Special Issue

Enhancement of cytokine expression in transiently transfected cells by magnetoliposome mediated hyperthermia

Isabelle Anne Bouhon, Masashige Shinkai, Hiroyuki Honda & Takeshi Kobayashi

Department of Biotechnology, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-01, Japan

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Abstract

We investigated the enhancement of cytokine expression by heat treatment in transiently transfected glioma cells. The cells were transfected with plasmid bearing the interferon (IFN)- β gene under control of the MMTV promoter, which is inducible by glucocorticoid (dexamethasone). Then magnetite particles (10 nm diameter) as intracellular heating material were incorporated to the cells as the form of magnetoliposome. After 5 hours of incorporation, alternative magnetic field (384Oe, 118kHz) was applied for intracellular heating. IFN- β secreted in the medium was assayed and its concentration was compared to the extracellular heating induced expression, both in the presence and absence of dexamethasone. Higher IFN- β concentration was detected in intracellular heating even at lower temperature, 39 °C, than 43 °C in extracellular heating. The IFN- β expression level reached in the presence of dexamethasone was about three times higher than in the absence of inducer. In intracellular heating of 60 min, the surviving cell number reduced until 20%.

Introduction

Local heating in well-defined region is a challenging point to be achieved in hyperthermia (Dewhirst, 1994). For the treatment of tumor characterized by uneasy access or by deep localization, the development of less invasive heat generating devices is desired. Ideally, the administration of an antibody-coated heating material remains as the optimum strategy for the preservation of intact healthy tissues.

Since magnetic fluids generate heat by hysteresis loss in an alternative magnetic field, intracellular incorporation of magnetite particles as thermoinducible material has been investigated (Viroonchatanpan, 1996). We have prepared intracellular heating materials using fine magnetite particles, which are 10 nm in size, in order to kill specifically tumor cells (Shinkai *et al.*, 1994; Suzuki *et al.*, 1995). We have also developed cationic magnetoliposome and reported that it could adsorb and accumulate at higher level than neutral magnetoliposomes (Shinkai *et al.*, 1996).

In the present paper, we report on the enhancement of cytokine gene expression by intracellular heating with incorporation of cationic magnetoliposomes and on the cell death. The interferon (IFN)- β was chosen as cytokine for showing high cytotoxicity from endogenous expression in glioma cells as demonstrated by previous researches (Mizuno *et al.*, 1990a; Mizuno *et al.*, 1990b). We chose the MMTV (Mouse Mammary Tumor Virus) promoter, which is induced by a glucocorticoid and of which the induction is enhanced by heat treatment at 39 °C (Sanchez *et al.*, 1994). As an inducer, dexamethasone was used in this study, which has been clinically used for its anti-inflammatory properties (Grabb *et al.*, 1995). The enhancement of IFN- β expression driven by the promoter and the killing effect on glioma cells were evaluated under several heating conditions. Results obtained by intracellular heating (magnetoliposomes incorporation and magnetic field induction) were compared with that by extracellular heating (water bath) conditions.

Materials and methods

Cell culture

The U87MG glioma cell line was used, which is highly resistant to exogenous IFN- β . The cell line exhibits a moderate glucocorticoid binding capacity of 50 fmol mg⁻¹ of protein. The cells were cultivated in complete minimal essential medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum, 5 mM non-essential amino acids, 100 μ g ml⁻¹ streptomycin and 100 U ml⁻¹ penicillin in a humidified atmosphere of 5% CO₂ and 95% air. Medium was changed every day.

Liposome preparation and incorporation

DLPC (Dilauroyl phosphatidylcholine) and DOPE (Dioleoyl phosphatidylethanolamine) were purchased from Sigma Chemical Co. (St. Louis, MO) and TMAG (N-(α -trimethylammonioacetyl)-didodecyl-D-glutamate chloride) bearing positive charge was provided by Sogo Pharmaceutical Co. (Tokyo, Japan). Lipid membrane, which consists of the molar ratio of 1:2:2 as TMAG:DLPC:DOPE (Koshizaki *et al.*, 1989), was prepared by evaporation of the chloroform solution in a round bottom flask. To prepare magnetoliposomes, an aqueous colloidal solution of magnetite particles (10 nm diameter) was added in the flask and then the lipids were dispersed by vortexing. After the osmolarity was adjusted with 10 $\times$ PBS (Phosphate Buffered Saline, pH 7.4), the liposomes with a final magnetite concentration of 5 mg ml⁻¹ were formed by sonication (Shinkai *et al.*, 1996).

To prepare plasmid-entrapped liposomes, an aqueous solution of pSVMTVIFN- β plasmid which was constructed by Toray Industries Inc. (Tokyo, Japan) was added in the flask mentioned above, instead of magnetite colloidal solution. To avoid the impairment of the plasmid, the sonication was replaced by vortexing. The final plasmid concentration was 50 μ g ml⁻¹. Both liposome solutions were freshly prepared before use.

For gene transfection, the cells were seeded in 10 cm diameter dishes and grown until close to confluence (about 10⁷ cells per dish). They were transfected with plasmid-entrapped liposomes under the plasmid concentration of 0.2 μ g ml⁻¹ which corresponds to 10 nM in lipids. After 8 hours, magnetoliposomes were added at 20 μ g ml⁻¹ in the medium for magnetite incorporation.

Heat treatment and induction

For extracellular heating using a water bath, the dishes were placed in an air tight vessel in contact with the hot water (43 °C) during 1 hour, according to the condition described for induction of MMTV promoter by Sanchez *et al.* (1994). For intracellular heating using magnetic field, the cells were collected as a cell pellet in a microcentrifugal tube at 5 hours after magnetite incorporation. The tube was placed inside the coil of a high frequency electromagnetic field generator (Dai-ichi High Frequency Co., Tokyo, Japan). The temperature was controlled at 39 °C under monitoring by an optical fiber thermometer (FX-9020, Anritsu Co., Tokyo, Japan), which was inserted in the center of the pellet (Shinkai *et al.*, 1996).

At 2 hours after heat treatment, dexamethasone, which was purchased from Wako Industries Ltd. (Osaka, Japan), was added in the medium at the concentration of 10⁻⁶ M. After treatment, the cells were reseeded in dishes and cultivated.

Assay

After 2 days from the reseeded (4 days after transfection), IFN- β concentration in the medium was determined by using an enzyme immunoassay kit (Toray Industries Inc., Tokyo, Japan), which is able to detect human interferon- β until 2.5 IU ml⁻¹.

At the same time, the viable cell number was measured by trypan blue exclusion method with a hemocytometer.

Results and discussion

Expression of Interferon- β

Table 1 shows the expression of IFN- β by extracellular or intracellular heating. In the control experiment without heating and without inducer addition, IFN- β expression was observed. This is considered to be due to the presence of inducer analog in the medium (Sanchez *et al.*, 1994). When the culture dishes were immersed in a water bath, it was observed that the expression level was less than 1.25 fold either with or without addition of dexamethasone. In the case of intracellular heating at lower temperature, 39 °C, however, the IFN- β expression was enhanced 1.8 fold in the absence of dexamethasone whereas 1.6 fold enhancement was yielded in the presence of inducer. It is con-

Table 1. Enhancement of interferon- β expression by extracellular (water bath) or intracellular heating (magnetic field induction)

Induction	Interferon- β concentration (–)				
	No heating	Extracellular heating (Water Bath) 43 °C 60 min	Intracellular heating (Mag. Field) 39 °C		
			20 min	40 min	60 min
Without Dex	1.0 $\pm$ 0.1	1.1 $\pm$ 0.1	1.3 $\pm$ 0.2	1.8 $\pm$ 0.3	1.1 $\pm$ 0.1
With Dex	2.8 $\pm$ 0.4	3.4 $\pm$ 0.5	4.5 $\pm$ 0.6	3.0 $\pm$ 0.4	2.1 $\pm$ 0.3

The figures are mean $\pm$ standard deviation (n = 6) of relative values against the interferon- β concentration detected in the case of no addition of dexamethasone and no heat treatment (taken as 1). The interferon- β assay was performed at 4 days after transfection. Medium was changed every day. The amount of interferon- β produced in the medium during 1 day was calculated as IU ml⁻¹ per viable cells, taking into account the diminution of cell number due to the interferon- β expression.

Table 2. Percentage of viable cells after extracellular or intracellular heating

Induction	Viable cell concentration (%)				
	No heating	Extracellular heating (Water Bath) 43 °C 60 min	Intracellular heating (Mag. Field) 39 °C		
			20 min	40 min	60 min
Without Dex	65 (100) $\pm$ 10	62 $\pm$ 10	46 $\pm$ 7	35 (58) $\pm$ 5	23 $\pm$ 3
With Dex	45 $\pm$ 7	43 $\pm$ 7	38 $\pm$ 5	34 $\pm$ 5	20 $\pm$ 3

The cell counting was performed on the same day as the interferon- β assay. Figures are mean $\pm$ standard deviation (n = 6) and those in parentheses show the results in untransfected cells. Cell number in the experiment of untransfected cells without addition of dexamethasone and without heat treatment was taken as 100%.

siderable that local hot spot occurred near by magnetite particles even though temperature of whole cell pellet was 39 °C. It is likely that IFN- β expression was strongly induced in the surroundings of this spot.

Moreover, the IFN- β production appeared to depend on the duration of heat treatment. The strongest influence was found to be 20 min in the presence of dexamethasone while 40 min of magnetic field induction gave the maximum expression without dexamethasone. The decrease of IFN- β expression in longer heat treatment was considered to be due to loss of expressing cells, because the population of untransfected cells become relatively high (see Table 2).

In both heat treatments, the expression level detected in the presence of inducer was about three times higher than in its absence.

Cytotoxicity

Table 2 shows the percentage of surviving cells at 2 days after heat treatment. In control experiment, only 65% of cells transfected by the plasmid pSVMTVIFN- β survived. This is due to the high sensitivity to endoge-

nous IFN- β although the cell line is highly resistant to exogenous IFN- β (Yoshida *et al.*, 1992). The influence on cell killing by external heating was not significant. The viable cell numbers for no heating and for extracellular heating fell to 45% and 43%, respectively. By intracellular heating, the surviving cell number remarkably decreased. With the longer duration of the magnetic field induction, i.e. 60 min, the viable cell number reduced until 20%. It should be also noted that the surviving cell numbers of untransfected and transfected cells were 58% and 35%, respectively. These results can be explained by a combined effect of IFN- β and intracellular heating on cell killing.

However, even if the expression of cytokine was higher with addition of dexamethasone, the viable cell number was similar to that without dexamethasone, especially in the case of 40 or 60 min treatment. It is likely that the surviving cells were not transfected. Then it is considered that the strong induction of IFN- β in the presence of inducer (4.5 times higher level as described in Table 1) was not necessary for cell killing under our experimental conditions as demonstrated in this study.

Conclusions

In order to enhance the expression driven by a thermoinducible promoter, the feasibility of the magnetoliposomes as heating materials was investigated. Intracellular heating after magnetoliposome incorporation triggered higher cellular expression of IFN- β gene in glioma cells even at lower temperature 39 °C, slightly above normal body temperature, compared with extracellular heating in 43 °C environment.

It was also found that the number of surviving cells decreased until 20% by the combination of magnetic heat generation for 60 min and cytokine gene transfection.

This means that intracellular heating using magnetite particles incorporation is one of the considerable protocols for successful combination with in gene therapy for cancer patients.

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References

- Dewhirst MW (1994) Future directions in hyperthermia biology. *Int. J. Hyperthermia* 10(3): 339–345.
- Grabb PA and Gilbert MR (1995) Neoplastic and pharmacological influence on the permeability of *in vitro* blood-brain barrier. *J. Neurosurg.* 82(6): 1053–1058.
- Koshizaki T, Hayashi Y and Yagi K (199) Novel liposomes for efficient transfection of β -galactosidase into COS-1 cells. *J. Clin. Biochem. Nutr.* 7: 73–77.
- Mizuno M, Yoshida J, Sugita K, Inoue I, Seo H, Hayashi Y, Koshizaki T and Yagi K (1990a) Growth Inhibition of glioma cells transfected with the human β -interferon gene by liposomes coupled with a monoclonal antibody. *Cancer Res.* 50: 7826–7829.
- Mizuno M, Yoshida J, Sugita K and Yagi K (1990b) Growth inhibition of glioma cells of different cell lines by interferon- β produced in the cells transfected with its gene by means of liposomes. *J. Lin. Biochem. Nutr.* 9: 73–77.
- Sanchez ER, Hu JL, Zhong S, Shen P, Greene MJ and Housley PR (1994) Potentiation of glucocorticoid receptor-mediated gene expression by heat and chemical shock. *Mol. Endo* 8(4): 408–421.
- Shinkai M, Suzuki M, Iijima S and Kobayashi T (1994) Antibody-conjugated magnetoliposomes for targeting cancer cells and their application in hyperthermia. *Biotechnol. Appl. Biochem.* 21: 125–137.
- Shinkai M, Yanase M, Honda H, Wakabayashi T, Yoshida J and Kobayashi T (1996) Intracellular hyperthermia for cancer using magnetite cationic liposomes: *in vitro* study. *Jpn. J. Cancer Res.* 87(11): 1179–1183.
- Suzuki M, Shinkai M, Kamihara M and Kobayashi T (1995) Preparation and characteristics of magnetite-labelled antibody with the use of poly(ethylene glycol) derivatives. *Biotechnol. Appl. Biochem.* 21: 335–345.
- Viroonchatapan E, Sato H, Ueno M, Adachi I, Tazawa K and Horikoshi I (1996) Magnetic targeting of thermosensitive liposomes to mouse livers in an *in situ* on-line perfusion system. *Life Sci.* 58(24): 2251–2261.
- Yoshida J, Mizuno M and Yagi K (1992) Cytotoxicity of human β -interferon produced in human glioma cells transfected with its gene by means of liposomes. *Biochem. Int.* 28(6): 1055–1061.

Address for correspondence: T. Kobayashi, Department of Biotechnology, Graduate School of Engineering, Nagoya University, Furocho, Chikusa-ku, Nagoya 464-01, Japan