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Laboratory Investigation

Inhibitory Effect of IFN-β, on the Antitumor Activity of Celecoxib in U87 Glioma Model

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Objective : Interferon-β, (IFN-β) has been used in the treatment of cancers. Inhibition of the enzyme cyclooxygenase (COX) with celecoxib had a significantly suppressive effect on tumor growth, angiogenesis, and metastasis in a variety of tumors. The aim of this study was to elucidate the antiglioma effect of combined treatment with IFN-β and celecoxib in U87 glioma model.

Methods : The *in vitro* effects of IFN-β (50-1,000 IU/mL) and celecoxib (50-250 μM) alone or combination of both on the proliferation and apoptosis of U87 cells were tested using MTT assay, FACS analysis and DNA condensation. To determine the i*n vivo* effect, nude mice bearing intracerebral U87 xenograft inoculation were treated with IFN-β intraperitoneally (2×10^5 IU/day for 15 days), celecoxib orally (5, 10 mg/kg) or their combination.

Results : IFN-β or celecoxib showed an inhibitory effect on the proliferation of U87 cells. When U87 cells were treated with IFN-β and celecoxib combination, it seemed that IFN-β interrupted the antiproliferative and apoptotic activity of celecoxib. No additive effect was observed on the survival of the tumor bearing mice by the combination of IFN-β and celecoxib.

Conclusion : These results suggest that IFN-β seems to inhibit the antiglioma effect of celecoxib, therefore combination of IFN-β and celecoxib may be undesirable in the treatment of glioma.

KEY WORDS : Celecoxib · Cyclooxygenase-2 inhibitor · Glioma · Interferon-beta.

INTRODUCTION

Malignant gliomas, the most common primary brain tumor, are very invasive, proliferative and angiogenic. These are very aggressive tumors with a dismal prognosis despite advances in surgery, radiation therapy and chemotherapy[12], so further development of more effective therapy is urgent.

Interferons (IFNs), a family of natural glycoproteins that consist of IFN-α, -β and -γ are known to have pleiotropic effects on tumor growth, angiogenesis and immune system[6,7]. IFN-β induces suppression of matrix metalloproteinase-9 (MMP-9) via signal transducers and activators of transcription 1 (STAT1) activation[15], evokes apoptosis via caspase and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)[10], and activates T lymphocytes, natural killer cells, and macrophages in human glioma implanted nude mouse model[13,19,28]. However, their antitumor effect was known to be only marginal in glioma patients[3,24], and combination therapy is thought to be one of the strategies to improve their antitumor effect[4,20]. We previously reported that combined therapy of IFN-β and temozolomide (TMZ) enhanced antitumor effect in murine glioma model[21].

Cyclooxygenase-2 (COX-2) is commonly overexpressed in both rodent and human tumors and is an important determinant of tumor behavior[26,27]. Its level of expression in tumors correlates with prognosis[5,9,23,25]. Interruption of COX-2 pathway, therefore, might suppress the tumor growth and improve the prognosis of patients[9]. In a number of different types of cells and tumor xenografted animal models, induction of COX-2 has been shown to promote cell growth, inhibit apoptosis, and enhance cell motility and adhesion; however, the mechanisms of these activities

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are largely unknown[2]. Celecoxib, a COX-2 inhibitor, was known to impair human glioma cell growth and metastasis of cancer cells *in vitro*[9,29] and showed antitumor effects in rat glioma model[18]. Presently, celecoxib is being tested in clinical trials for its therapeutic activity against various cancers as a single agent and also in combination with other agents. COX-2 inhibitors were known to impair malignant glioma-derived cell line growth *in vitro* and *in vivo*[2,11]. Here, we examined the *in vitro* and *in vivo* antitumor activity of treatment combined with IFN-β and celecoxib in U87 glioma model.

MATERIALS AND METHODS

Animals and cell lines

Six to eight-week-old athymic nude (*nu/nu*) mice (BALB/*nu*-c, Shizuoka, Japan) were housed in laminar-flow cabinets under specific-pathogen-free conditions. Human glioma cells (U87; American Type Culture Collection, Rockville, MD, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL Co., Grand Island, NY, USA) containing 5% fetal bovine serum (FBS, Gibco BRL Co.).

Glioma cell proliferation assay

MTT assay was used to examine cell proliferation. Briefly, cells (U87 : 4×10^3 cells/well) were suspended with 200 μL media and plated on 96-well cell culture plates. After 24 h, the cells were treated with control media, 50-800 IU/mL recombinant human IFN-β (Schering AG, Berlin, Germany) or 50-250 μM celecoxib (Searle Korea Ltd., Korea) for 3 days to monitor dose-response. The cells were also treated with control media, 50 IU/mL, 100 IU/mL, 200 IU/mL, 400 IU/mL, 800 IU/mL, 1000 IU/mL IFN-β and 25 μM, 50 μM, 100 μM, 200 μM, 400 μM celecoxib or each combination for 1-3 days to examine the effects of the combinations. The cells were incubated with MTT solution (Sigma Chemical CO., Steinheim, Germany) and then the optical density was measured at 550 nm using an ELISA reader (Molecular Devices, San Francisco, CA, USA).

Cell apoptosis assay

U87 cells were treated with IFN-β (50 IU/mL), celecoxib (25 μM) or combination for 72 h. Then U87 cells were washed with phosphate buffer solution (PBS). Apoptosis of U87 cells was quantified using the annexin V-PE apoptosis Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. The cells were incubated at room temperature for 15 min in the dark room. Afterwards, cells were analyzed by FACScan (Becton-Dickinson, Franklin Lakes, NJ, USA) and the results were processed using CellQuest software (Becton-Dickinson). To examine the apoptotic cells by fluorescence microscopy, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, stained by Hoechst 33258 (2 μg/mL) for 1 h, and observed by fluorescence microscopy (Axiovert200, Zeiss, Germany).

Animal experiment

The animals were anesthetized by intraperitoneal (i.p.) injection of xylazine (Rompun; Cutter Laboratories, Shawnee, KS, USA) 12 mg/kg and ketamine (Ketalar; Parke-Davis & Co., Morris Plains, NJ, USA) 30 mg/kg. The mice were held in a stereotactic frame with ear bars. A brain tumor animal model was prepared as described previously[21]. U87 cells (2×10^5) in a volume of 3 μL PBS was injected slowly into the brain of athymic nude mice with a Hamilton syringe. Mice bearing glioma were randomly divided into 4 groups (n = 5 in each group). Mice of each group were treated with i.p. injection of PBS (control group), IFN-β (2×10^5 IU/day) 4 days after the tumor inoculation for 15 days, celecoxib (50 mg/kg) from day 4 for 27 days, or IFN-β and celecoxib combination (IFN-β2×10^5 IU/day from day 4 for 15 days + celecoxib 50 mg/kg from day 4 for 27 days), respectively. Survival and changes of neurologic signs and weight were recorded.

Statistical analysis

Statistical analysis was carried out using the Student's t-test (two-tailed); survival curves and mean values were generated by the Kaplan-Meier product-limit estimate. All data measurements were reported as the mean ± SE. $p < 0.05$ is statistically significant.

RESULTS

Anti-proliferative effect of IFN-β, celecoxib, and their combination

IFN-β and celecoxib showed antiproliferative activity on U87 human glioma cells in a dose-dependent manner (Fig. 1A, B). From these results, IFN-β of 50 IU/mL and celecoxib 25 μM which were the lower concentrations than each IC_{50} were chosen for the following *in vitro* combination experiments. As shown in Fig. 1C, the combination of IFN-β and celecoxib also had an antiproliferative effects on U87 cells. However, celecoxib alone had higher antiproliferative effects than that of combined IFN-β and celecoxib.

We investigated whether antiproliferative effect of celecoxib might be affected by addition of low-dose (50 IU/mL) or high-dose (1,000 IU/mL) of IFN-β. As shown in Fig. 2, exposure of cells to IFN-β 50 IU, 1,000 IU (28%, 34%) or celecoxib (84%) alone for 72 h inhibited cell proliferation.

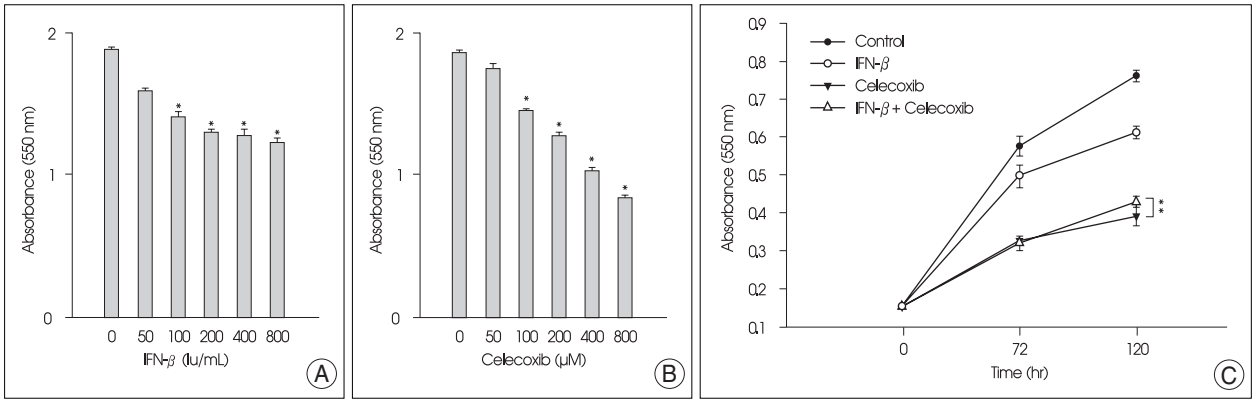

Fig. 1. Antiproliferative effect of IFN-β and celecoxib. U87 cells (Human glioma cell line) were treated with IFN-β and/or celecoxib. Cell viability was determined by MTT assay. A : IFN-β antiproliferative effect in a dose dependent manner at 72 h (*p < 0.01). B : Celecoxib antiproliferative effect in a dose dependent manner at 72 h (*p < 0.01). C : Combination therapy of IFN-β and celecoxib at 120 h (**p < 0.05).

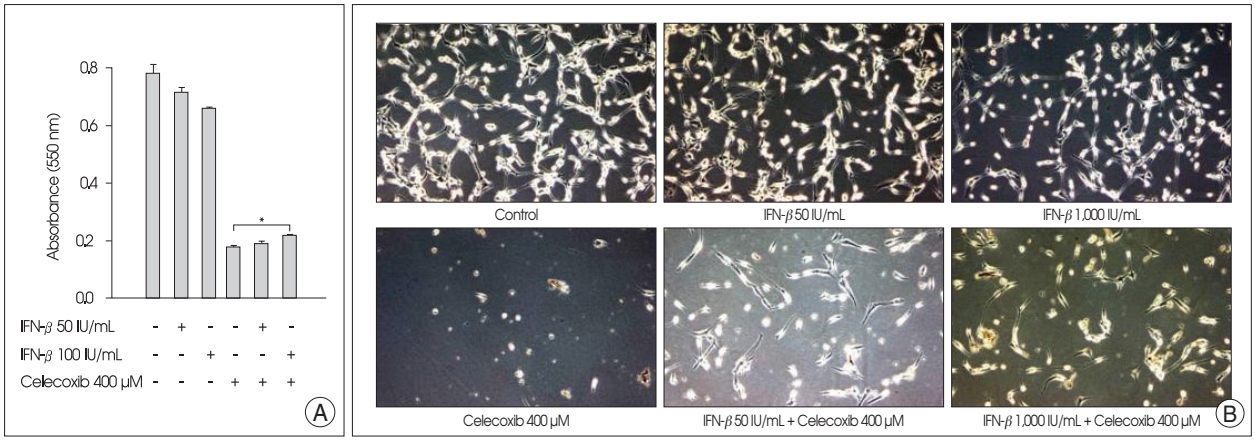

Fig. 2. Variation of cell viability by dose-dependent IFN-β. A : U87 cells were treated with IFN-β (50, 1,000 IU/mL), celecoxib (400 μM) or IFN-β and celecoxib for 72 h (*p < 0.002). Cell growth inhibition was assessed by the MTT assay. B : Cells are viewed by phase-contrast microscopy at 100×.

Antiproliferative effect of IFN-β at 50 IU/mL plus celecoxib (79%) or IFN-β at 1,000 IU/mL plus celecoxib (76%) was lower than that of celecoxib alone (84%).

Apoptotic activity of IFN-β, celecoxib, and their combination

Apoptosis induced by IFN-β and celecoxib was evaluated with FACS assay and Hoechst 33258 staining. FACS analysis showed that apoptosis rate by IFN-β and celecoxib combination (20.95%) was lower than that by celecoxib alone (28.46%) (Fig. 3A). Also as shown in Fig. 3B, the induction rate of DNA condensation by combination was lower than that by celecoxib alone. These results suggest that the percentage of apoptotic cells induced by IFN-β and celecoxib combination treatment is lower than that of celecoxib alone treatment.

Survival of tumor-bearing animals treated with IFN-β, celecoxib, and their combination

Control mice that received i.p. injections of PBS died on 30-39 days after tumor implantation. Animals treated with either IFN-β (2×10^5 IU/day) or celecoxib (50 mg/kg) alone survived for 34-40 days ($p < 0.469$ vs. control) and 38-46 days ($p < 0.016$ vs. control), respectively. Mice treated with IFN-β and celecoxib combination survived for 30-42 days ($p < 0.324$ vs. control) (Fig. 4). These results suggest that IFN-β might decrease the antitumor effect of celecoxib in the brain tumor-bearing mice.

DISCUSSION

Malignant gliomas are very aggressive tumors with a poor prognosis despite the multimodality therapy including surgery, radiotherapy and chemotherapy[12], so further development of more effective therapy by combined use of various molecules such as chemotherapeutic, antiangiogenic, and immune modulating agents has been one of the important issues in neuro-oncology field[16,22]. We previously reported that an antiglioma effect could be potentiated by the combination of TMZ and IFN-β both *in vitro* and *in*

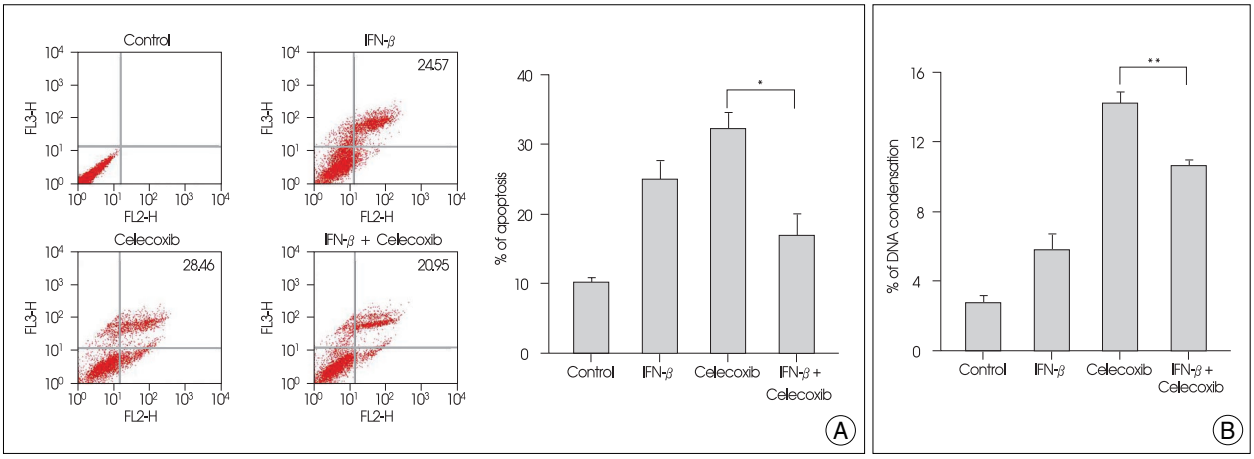

Fig. 3. Apoptosis rate of IFN-β and celecoxib combination therapy. A : U87 cells were treated with IFN-β (50 IU/mL), celecoxib (25 μM) or IFN-β and celecoxib combination for 72 h. Apoptosis of U87 cells were analyzed by FACS after staining with Annexin V-PE and 7-AAD. IFN-β and celecoxib induced apoptosis by 24.57% and 28.46%, respectively. The combined treatment decreased the number of apoptotic cells (20.95%) (*p < 0.05). B : Morphological changes of DNA condensation of the nucleus using Fluorescence microscopy dye, Hoechst33258, in U87 cells at 72 h after treatment with IFN-β and celecoxib (**p < 0.05).

vivo[21]. We expected a potential synergistic effect of TMZ, IFN-β and celecoxib combination, but no synergistic effect of combination in U87 glioma model were observed (data not shown). Here, our interests focused on the combination effect of IFN-β and celecoxib.

IFN has been reported to improve its antitumor effect by combined use with non-steroidal anti-inflammatory drugs (NSAIDs). Lee et al.[14] demonstrated an increased antiproliferative effect by the combination of IFN-α (1,000 IU/mL) and celecoxib (10-50 μM/mL) or curcumin (10-50 μM/mL) in non-small cell lung cancer (NSCLC). Nakamoto et al.[17] showed an enhanced antitumor effect by the combined use of IFN-β (5×10^4 IU, 3/week) and NSAID, NS-398 (15 mg/kg, every day) in nude mice bearing hepatocellular carcinoma (HCC). In the present study, IFN-β and celecoxib demonstrated an antiproliferative activity on U87 human glioma cells in a dose-dependent manner, respectively. However, the antitumor effect was not enhanced by their combination, and even IFN-β appeared to inhibit the antiglioma effect of celecoxib.

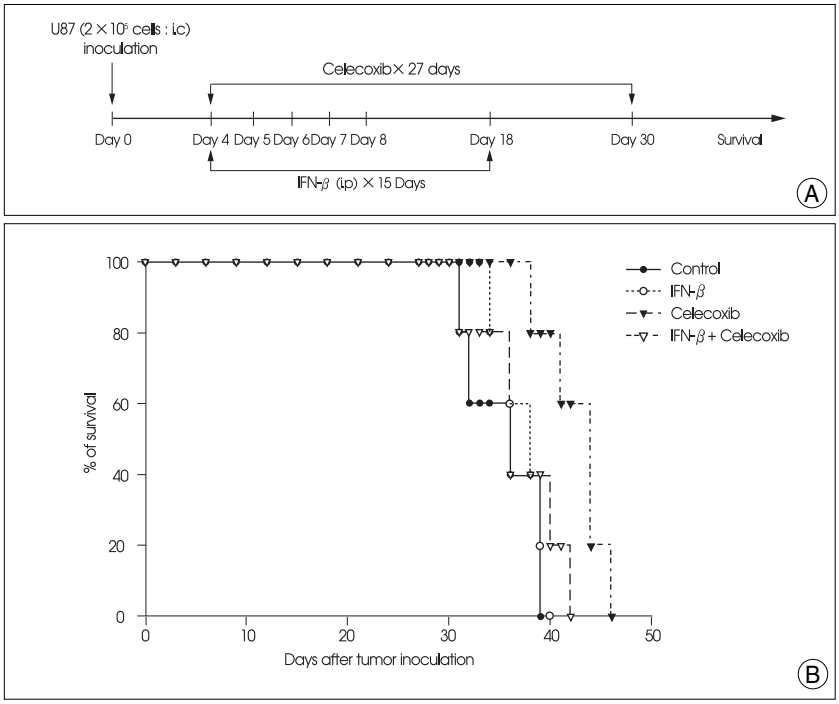

Fig. 4. Survival of U87 brain tumor-bearing mice with IFN-β and celecoxib combination therapy. A : The experimental design of survival study. Mice were intraperitoneally injected with IFN-β (2×10^5 IU/day) from 4 to 18 days and orally injected with celecoxib (50 mg/kg/day) from day 4 until the first mouse in control group was dead. B : Survival of tumor-bearing mice treated with IFN-β and celecoxib.

Antitumor effect of IFN was known to be related to the dose of IFN. Huang et al.[8] evaluated the antitumor effect of IFN-α-2b at different doses (3.5×10^4 IU/week, 7×10^4 IU/week, or 35×10^4 IU/week) in nude mice bearing human prostate cancer and found a variable antitumor effect depending on the doses of IFN-α-2b, most potent at 7×10^4 IU and less potent at 35×10^4 IU. Cao et al.[1] also reported a similar result in nude mice bearing HCC after treatment with several doses of IFN-α-2b (1×10^4 IU/d, 2×10^4 IU/d, 4×104 IU/d). In our experiment, IFN-β (50 IU/mL-1,000 IU/mL) decreased the proliferative activity of U87 cells in a dose-dependent manner. However, when IFN-β was used in combination with celecoxib, antiproliferative activity of celecoxib on U87 cells decreased remar-

kably at 1,000 IU/mL than at 50 IU/mL. *In vivo* antiglioma effect of celecoxib was also decreased by addition of IFN-β at 2×10^5 IU/day. These suggest that dose optimization might be a very important factor in the development of IFN-based antiglioma therapy in a single or combination strategy.

The mechanism underlying the inhibitory effect of IFN-β on antiglioma activity of celecoxib is unclear. It has been reported that IFN-α/β up-regulates the amount of COX-2 protein and mRNA levels via STAT activation in human hepatoma cells or NSCLC, A549 cells[1,14,17]. The relationship between maximal tolerated doses of IFN-β and the induction of down-regulation of STAT1 signaling has not yet been fully elucidated. In our study, inhibitory effect of IFN-β on the apoptotic activity of celecoxib was demonstrated in U87 cells. Further study of mechanism about inhibitory effect would be needed.

CONCLUSION

We showed that either IFN-β and celecoxib has an antiglioma activity in U87 glioma model, but their combination has no additive effect. IFN-β seems to interrupt antiglioma activity of celecoxib, and this mechanism of actions is somewhat complicated to overcome a development of a new antiglioma therapy by their combination. Therefore, further study is needed to find a more effective combination strategy for IFN-based glioma therapy.

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References

1. Cao B, Chen XP, Zhu P, Ding L, Guan J, Shi ZL : Inhibitory effect of interferon-α-2b on expression of cyclooxygenase-2 and vascular endothelial growth factor in human hepatocellular carcinoma inoculated in nude mice. **World J Gastroenterol 14** : 6802-6807, 2008
2. Cao Y, Prescott SM : Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. **J Cell Physiol 190** : 279-286, 2002
3. Chan JL, Lee SW, Fraass BA, Normolle DP, Greenberg HS, Junck LR, et al. : Survival and failure patterns of high-grade gliomas after three-dimensional conformal radiotherapy. **J Clin Oncol 20** : 1635-1642, 2002
4. Chiu HW, Ho SY, Guo HR, Wang YJ : Combination treatment with arsenic trioxide and irradiation enhances autophagic effects in U118-MG cells through increased mitotic arrest and regulation of PI3K/Akt and ERK1/2 signaling pathways. **Autophagy 5** : 472-483, 2009
5. Deininger MH, Meyermann R, Trautmann K, Morgalla M, Duffner F, Grote EH, et al. : Cyclooxygenase (COX)-1 expressing macrophages/microglial cells and COX-2 expressing astrocytes accumulate during oligodendroglioma progression. **Brain Res 885** : 111-116, 2000
6. Dong Z, Greene G, Pettaway C, Dinney CP, Eue I, Lu W, et al. : Suppression of angiogenesis, tumorigenicity, and metastasis by human prostate cancer cells engineered to produce interferon-beta. **Cancer Res 59** : 872-879, 1999
7. Hong YK, Chung DS, Joe YA, Yang YJ, Kim KM, Park YS, et al. : Efficient inhibition of in vivo human malignant glioma growth and angiogenesis by interferon-beta treatment at early stage of tumor development. **Clin Cancer Res 6** : 3354-3360, 2000
8. Huang SF, Kim SJ, Lee AT, Karashima T, Bucana C, Kedar D, et al. : Inhibition of growth and metastasis of orthotopic human prostate cancer in athymic mice by combination therapy with pegylated interferon-alpha-2b and docetaxel. **Cancer Res 62** : 5720-5726, 2002
9. Joki T, Heese O, Nikas DC, Bello L, Zhang J, Kraeft SK, et al. : Expression of cyclooxygenase 2 (COX-2) in human glioma and in vitro inhibition by a specific COX-2 inhibitor, NS-398. **Cancer Res 60** : 4926-4931, 2000
10. Juang SH, Wei SJ, Hung YM, Hsu CY, Yang DM, Liu KJ, et al. : IFN-beta induces caspase-mediated apoptosis by disrupting mitochondria in human advanced stage colon cancer cell lines. **J Interferon Cytokine Res 24** : 231-243, 2004
11. Kardosh A, Blumenthal M, Wang WJ, Chen TC, Schönthal AH : Differential effects of selective COX-2 inhibitors on cell cycle regulation and proliferation of glioblastoma cell lines. **Cancer Biol Ther 3** : 55-62, 2004
12. Kornblith PK, Welch WC, Bradley MK : The future of therapy for glioblastoma. **Surg Neurol 39** : 538-543, 1993
13. Krown SE : Interferons in malignancy : biological products or biological response modifiers? **J Natl Cancer Inst 80** : 306-309, 1998.
14. Lee J, Jung HH, Im YH, Kim JH, Park JO, Kim K, et al. : Interferon-alpha resistance can be reversed by inhibition of IFN-alpha-induced COX-2 expression potentially via STAT1 activation in A549 cells. **Oncol Rep 15** : 1541-1549, 2006
15. Ma Z, Qin H, Benveniste EN : Transcriptional suppression of matrix metalloproteinase-9 gene expression by IFN-gamma and IFN-beta : critical role of STAT-1alpha. **J Immunol 167** : 5150-5159, 2001
16. Mathieu V, De Nève N, Le Mercier M, Dewelle J, Gaussin JF, Dehoux M, et al. : Combining bevacizumab with temozolomide increases the antitumor efficacy of temozolomide in a human glioblastoma orthotopic xenograft model. **Neoplasia 10** : 1383-1392, 2008
17. Nakamoto N, Higuchi H, Kanamori H, Kurita S, Tada S, Takaishi H, et al. : Cyclooxygenase-2 inhibitor and interferon-beta synergistically induce apoptosis in human hepatoma cells in vitro and in vivo. **Int J Oncol 29** : 625-635, 2006
18. Nam DH, Park K, Park C, Im YH, Kim MH, Lee S, et al. : Intracranial inhibition of glioma cell growth by cyclooxygenase-2 inhibitor celecoxib. **Oncol Rep 11** : 263-268, 2004
19. Natsume A, Mizuno M, Ryuke Y, Yoshida J : Antitumor effect and cellular immunity activation by murine interferon-beta gene transfer against intracerebral glioma in mouse. **Gene Ther 6** : 1626-1633, 1999
20. Ohno M, Natsume A, Fujii M, Ito M, Wakabayashi T : Interferon-beta, MCNU, and conventional radiotherapy for pediatric patients with brainstem glioma. **Pediatr Blood Cancer 53** : 37-41, 2009
21. Park JA, Joe YA, Kim TG, Hong YK : Potentiation of antiglioma effect with combined temozolomide and interferon-beta. **Oncol Rep 16** : 1253-1260, 2006
22. Peres E, Wood GW, Poulik J, Baynes R, Sood S, Abidi MH, et al. : High-dose chemotherapy and adoptive immunotherapy in the treatment of recurrent pediatric brain tumors. **Neuropediatrics 39** : 151-156, 2008
23. Prayson RA, Castilla EA, Vogelbaum MA, Barnett GH : Cyclooxygenase-2 (COX-2) expression by immunohistochemistry in glioblastoma multiforme. **Ann Diagn Pathol 6** : 148-153, 2002
24. Ruggiero A, Cefalo G, Garré ML, Massimino M, Colosimo C, Attinà G, et al. : Phase II trial of temozolomide in children with recurrent

high-grade glioma. **J Neurooncol** 77 : 89-94, 2006
25. Shono T, Tofilon PJ, Bruner JM, Owolabi O, Lang FF : Cyclooxygenase-2 expression in human gliomas : prognostic significance and molecular correlations. **Cancer Res 61** : 4375-4381, 2001
26. Taketo MM : Cyclooxygenase-2 inhibitors in tumorigenesis (Part I). **J Natl Cancer Inst 90** : 1529-1536, 1998
27. Taketo MM : Cyclooxygenase-2 inhibitors in tumorigenesis (Part II). **J Natl Cancer Inst 90** : 1609-1620, 1998
28. Thomas H, Balkwill FR : Effects of interferons and other cytokines on tumors in animals : a review. **Pharmacol Ther 52** : 307-330, 1991
29. Wei D, Wang L, He Y, Xiong HQ, Abbruzzese JL, Xie K : Celecoxib inhibits vascular endothelial growth factor expression in and reduces angiogenesis and metastasis of human pancreatic cancer via suppression of Sp1 transcription factor activity. **Cancer Res 64** : 2030-2038, 2004