Superinduction of Cytotoxic Interferon- β in Glioma Cells

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Abstract

The possibility for new interferon therapy was investigated using the effect of endogenous human interferon- β (HuIFN- β) on various culture cell lines. Cell lines were exposed to superinduction agents (poly I: poly C, cycloheximide, and actinomycin D) and the production of endogenous interferon analyzed. Quantitative determination of HuIFN- β and messenger ribonucleic acid (mRNA) showed HuIFN- β was induced in all of five glioma cell lines, one of two melanoma cell lines, and all of three lung carcinoma cell lines as well as fibroblasts. Northern blot analysis showed HuIFN- β mRNA induced in glioma cells was identical to that from fibroblasts. Endogenous HuIFN- β induced from glioma cells had a cytostatic or cytotoxic effect against various human glioma cell lines, even those resistant to fibroblast-derived HuIFN- β . These results show it may be possible to use the induction of excess endogenous cytotoxic HuIFN- β in human glioma tissue itself.

Key words: human interferon- β , superinduction, glioma

Introduction

The extensive research by Gresser *et al.*^{5,6)} and the clinical studies by Strander *et al.*¹⁶⁾ show that interferon has potent antitumor activity against various human neoplasms. β -type interferon has been useful in the treatment of malignant glioma.¹²⁾ Our previous experimental studies^{18,20)} demonstrated that human interferon- β (HuIFN- β) has cytostatic or cytotoxic direct and host-mediated indirect effects against human malignant glioma *in vitro* and *in vivo*.

Generally, HuIFN- β is induced in human skin fibroblast by viral infection and double-strand ribonucleic acids (RNAs) such as poly I: poly C. Vilcek *et al.*^{7,17)} established the superior method of superinduction (SI) which obtains large amounts of HuIFN- β . Larsson *et al.*¹⁰⁾ also demonstrated that SI induces high levels of interferon- β in glia or glioma cell lines.

This study analyzed the SI of HuIFN- β and its

messenger RNA (mRNA) in various cell lines including human glioma and investigated the antitumor activity of this interferon.

Materials and Methods

I. Cell lines and cell culture

Twenty-eight human malignant cell lines including seven human glioma cell lines and one skin fibroblast used in the present study are listed in Table 1. The F-1 cell line was established in our laboratory from a human skin specimen. The origins of other cell lines are described elsewhere.^{4,14,15)}

Cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), streptomycin (100 μ g/ml), penicillin (100 U/ml), 2 mM L-glutamine, and non-essential amino acids. All cultures were incubated in humidified air containing 5% CO₂ at 37°C.

II. SI method

When plating cells became confluent in the flasks, the culture was primed with 100 IU/ml of human

Table 1 List of cell lines

Fibroblast	Lymphoma
F-1	T cell
Neural tumor	CCRF-CEM
glioma	HPB-ALL
SK-MG-1	B cell
SK-MG-4	Daudi
SK-MG-17	Monca
U-251MG	non T, non B
U-251SP	NALL-1
T-98	Carcinoma
MG-178	lung
neuroblastoma	SBC-3
NB-1	SK-MES-1
SK-N-MC	Calu-1
SK-N-SH	pancreas
Melanoma	Paca-2
Levy	Panc-1
Murawski	liver
Teratoma	HepG2
Tera-2	colon
Sarcoma	HT-29
U-20S	cervix
	ME180

fibroblast-derived HuIFN- β (Toray Industries, Inc., Tokyo). After 24 hours, the medium was changed to MEM containing 2% FCS, 5 μ g/ml cycloheximide, and 100 μ g/ml poly I: poly C, and cultured for 5 hours at 37°C. Actinomycin D was added during the last hour to a final concentration of 4 μ g/ml. The culture was washed three times with phosphate buffer saline at 37°C to remove the inducers. The medium was then changed to MEM containing 2% FCS. After incubation for 24 hours, the medium and cells were collected for the quantitative determination of HuIFN- β and mRNA. The control culture was incubated by the same method but not exposed to any SI agents.

III. Quantitative determination of HuIFN- β

HuIFN- β in the medium and intracellular HuIFN- β extracted with 0.5% Nonidet P-40 were measured quantitatively using an enzyme HuIFN- β enzyme immunoassay kit (Fuji Rebio, Inc., Tokyo). This assay uses rabbit anti-HuIFN- β polyclonal antibody and peroxidase-labeled mouse anti-HuIFN- β monoclonal antibody to detect a minimum HuIFN- β amount of one interferon unit (IU)/ml.

Protein concentrations in the medium and the cell extract were determined by a modified Lowry's method,¹¹⁾ and HuIFN- β production measured as IU/mg protein.

IV. Quantitative determination of HuIFN- β mRNA

HuIFN- β gene fragment cloned into plasmid pSV2 (Basic Research Laboratories, Toray Industries, Inc.) was labeled with ³²P dCTP (3000 Ci/mmol) (Du Pont Company New England Nuclear Research Products, Boston, Mass., U.S.A.) by the random primed labeling method.³⁾ The deoxyribonuclei acid (DNA) fragment of 575bp included the whole coding region for HuIFN- β gene,¹³⁾ a fragment coding for mature peptide, and a synthetic fragment coding for the signal peptide of HuIFN- β (personal communication from M. Naruto *et al.*, Basic Research Laboratories, Toray Industries, Inc.).

All intracellular RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method.²⁾ For northern blot analysis, an 20 μ g aliquot of the total RNA was denatured in 50% dimethylsulfoxide, 1 M glyoxal, and 0.01 M phosphate buffer (pH 7.5) at 50°C, followed by electrophoresis in 0.9% agarose gel and transfer to Gene Screen Plus membrane (GSP) (New England Nuclear, Inc.). For dot blot analysis, the total RNA was denatured by the same protocol, serially diluted, and dotted on GSP. The membranes were prehybridized at 42°C for 2 hours and then hybridized with the labeled probe. The hybridization conditions were 50% formamide, 5 $\times$ Denhardt's solution, 5 $\times$ SSPE (0.9 M NaCl, 0.05 M NaH₂PO₄, pH 7.4, 0.005 M ethylenediamine tetra-acetic acid, pH 7.4), 1% sodium dodecyl sulfate (SDS), and 0.1 mg/ml hering sperm DNA at 42°C for 20–24 hours. The hybridized blots were washed in 2 $\times$ SSC (0.3 M NaCl, 0.03 M trisodium citrate) at room temperature for 5 minutes twice, in 2 $\times$ SSC and 1% SDS at 65°C for 30 minutes twice, and then in 0.1 $\times$ SSC at room temperature for 30 minutes twice. After autoradiography at –80°C, the amounts of HuIFN- β mRNA were quantified by image analyzer and corrected by known amounts of RNA.

V. Cytotoxic activity of HuIFN- β derived from U-251MG glioma

Five human glioma cell lines, U-251MG, SK-MG-1, SK-MG-4, MG-178, and T-98, were plated at 1 $\times$ 10⁴ cells per well in 24 well dishes and incubated in MEM containing 10% FCS at 37°C for 24 hours. Then the medium was changed to MEM containing 2% FCS and HuIFN- β superinduced from U-251MG glioma cells at 1000 IU/ml. The controls were culture medium with 2% FCS and medium containing U-251MG cells cultured for 54 hours without any SI agent. For positive cytotoxic control, HuIFN- β from fibroblasts purified using columns conjugated

with anti-HuIFN- β monoclonal antibody (Toray Industries, Inc.) was used at 1000 IU/ml in the culture medium. Surviving cells in each well were counted after trypan blue staining 0, 3, and 6 days after incubation.

Results

I. Quantitative determination of HuIFN- β and mRNA

The northern blot analysis autoradiograph of RNA extracted from fibroblast (F-1) and human glioma cell lines (SK-MG-1 and U-251MG) showed no hybridization to a HuIFN- β DNA probe and the control mediums contained no HuIFN- β . However, a single band was demonstrated at 0.9 kb in all three SI group cell lines (Fig. 1). The band intensities correlated well with the HuIFN- β titer in the culture medium: F-1 (60,900 IU/ml), SK-MG-1 (534 IU/ml), and U-251MG (15,800 IU/ml).

HuIFN- β was superinduced in the culture medium of all glioma cell lines (26,085–145,235 IU/mg protein), one of two melanoma cell lines (283,075 IU/mg protein), and all three lung carcinoma cell lines (21,650–43,860 IU/mg protein). The corresponding expression of HuIFN- β mRNA was also observed by RNA dot blot analysis. However, neither

Fig. 1 Northern blot analysis autoradiograph of HuIFN- β mRNA. The band at 0.9 kb is observed in each lane of the SI group. The band intensities correlate with the HuIFN- β titer in each medium. C: control group, F: fibroblast, S: SK-MG-1 glioma cells, SI: SI group, U: U-251MG glioma cells.

Table 2 HuIFN- β and mRNA induced by SI

	HuIFN- β			mRNA (AU/ μ g RNA*)
	Medium (IU/ml)	Medium (IU/mg protein)	Intracellular (IU/mg protein)	
Glioma				
SK-MG-4	6855	145,235	138	100
U-251MG	4106	26,085	203	16
U-251SP	6178	115,260	41	64
SK-MG-17	1445	×	×	×
Neuroblastoma				
NB-1	—	—	—	—
SK-N-MC	—	—	—	—
SK-N-SH	—	—	—	—
Melanoma				
Levy	—	—	—	—
Murawski	368	283,075	×	1
Teratoma				
Tera-2	—	—	—	—
Sarcoma				
U-20S	—	—	—	—
Lymphoma				
CCRF-CEM	—	—	—	—
HPB-ALL	—	—	—	—
Daudi	—	—	—	—
Monca	—	—	—	—
NALL-1	—	—	—	—
Carcinoma				
SBC-3	345	34,500	—	—
SK-MES-1	394	21,650	—	5
Calu-1	5671	43,860	—	13
Paca-2	—	—	—	—
Panc-1	—	—	—	—
HepG2	—	—	—	—
HT-29	—	—	—	—
ME180	—	—	—	—

*The intensity of each dot was quantified using image analyzer, considering that of SK-MG-4 as 100 arbitrary unit (AU)/ μ g RNA. —: not detected, ×: not measured.

HuIFN- β nor RNA were detected in the other neuroblastoma, sarcoma, teratoma, various lymphoma, and carcinoma cell lines (Table 2).

II. Cytotoxic activity of HuIFN- β derived from U-251MG glioma

Compared to growth in the control medium, all glioma cells (U-251MG, SK-MG-1, SK-MG-4, MG-178, and T-98) were markedly inhibited in culture medium containing 1000 IU/ml HuIFN- β derived from U-251MG cells by SI (Fig. 2). SK-MG-1 and T-98 cells were apparently more severely inhibited than others. The cytotoxicity of the medium containing superinduced HuIFN- β was greater than that containing purified HuIFN- β derived from fibroblasts at the same concentration.

Fig. 2 Cell growth in the medium containing endogenous HuIFN- β derived from U-251MG glioma cells by SI. After plating at 1×10^4 cells/well and 1 day incubation, U-251MG, SK-MG-1, SK-MG-4, MG-178, and T-98 glioma cells were exposed to medium containing 1000 IU/ml HuIFN- β derived from U-251MG glioma cells by SI ($\blacktriangle$), to medium containing fibroblast HuIFN- β at the same concentration ($\circ$), and to control MEM containing 2% FCS ($\bullet$). Marked inhibition of the growth of all glioma cell lines was observed in the culture containing HuIFN- β from U-251MG glioma cells.

Discussion

Human glioma cells may produce HuIFN- β acting as a cytostatic or cytotoxic agent against human glioma cells.¹⁰ In this study, the northern blot analysis autoradiograph of fibroblast and glioma cells treated by SI demonstrated a single band 0.9 kb mRNA which hybridized with radioisotope-labeled HuIFN- β DNA fragment. The size corresponds well with that reported for HuIFN- β mRNA.¹⁹ HuIFN- β was also present in the culture medium. This suggests that SI causes glioma cells to produce HuIFN- β almost identical to that of fibroblast. The assay of HuIFN- β in various cell lines showed that the ability to produce HuIFN- β was restricted. Glioma, melanoma, and lung carcinoma cells produced large amounts of HuIFN- β by SI, and the corresponding expression of HuIFN- β mRNA was also observed. However, neither HuIFN- β nor mRNA were detected in other cell lines of the SI group and all cell lines in the control group. Intracellular HuIFN- β was detected in glioma cells only, not in melanoma or lung carcinoma cells. This may result from the rapid release of HuIFN- β from the cells.

We also demonstrated that the medium containing HuIFN- β derived from U-251MG cell lines by SI markedly inhibited the growth of U-251MG, SK-MG-1, SK-MG-4, MG-178, and T-98 glioma cell lines. The cytotoxic activity was much higher than that of HuIFN- β produced by human fibroblast. This suggests that either HuIFN- β produced by SI in glioma cell lines is different from that in fibroblasts, for example, at the glycosylation site, or glioma cell

lines produce another unidentified cytotoxic factor during SI.

HuIFN- β has two major antitumor activities, a direct effect on the tumor and an indirect effect through stimulation of the immune system. Previously, we showed that HuIFN- β has a direct cytotoxic effect against human glioma cells and also activates natural killer cells in peripheral blood lymphocytes.¹⁸ However, clinical studies showed that the antitumor effect by direct intravenous or local administration of HuIFN- β was inadequate in patients with malignant glioma. This may be due to the instability of HuIFN- β in the blood and inadequate penetration of the blood-brain barrier.^{1,8)}

The present study demonstrated that endogenous HuIFN- β and/or other antitumor factors superinduced in glioma cells had a cytotoxic or cytostatic effect against various human glioma cell lines. Moreover, in comparison with exogenous HuIFN- β , the cytotoxic effect was much stronger even if cells were resistant to fibroblast-derived HuIFN- β .

The clinical use of endogenous HuIFN- β , however, raises many problems, such as the administration of SI agents. Although it has been reported that poly I: poly C can be administered intravenously without serious side effects, a selective method of delivery for poly I: poly C, cycloheximide, and actinomycin D to glioma cells should be developed. We are now planning to use liposomes and monoclonal antibody for this purpose. Previously, we demonstrated that a monoclonal antibody (G-22) for glioma-associated antigen is accumulated into human glioma tissues.²¹ Agents may also be ab-

sorbed selectively in glioma cells by liposome coupled with a monoclonal antibody (G-22).^{9,22)}

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