZ had a greater effect on the apoptosis of C6 glioma cells than TMZ therapy alone. The combination of bevacizumab and TMZ maintained better walking function of the hind limbs and reduced tumor mass than TMZ treatment alone. Histological analyses and MRI findings showed that combined treatment with TMZ and bevacizumab reduced tumor mass. In this study, bevacizumab, which is an anti-angiogenic drug with a different mechanism of action, could be useful in combination with temozolomide to increase therapeutic benefit in intramedullary spinal cord tumor model.

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ABSTRACT (IN KOREAN)

척수 신경교종 치료를 위한 테모졸로마이드 (temozolomide)와 베바시쭈맵 (bevacizumab) 병행치료의 효과

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양 문 술

연구 설계: 현재 악성 척수 신경교종 치료를 위한 표준 치료법으로 수술요법, 화학요법 및 방사선요법 등이 있다. 최근에는 새롭게 악성 척수 신경교종의 치료제로 temozolomide (TMZ)를 병용하거나 보조치료제로 사용하는 새로운 치료법이 검토되고 있다. TMZ는 척수 종양 환자를 완치할 수는 없지만 환자의 생존율을 높이고 삶의 질을 향상 시킨다.

목적: 본 연구에서는 척수 신경교종 동물 모델에서 TMZ를 단독 치료제로 사용한 경우보다 bevacizumab을 병용 치료약물로 투여함으로써 치료효과를 촉진시킨다는 가설을 세웠다.

방법: 신경 교종세포 C6를 척수 신경절의 T5부위에 이식하고 TMZ와 Bevacizumab은 C6 이식 후 5일째에 투여 했다. 접종 후 13일째에 종양크기를 조직학적 분석방법 및 자기공명영상 (MRI) 을

이용하여 분석했다.

결과: 조직학적 분석 및 MRI 소견상 TMZ 와 bevacizumab을 복합투여 한 경우 종양크기가 줄어드는 것을 볼 수 있다. 신경학적 결과에서 TMZ를 단독으로 처리한 그룹 또는 대조군 보다 복합 투여한 경우 뒷다리 운동기능개선에 효과적임을 보여 준다.

결론 : 본 연구의 결론은 척수 종양치료에 TMZ의 단독투여보다 bevacizumab의 병행치료가 더 효과적이다.

핵심되는 말 : 베바시쭈맵, 테모졸로마이드, 골수 내 척수종양