The Effectiveness of Combination Therapy with Hu IFN- β and ACNU against Malignant Glioma

Hitomi ENOMOTO, Jun YOSHIDA and Naoki KAGEYAMA

Department of Neurosurgery, Nagoya University, School of Medicine, Nagoya

Abstract

Combination therapy with human fibroblast interferon (Hu IFN- β) and 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) was administered against a cell line of human malignant glioma transplanted into nude mice, and the histological changes were examined. The mice were divided into four groups (control, Hu IFN- β , ACNU, and combination therapy). The drugs were administered intraperitoneally.

As in an earlier *in vitro* study,²²⁾ the anti-proliferative effect in the combination therapy group was significantly greater than that in the other groups. In the combination therapy group the histological findings also revealed more degenerative changes, such as a tendency for tumor cells to separate from each other, an increase in multinucleate giant cells, and extensive necrosis.

As an adjunct to surgery, combination therapy with Hu IFN- β and ACNU may be a significantly effective treatment for malignant gliomas.

Key words: interferon, ACNU, brain neoplasms, immunochemotherapy

Introduction

It is difficult to totally remove a malignant glioma because of its infiltrative nature. Therefore, multimodality treatment involving surgery, radiation, and chemotherapy with nitrosourea derivatives has been tried, with some effectiveness.¹⁹⁾ In Japan, ACNU, a water-soluble nitrosourea derivative, is used exclusively, but the mean survival period is reported to be only about 2 years.²¹⁾ Recently, however, IFN, whose anti-tumor activity has been demonstrated in many studies,³⁾ has been applied clinically,¹⁶⁾ and the effectiveness of Hu IFN- β against malignant glioma has been suggested.¹¹⁾

We report the results of a study of combination therapy with ACNU and Hu IFN- β on a cell line of malignant glioma that was transplanted subcutaneously into nude mice. A summary of a separate, *in vitro* study²²⁾ of combination therapy is also presented.

Materials and Methods

I. Materials

We used U-251-SP, a glioma cell line that was established from a human glioma by Ponten *et al.* of the University of Uppsala. It was successfully transplanted into the subcutaneous space of female nude mice in our laboratory. The nude mice, BALB nu/nu Jcl, 8 to 9 weeks of age and weighing 30 to 35 g, were kept in a barrier system and given a diet of sterilized pellets and tap water *ad libitum*.

Hu IFN- β was prepared on diploid human foreskin cells by the use of poly I:poly C and provided by Toray Industries Inc., Tokyo. ACNU was provided by Sankyo Co., Tokyo.

A tumor mass was obtained under sterile conditions from a nude mouse and cut into pieces of about 2 $\times$ 2 mm. A tumor fragment was then inoculated into the subcutaneous space in the abdominal region of a nude mouse using a trocar needle. When the tumors had reached about 6 mm in diameter, approximately 3 weeks later, the mice were randomly separated into four groups.

Received November 15, 1985; Accepted April 3, 1986

Author's present address: H. Enomoto, M.D., Department of Neurosurgery, Chukyo Hospital, Nagoya, Japan.

II. Drug administration

The control group ($n = 5$) was given 0.1 ml of physiologic saline intraperitoneally three times a week for 4 weeks. The Hu IFN- β group ($n = 6$) received Hu IFN- β 50×10^4 IU dissolved in 0.1 ml of distilled water intraperitoneally three times a week for 4 weeks. In the third group ($n = 5$), ACNU, 5 mg/kg dissolved in 0.1 ml of distilled water, was administered intraperitoneally three times a week for 2 weeks. The combination therapy group ($n = 8$) received Hu IFN- β 50×10^4 IU and ACNU 5 mg/kg, each dissolved in 0.1 ml of distilled water, intraperitoneally three times a week.

The subcutaneous tumors were measured in two dimensions twice a week for 5 weeks, and their weights were calculated according to the following formula: Tumor weight (mg) = $1/2$ length (mm) $\times$ width² (mm). The relative tumor weights were calculated according to $T_n/T_o \times 100$ (%), where T_o = tumor weight immediately before drug administration and T_n = tumor weight after drug administration.

For statistical analysis, Student's *t*-test was used to compare tumor weight values in the control and treatment groups.

At the end of the experiment, the tumor masses in each group were removed and fixed in a 10% formalin solution for microscopic examination by the hematoxylin-eosin staining method.

Results

I. Evaluation of anti-tumor effects

No nude mice died as a result of side effects of drugs or miscellaneous causes, and all animals were living at the end of the experiment, with no significant loss in body weight. Table 1 and Fig. 1 show the mean tumor weight ratios for each day in comparison with the values on Day 0, *i.e.*, immediately

Fig. 1 Mean tumor weight ratios during treatment in comparison with values on Day 0, expressed as percents. a): Intraperitoneal injection of 50×10^4 IU/mouse Hu IFN- β , b): Intraperitoneal injection of 5 mg/kg ACNU.

before the administration of drugs. In the control and Hu IFN- β groups, there was a progressive increase in the tumor weight ratio, which was slightly or not at all influenced by the administration of drugs. At the end of the experiment, Day 32, the mean tumor weight was about 9 and 7 times heavier in the control and Hu IFN- β groups, respectively, than the initial tumor weight. In the ACNU group, from Day 13 to Day 18, a decrease in the tumor weight ratio was observed, but thereafter the values progressively increased, finally reaching about 200% of the value on Day 0. In the combination therapy group, from Day 11 to Day 18 there was a gradual decrease and, thereafter, no increase in the tumor weight ratio; this trend persisted for at least 1 week after cessation of drug administration. At the end of the experiment, the mean tumor weight ratio was 36% of the value on Day 0. On Day 11 and thereafter, significant differences in the mean tumor weight ratios were observed between the control and

Table 1 Mean tumor weight ratios during treatment, relative to weights on Day 0

	Day 0	Percentage of value on Day 0								
		4	6	11	13	18	20	25	27	32
Control ($n=5$)	100	144	160	269**	324*	475**	488*	539**	617**	894**
Hu IFN- β ($n=6$)	100	180	203	310***	258**	278***	332***	476***	612**	719***
ACNU ($n=5$)	100	148	205	199***	148***	86	99	155***	162***	205**
Combination ($n=8$)	100	113	124	76	69	40	41	41	41	36

* $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$ versus combination therapy group.

combination therapy groups and between the Hu IFN- β and combination therapy groups. Also, on Day 25 and thereafter, there was a significant difference between the ACNU and combination therapy groups.

II. Histological findings

Control group (Fig. 2A): The tumor consisted exclusively of polyhedral or fusiform cells, which were arranged in a cord-like or reticular pattern and exhibited hypercellularity. There were about five to ten mitotic figures per microscopic field of 100 magnifications. There were also some small necrotic foci with surrounding pseudopalisade patterns.

Hu IFN- β group (Fig. 2B): The arrangement and the shape of tumor cells were, as a rule, similar to those in the control group, although there was greater variability in the size of nuclei. Five to ten mitotic figures were observed per microscopic field of 100 magnifications. In addition, there were more vessels and connective tissues within the tumor and more infiltration by round cells in the periphery

beneath the capsule. Small necrotic foci were also seen in this group.

ACNU group (Fig. 2C): In this group, there was a large necrotic area in the central part of the tumor and, in the periphery, polyhedral cells were arranged irregularly, with five to ten mitotic figures per microscopic field and little or no degenerative change.

Combination therapy group (Fig. 2D): In these tumors there were large necrotic areas in the central part. In the periphery the findings were different from those of the other groups, especially the ACNU group. There were round, irregularly arranged cells, with individual cells tending to be separated. Also, there were more giant and multinucleate cells and fewer mitotic figures.

Discussion

ACNU crosses the blood-brain barrier without difficulty due to its lipid solubility, and its anti-tumor activity depends upon its concentration in tissues. Therefore, it would seem favorable to administer

Fig. 2 Histological sections. HE stain, $\times 200$. A: Transplanted tumor from the control group, showing mitoses (arrows). B: Transplanted tumor from the Hu IFN- β group, revealing mitoses (arrows) and round cell infiltration (R). C: Transplanted tumor from the ACNU group, showing mitoses (arrows) and necrosis (N). D: Transplanted tumor from the combination therapy group, showing a multinucleate giant cell (arrow) and separation of tumor cells (S).

ACNU in as large a dose as possible. However, there is a dosage limitation because of its persistent and cumulative suppression of bone marrow function. Thus, intravenous administration of 2 to 3 mg/kg ACNU is usually advised, with its optimal intratumoral concentration being 5 μ g/g tissue.¹⁰⁾ In one *in vitro* experiment,²²⁾ only two of 13 glioma cell lines demonstrated a cytotoxic effect at more than 2 μ g/ml, and eight cell lines demonstrated a slight cytostatic effect with a 5 μ g/ml concentration of ACNU. Thus, it is thought that ACNU therapy alone is not fully effective against malignant glioma. IFN is well known for its anti-tumor action, the mechanism of which is believed to involve both direct action on tumor cells and indirect action through the host immunological system.¹²⁾ In clinical trials on human glioma, Nagai¹¹⁾ reported its effectiveness in 7 of 42 cases of malignant glioma. In their study of combination therapy with ACNU and IFN, Yoshida *et al.*²²⁾ reported that combination treatment with a 5 μ g/ml concentration of ACNU and a 10³ IU/ml concentration of Hu IFN- β against 13 malignant glioma cell lines resulted in increased cytotoxic or cytostatic effects in all cell lines investigated. Further, alteration of the DNA pattern showed more remarkable and persistent accumulation in the G₁S phase in comparison with treatment using a single drug.

Since it is known that human tumors transplanted into nude mice maintain the histological and biochemical characteristics of the original tumor after transplantation, many studies on the anti-tumor effects of IFN or other drugs, including nitrosoureas, have been reported.^{1,2,4-7,9,13-15,17,18,20)} Suppressive effects on the proliferation of sarcoma 180²⁰⁾ and breast carcinoma¹⁾ have been reported following intraperitoneal injections of Hu IFN- β , 2 to 2.5 $\times$ 10⁴ IU/mouse. In studies with glioma-transplanted nude mice,^{9,13)} daily intratumoral injections of Hu IFN- β 60 $\times$ 10⁴ IU/mouse had a remarkable effect, whereas daily intraperitoneal injections of Hu IFN- β 50 $\times$ 10⁴ IU/mouse produced only temporary suppression.⁹⁾ Other investigators¹³⁾ reported slight diminution following intraperitoneal injections of Hu IFN- β , 500 $\times$ 10⁴ IU/kg. It is generally considered that intratumoral injections of Hu IFN- β result in greater anti-tumor effects than do intraperitoneal injections.^{4,7,18)} In their study using ACNU with breast cancer-transplanted nude mice, Inoue *et al.*⁶⁾ reported the greater effectiveness of an intraperitoneal injection of ACNU, 40 mg/kg, accompanied by a greater decrease in body weight, in comparison with intraperitoneal injections of ACNU of 10 or 20 mg/kg.

There have also been a few reports^{14,15)} concerning

combination treatment of nude mouse tumors with IFN and other chemotherapeutic agents. Nosoh *et al.*,¹⁵⁾ using nude mice with transplanted gastric cancer or melanoma, examined the effect of Hu IFN- β , 5 $\times$ 10⁵ IU/mouse intramuscularly injected, combined with mitomycin C, adriamycin, cyclophosphamide, ACNU, or neothramycin, intraperitoneally or intravenously administered. They reported a significant suppressive effect and fewer side effects in one cell line of gastric cancer subjected to a combination of Hu IFN- β and mitomycin C or adriamycin. Excellent results were also reported¹⁴⁾ when nude mice who received transplants of HeLa cells were treated with a combination of IFN plus 5-fluorouracil.

In our investigation the effect of combination therapy with Hu IFN- β and ACNU against the glioma cell line U-251-SP was evaluated. As shown in Fig. 1, tumor proliferation in the combination therapy group was significantly suppressed on the eleventh day and thereafter, in comparison with the control and Hu IFN- β groups. Combination therapy also produced greater suppression of tumor proliferation than did ACNU treatment alone, although on Days 18 and 20 no significant difference was observed. The drug dosages in this study—5 mg/kg 6 times for ACNU and 50 $\times$ 10⁴ IU/mouse 12 times for Hu IFN- β —are not considered high, and there were few or no side effects.

Histological findings in studies of transplanted tumors suggest that the suppressive effect against tumor proliferation is greater with intratumoral than with intraperitoneal injections.^{4,18)} Some reports^{9,18)} concerning histological changes following intratumoral injections of Hu IFN- β into human glioma have mentioned a decrease in cell components, an infiltration of round cells, increased multinuclear giant cells, increased collagenous fibers within the tumor, scant mitotic figures, and intracellular mucoid degeneration. One group,⁸⁾ reporting on changes following intraperitoneal injection of IFN, noted pyknosis, fibrosis, and a tendency for tumor cells to be separated from each other in one type of osteogenic sarcoma. Other investigators,⁴⁾ who used malignant melanoma, commented on the relative ineffectiveness of intraperitoneal injections of Hu IFN- β compared with intratumoral injections, following which they observed increased connective tissue, cell degeneration, and decreased mitotic figures.

The histological evaluation in our experiment, in which injections were given intraperitoneally, showed that a single treatment with Hu IFN- β caused only a slight increase in connective tissue and

a minimal degree of subcapsular infiltration of round cells. However, with combination treatment, in addition to the changes due to the administration of ACNU, there were degenerative changes in tumor cells, increased multinuclear cells, a tendency for tumor cells to separate, a marked decrease in mitotic figures, and an increase in connective tissue. These changes appear to be similar to those reported with intratumoral injections of Hu IFN- β .

Thus, not only in *in vitro* work²²⁾ but also in *in vivo* studies employing nude mice, histological findings strongly suggest the effectiveness of combination therapy with Hu IFN- β and ACNU against human malignant gliomas. This combination therapy, used together with surgery, may become important in the treatment of malignant glioma.

References

- 1) Balkwill F, Tailor-Paradimitriou J, Fantes KH, Sebesteny A: Human lymphoblastoid interferon can inhibit the growth of human breast cancer xenografts in athymic (nude) mice. *Eur J Cancer* 16: 569-573, 1980
- 2) Fujimoto S, Ogawa M: A new nitrosourea derivative TA-077, 1-(2-chloroethyl)-3-(β -maltosyl)-1-nitrosourea. 1. Comparative study on anti-tumor activity. *Cancer Chemother Pharmacol* 9: 134-139, 1982
- 3) Gresser I, Bourali C: Anti-tumor effects on interferon preparations in mice. *J Natl Cancer Inst* 45: 365-376, 1970
- 4) Hashizume S, Nagao S, Tanaka N, Sekiguchi F, Kohno M, Ogawa H, Yoshino Y, Seki K, Ishihara K: Effects of human fibroblast interferon on human tumors transplanted into nude mice: Sensitivity of malignant melanoma. *Jpn J Exp Med* 53: 77-85, 1983
- 5) Ida N, Uenishi N, Kajita A, Satoh Y: Anti-tumor effects of human fibroblast interferon on the growth of human melanoma cells implanted in nude mice. *Gan* 73: 952-960, 1982
- 6) Inoue K, Fujimoto S, Ogawa M: Comparison of anti-tumor activities of nitrosourea derivatives against mammary breast carcinoma (MX-1) in nude mice. *Gan* 74: 308-316, 1983
- 7) Kohno M, Ida N, Nagao S, Tanaka N, Sekiguchi F, Uenishi N, Kajita A, Ogawa H: Effects of human fibroblast interferon on human tumors transplanted into nude mice: Sensitivity of human tumors to interferon. *Gan* 73: 945-951, 1982
- 8) Masuda S, Fukuma H, Beppu Y: Anti-tumor effects of human leucocyte interferon (HL-IF). *Rinsho Seikei Geka* 14: 131-138, 1979 (in Japanese)
- 9) Miki Y: Anti-neoplastic effect of human fibroblast interferon on human malignant gliomas transplanted into nude mice and its clinical application. *Neurol Med Chir (Tokyo)* 22: 785-796, 1982 (in Japanese)
- 10) Muraoka K: ACNU delivery to malignant brain tumor tissue and serum; route of administration and effects of phenobarbital. *No To Shinkei* 35: 1199-1206, 1983 (in Japanese)
- 11) Nagai M: Clinical trial of human fibroblast interferon (Hu IFN- β) on malignant brain tumors. *J Jpn Soc Cancer Ther* 18: 60-68, 1983
- 12) Nagai M, Arai T: Interferon therapy for malignant brain tumors. Present and future. *No Shinkei Geka* 10: 463-476, 1982 (in Japanese)
- 13) Nakamura O, Teramoto A, Yamamoto H, Ochiai S, Takakura K, Maruo Y, Kamiyama Y, Shimamura K: Anti-neoplastic effects of Hu IFN- β on human malignant brain tumors. *No To Shinkei* 35: 905-911, 1983 (in Japanese)
- 14) Nanba M, Yamamoto S, Miyoshi T, Nobuhara M: Potentiation of cytotoxic effects of anti-cancer drugs by interferon on human neoplastic cells. *Soshiki-baiyo* 10: 2-6, 1984 (in Japanese)
- 15) Nosoh Y, Yamaguchi M, Tani T, Tohge T, Aramoto M, Hattori T: Increased cytotoxic effects of various anticancer drugs by interferon (HLBI) on human tumor xenografts in the nude mice. *Gan To Kagaku Ryoho* 11: 1623-1628, 1984 (in Japanese)
- 16) Strander H: Anti-tumor effect of interferon and its possible use as anti-neoplastic agent in man. *Tex Rep Biol Med* 35: 429-435, 1977
- 17) Suzuki Y, Tanaka R: Anti-tumor effect of ACNU on experimental mouse brain tumors. *Neurol Med Chir (Tokyo)* 20: 405-413, 1980 (in Japanese)
- 18) Tanaka N, Nagao S, Tohgo A, Sekiguchi F, Kohno M, Ogawa H, Matsui T, Matsutani M: Effects of human fibroblast interferon on human gliomas transplanted into nude mice. *Gan* 74: 308-316, 1983
- 19) Wilson CB, Gutin E, Boldrey B: Single agent chemotherapy of brain tumors: A five-year review. *Arch Neurol* 33: 739-744, 1976
- 20) Yokota Y, Kishida T: Anti-tumor effects of interferon on transplanted tumors in congenitally athymic nude mice. *Biken J* 19: 125-127, 1976
- 21) Yoshida J, Kobayashi T, Kageyama N: Multimodality treatment of malignant glioma. Effect of chemotherapy with ACNU and immunotherapy with N-CWS. *Neurol Med Chir (Tokyo)* 24: 19-26, 1984
- 22) Yoshida J, Wakabayashi T, Inoue I, Kageyama N: Combination therapy with Hu IFN- β and ACNU against malignant brain tumors. Part 1. Experimental study in vitro. *Gan To Kagaku Ryoho* 12: 99-104, 1985 (in Japanese)

Address reprint requests to: H. Enomoto, M.D., Department of Neurosurgery, Chukyo Hospital, 1-1-10 Sanjo-cho, Minami-ku, Nagoya 457, Japan.