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 in vivo effect, nude mice bearing intracerebral U87 xenograft inoculation were treated with IFN-β intraperitoneally (2 × 10⁵ 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, 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. IFN-β induces suppression of matrix metalloproteinase-9 (MMP-9) via signal transducers and activators of transcription 1 (STAT1) activation, evokes apoptosis via caspase and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and activates T lymphocytes, natural killer cells, and macrophages in human glioma implanted nude mouse model. However, their antitumor effect was known to be only marginal in glioma patients, and combination therapy is thought to be one of the strategies to improve their antitumor effect. We previously reported that combined therapy of IFN-β and temozolomide (TMZ) enhanced antitumor effect in murine glioma model.

Cyclooxygenase-2 (COX-2) is commonly overexpressed in both rodent and human tumors and is an important determinant of tumor behavior. Its level of expression in tumors correlates with prognosis. Interruption of COX-2 pathway, therefore, might suppress the tumor growth and improve the prognosis of patients. 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...
are largely unknown. Celecoxib, a COX-2 inhibitor, was known to impair human glioma cell growth and metastasis of cancer cells in vitro and in vivo, and showed antitumor effects in rat glioma model. 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. 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 × 10^4 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 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. 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 × 10^4 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 × 10^4 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 IC50 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.
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 × 10^7 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, 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. We previously reported that an antiglioma effect could be potentiated by the combination of TMZ and IFN-β both in vitro and in vivo.
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. 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. 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.

Antitumor effect of IFN was known to be related to the dose of IFN. Huang et al. 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. 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 × 10^4 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-

**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).

**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.
likely at 1,000 IU/mL than at 50 IU/mL. In vivo antglioma 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 antglioma therapy in a single or combination strategy.

The mechanism underlying the inhibitory effect of IFN-β on antglioma 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. 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 antglioma activity in U87 glioma model, but their combination has no additive effect. IFN-β seems to interrupt antglioma activity of celecoxib, and this mechanism of actions is somewhat complicated to overcome a development of a new antglioma 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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