Original Contribution

Induction of *TNF- α* Gene Expression by Heat Inducible Promoter *gadd* 153

AKIRA ITO, MASASHIGE SHINKAI, ISABELLE ANNE BOUHON, HIROYUKI HONDA and TAKESHI KOBAYASHI*

Department of Biotechnology, Graduate School of Engineering, Nagoya University, Nagoya 464-8603, Japan.

Abstract : We demonstrated the effectiveness of inducible expression of the *TNF- α* gene. The *TNF- α* gene located under the heat-inducible promoter, *gadd* 153, was introduced into the human glioma cell line, U251-SP. Without heat treatment, no cytotoxicity for the transfected cells was observed. When the transfected cells were treated with hyperthermia at 43℃ for 1 h or 45℃ for 1 h, inducible expression of the *TNF- α* gene was observed. The pattern of enhancement of the gene expression varied with temperature. The TNF- α production per cell on treatment at 43℃ reached 4.3 times the basal level at one day after the heat treatment while the maximum production on treatment at 45℃ was observed at 4 days after the heating. The cytotoxicity of the transfected cells treated at 43℃ and at 45℃ was 81% at 3 days after the heating and 99.9% at 4 days after the heating, respectively.

Key Words : hyperthermia, TNF- α , *gadd* 153, glioma, heat-inducible promoter.

Introduction

Complete resection of brain tumors, for instance gliomas, is generally difficult. Clinical outcome in patient with malignant glioma treated with other modalities including radiation, chemotherapy or hyperthermia, is rather poor.

Tumor necrosis factor (TNF)- α , first reported as a direct cytolytic effector of cancer cells, induces tumor cell death by apoptosis [1, 2]. It has been also reported that the immune system of the host animal was activated [3] and remarkable antitumor activity was obtained in *in vivo* experiments using TNF- α in murine. However, its clinical use in human has been greatly limited by a strong toxicity.

We have investigated the hyperthermia of brain tumor using fine magnetite particles as intracellular heating materials [4]. Complete regression was observed in an *in vivo* experimental model [5]. However, brain tumors were difficult to cure, because the heating is prevented by the cooling effect due to extremely high blood flow in the brain. Combination treatment of hyperthermia with gene expression of cytokine is a promising approach.

The *gadd* 153 (the Growth Arrest and DNA Damage) gene, which is one of the gadd transcription factor family, was originally identified by DNA damaging reagents in hamster cell lines [6]. It was reported that the *gadd* 153 gene was inducible under stress conditions that damage DNA integrity including heat stress [7, 8]. In our previous study [9], we investigated the induction of *gadd* 153 promoter-mediated gene expression by

Received 19 March 2000, Accepted 15 April 2000. *Corresponding author, Tel: 81-52-789-3213, Fax: 81-52-789-3214.

heat stress using a reporter gene. We also found that this promoter system works under the condition of mild hyperthermia. In the present study, we demonstrated the inducible expression of the TNF-α gene located under the *gadd* 153 promoter and its effect to tumor cell death.

Materials and Methods

Plasmid construction

The plasmid JymCAT0 including the *gadd* 153 promoter was kindly provided by Prof. Nikki Holbrook[6]. The plasmid pGadLuc is the vector which has the luciferase reporter gene located under the *gadd* 153 promoter[9]. The plasmid pGadTNF is the vector in which the luciferase gene of pGadLuc has been replaced by the TNF-α gene from the plasmid SKhTNFα (kindly provided by Dr. Hirohumi Hamada). These plasmids have no other promoters or enhancer elements.

Cell culture and transfection

U251-SP human glioma cells were grown in Minimum Essential Medium, supplemented with 10% fetal calf serum, 10 mM non-essential amino acids, 0.1 mg/ml streptomycin sulfate and 100 U/ml potassium penicillin G as antibiotics. The cells in the logarithmic growth phase were seeded one day before transfection at 1×10^6 cells per 100 mm-diameter dish and after heat stress treatment, were re-seeded at 1×10^5 cells per well in 6-well culture plates. The cells were incubated at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air.

The cells were transfected by the lipofection method using cationic liposomes including the plasmid[9]. The plasmid-liposome complex was added to the medium to reach 0.2 μg/ml in DNA concentration.

Heat stress treatment

As described in our previous paper[9], heat stress was applied at 60 h after transfection. The cell culture plates were wrapped individually with hermetic film and immersed directly in a water bath at 43℃ or 45℃ for 1 h. The temperature of the medium increased quickly and reached equilibrium within 5 minutes. The temperature in the medium was monitored with a fiber optic thermometer probe (FX-9020, Anritsu Meter Co., Tokyo, Japan).

Effect of TNF-α on U251-SP cells exposed to heat stress

Recombinant human TNF-α (Sigma Chemical Co., St. Louis, MO, USA) was used for the experiments of exogenous addition of TNF-α. In the experiments to study effect of endogenous TNF-α on U251-SP cells, the TNF-α concentration was determined by an enzyme-linked immunosorbent assay (ELISA) kit (ENDOGEN Co., Woburn, MA, USA). The standards were calibrated using WHO reference lot 87/ 650. One pg of the standards was equivalent to one WHO pg. Luciferase activity was determined with a luciferase assay kit (Promega Co., Madison, WI, USA).

Number of viable cell was evaluated by a Cell Counting Kit based on the modified MTT assay (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for the experiments of the exogenous TNF-α and by the trypan blue dye-exclusion method using a hemocytometer for the experiment of the endogenous TNF-α. The cell viability was determined as follows:

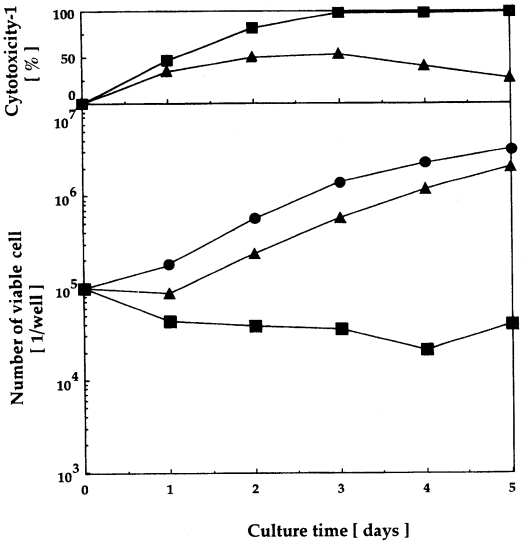

Fig. 1. Cell growth and the cytotoxicity-1 of pGadLuc transfected U251-SP cells after heat treatments. Cell growth was evaluated by counting number of viable cell by trypan blue staining. ●: 37℃, ▲: 43℃, 1 h, ■: 45℃, 1 h.

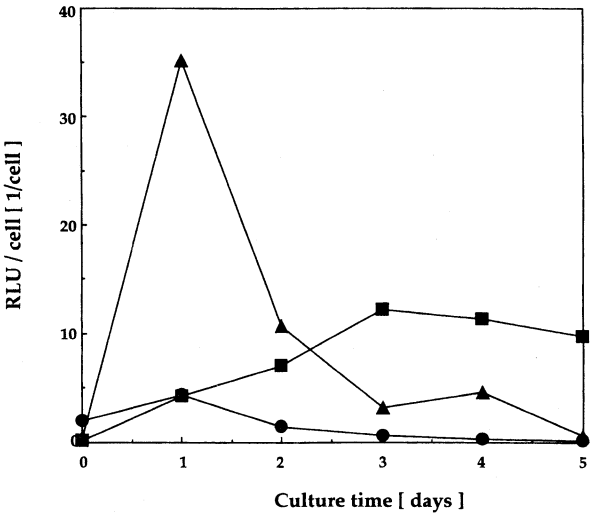

Fig. 2. Time course of induction of *gadd* 153 promoter-mediated luciferase gene expression by heating. Luciferase activity per cell was calculated as follows:

Luciferase activity per cell (1/cell) = (the luciferase activity in the culture supernatant) / (number of viable cell) ●: 37℃, ▲: 43℃, 1 h, ■: 45°C, 1 h. RLU: relative luciferase unit.

Cell viability [%] = 100×(number of viable cell in the tested dish)/(number of viable cell in the dish at 37℃ without TNF-α addition)

The cytotoxicity-1 represents the overall cytotoxicity and was determined as:

Cytotoxicity-1 [%] = 100×{1 - (number of viable cell in the tested dish)/(number of viable cell in the control dish at 37℃)}

The cytotoxicity-2 represents the cytotoxicity induced by TNF-α and was determined as:

Cytotoxicity-2 [%] = 100×{1 - (number of viable cell in the tested dish)/(number of viable cell in the control dish at each temperature)}

Results

Induction profiles of gadd 153 promoter in U251-SP cell line

At first, in order to investigate the gene expression level induced by the *gadd* 153 promoter after heat treatment, the pGadLuc plasmid containing the luciferase gene was used for transfection. Fig. 1 and 2 show the number of viable cell, the cytotoxicity-1 and the luciferase activity of the transfected cells after hyperthermia treatment, respectively. Growth inhibition of transfected cells was observed at one day after the heat treatment at 43℃. After that, the cell growth recovered. The cytotoxicity-1 was 35% at day 1, increased to 53% at day 3 and then gradually decreased to 27% at day 5. When the cells were heated at 45℃ for 1 h, the cell growth was strongly inhibited for 4 days. The cytotoxicity-1 increased to 98% at day 3 and was almost the same at days 4 and 5.

As shown in Fig. 2, the luciferase activity at one day after heating to 43℃ was 7 times that in the case

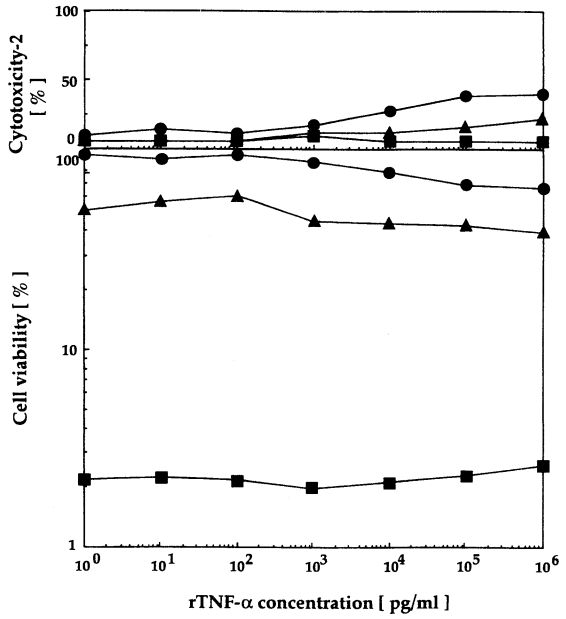

Fig. 3. Effect of exogenous TNF- α on U251-SP cells exposed to different stresses. Cell viability was evaluated by a Cell Counting Kit based on the modified MTT assay. ●: 37℃, ▲: 43℃, 1 h, ■: 45℃, 1 h.

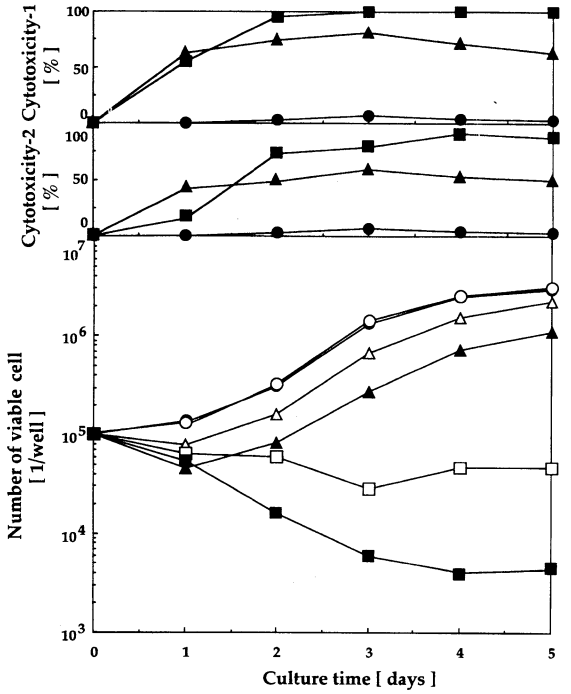

Fig. 4. Cell growth, and the cytotoxicity-1 and -2 of pGadTNF transfected or non-transfected U251-SP cells after heating. Cell growth was evaluated by counting number of viable cell by trypan blue staining. ○: 37℃ incubation, ●: pGadTNF+37℃, △: 43℃, 1 h, ▲: pGadTNF + 43℃, 1 h, □: 45℃, 1 h, ■: pGadTNF + 45℃, 1 h.

without heat treatment (the basal level). Then the activity gradually decreased to near the basal level. On the other hand, the expression level gradually increased after heating to 45℃, with a maximum value of 31 times the basal level obtained at 3 days after the treatment.

Effect of exogenous TNF- α on U251-SP cells exposed to heat stress

As a preliminary experiment, the effect of exogenous addition of TNF- α on cell viability was investigated (Fig. 3). TNF- α was added within a wide range of between 1 pg/ml and 1 μg/ml. However, no significant cytotoxicity was observed with or without heat stress. In the case without heating, a slight decrease of the cell viability or a slight increase of the cytotoxicity-2 was seen within the range from 10 ng/ml to 1 μg/ml. Even when the TNF- α concentration was 1 μg/ml, only about 35% cytotoxicity-2 was observed. However, no obvious dose response to the exogenous TNF- α concentration was observed in combination with 43℃ or 45℃ heating, and the cytotoxicity-2 was 17% and 0%, respectively. Here, the synergism between hyperthermia and the exogenous TNF- α was not observed.

Induction of TNF- α gene expression by heating and evaluation of its cytotoxicity

The cell killing effect both of the TNF- α gene expression and of hyperthermia is shown in Fig. 4. Without heating, the growth of the cells transfected with pGadTNF was almost the same as that of non-transfected cells. This means that the TNF- α gene expression was negligibly low and the cytotoxicity-1

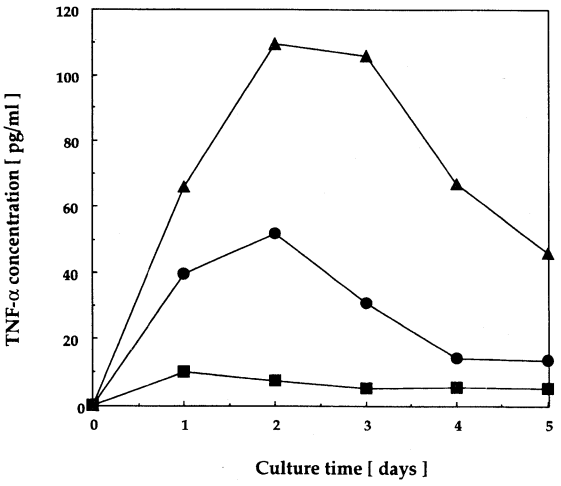

Fig. 5. The time course of the TNF- α concentrations in the culture supernatant of the pGadTNF transfected cells. The TNF- α concentrations in the culture supernatant were determined by ELISA. ●: 37℃, ▲: 43℃, 1 h, ■: 45℃, 1 h.

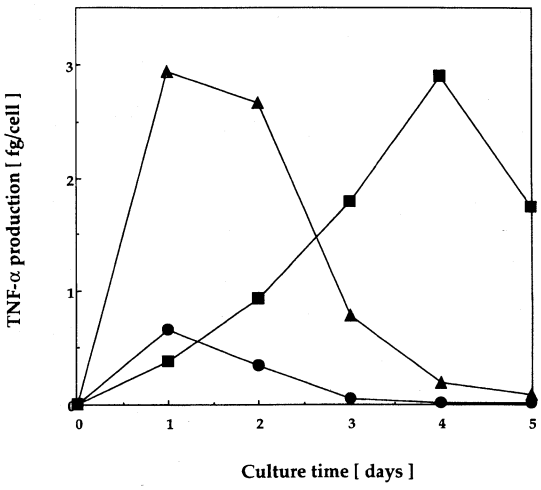

Fig. 6. The time course of the TNF- α production of the pGadTNF transfected cell. The TNF- α production per cell was calculated as follows:

TNF- α production per cell (fg/cell) = (the TNF- α concentration in the culture supernatant) / (number of viable cell)

●: 37°C, ▲: 43°C, 1 h, ■: 45°C, 1 h.

cannot be detected when the transfected cells are not exposed to heat stress. On the other hand, when the cells were heated at 43℃ or 45℃, significant increase in cytotoxicity-1 or -2 was observed as compared with non-transfected cells. In the case of heating at 43℃, the cell growth of non-transfected cells was suppressed by about half that without the heat treatment. Here, the cytotoxicity-1 is not shown in Fig. 4, but it was 58% at day 3, which was similar to that (53%) of the reporter gene experiment shown in Fig. 1. However, in the cells transfected with pGadTNF, the cytotoxicity-1 was 81% at day 3 as shown in Fig. 4.

For heating at 45℃, the cell growth of both transfected and non-transfected cells was strongly inhibited for over 4 days. In the case of transfected cells, the viable cell number decreased exponentially and the cytotoxicity-1 was 99.9% on day 4.

Time courses of the change in TNF- α concentration are shown in Fig. 5. Without heating, the concentration was 20~50 pg/ml. When transfected cells were heated at 43℃ for 1 h, TNF- α accumulated to over 100 pg/ml. However, for heating to 45℃, the TNF- α concentration was very low (less than 10 pg/ml). Time courses of the TNF- α production per viable cell are shown in Fig. 6. At 43℃, the maximum TNF- α expression was observed one day after heating. For heating to 45℃, the gene expression was not high at one day after heating but gradually increased from day 2 to day 3 and reached a maximum on day 4.

The maximum TNF- α expression level at 43℃ and 45℃ was 4.4 and 240 times the basal level, respectively. The former value was comparable with that (6.9 times) for the case of luciferase expression under the same heating condition. Both expression profiles were also similar. On the other hand, the latter value was much higher than that (31 times) in the case of the luciferase expression and the expression profile of TNF- α was obviously peaky.

The toxicity induced by TNF- α gene expression is clearly discussed by the cytotoxicity-2 rather than the cytotoxicity-1. For heating at 43℃, the maximum TNF- α expression level was observed at day 1

(Fig. 6), and the strong cytotoxicity-2 was also observed at day 1 (Fig. 4). The expression profiles after day 2 decreased gradually as described in Fig. 6, and the cytotoxicity-2 after day 2 was almost similar to that at day 1. On the other hand, the TNF-α expression level in the case of 45℃ treatment was low at day 1 (Fig. 6), and the weak cytotoxicity-2 was also observed at day 1 (Fig. 4). After that, the cytotoxicity-2 markedly increased, which corresponded to the increased expression of the TNF-α after day 2 (Fig. 6). Here, low TNF-α concentrations were detected under all conditions (Fig. 5), and the cytotoxicity-2 was not increased when these TNF-α concentrations were exogenously added as shown in Fig. 3. The cytotoxicity-2 was increased with the temperature for heat treatment when the TNF-α was expressed endogenously. This unique system of TNF-α gene located under *gadd* 153 promoter is very attractive for hyperthermia.

Discussion

Exogenous TNF-α has a growth inhibition effect on some human glioma cells, although other glioma cells are resistant [10]. U251-SP cells showed strong resistance to exogenous TNF-α. In the case of tumor tissue with resistance, the administration of TNF-α is meaningless for cancer therapy and the risk to normal tissue remains due to the insufficient localization of the TNF-α. Moreover, synergism between hyperthermia and the exogenous TNF-α was not observed as shown in Fig. 3. In these ways, the heat treatment decreased the cytotoxicity due to the exogenous TNF-α, which has been reported by Jäättelä *et al* [11].

We demonstrated the effectiveness of inducible expression of the TNF-α gene. Much lower TNF-α concentrations than those required to promote the same cytotoxic effect of exogenous TNF-α were detected (Figs. 3 and 5). Mizuno *et al.* reported that cells transfected with the TNF-α gene were more strongly inhibited than those treated with exogenous TNF-α, including U251-SP cells [12]. Tos *et al.* reported that continuous release of the TNF-α from cells transfected with the TNF-α gene generated a cytotoxic effect to the surrounding cells [13]. In the present study, these phenomena seemed to occur. As the temperature for heat treatment was increased, continuous release of the TNF-α per cell was also increased (Fig. 6), so that the cell killing effect was enhanced.

The induction of the *gadd* 153 promoter increased with the temperature for heat treatment, which are similar to our previous results [9]. However, the reason for the high expression level of TNF-α at 45℃ was not the characteristics of the *gadd* 153 promoter. It has been reported that TNF-α induces the production of reactive oxygens which are DNA damaging products [1]. U251-SP cells showed resistant to exogenous TNF-α, but it was not caused by the lack of TNF receptor according to the results by Mizuno *et al* [12]. The reactive oxygens seemed to be produced by TNF-α signal in U251-SP cells. Therefore, it seems that TNF-α itself induced the activity of the *gadd* 153 promoter and enhanced the TNF-α expression.

Conclusion

We have investigated the inducible expression of the TNF-α gene under the heat inducible promoter *gadd* 153 and its enhanced effect during hyperthermia for cancer therapy. TNF-α expression levels at 43℃ and 45℃ were 4.4 and 240 times the basal level, respectively. On heating at 43℃ and 45℃, the cytotoxicity-1 was 81% on day 3 and 99.9% on day 4, respectively. The cytotoxicity-2 was increased with the temperature for heat treatment. It was concluded that the *gadd* 153 promoter system is very useful for cancer hyperthermia.

Acknowledgments

We are grateful to Prof. Nikki Holbrook (National Institute on Aging, Baltimore, MD, USA) for providing the JymCAT0 plasmid including the *gadd* 153 promoter and Dr. Hirohumi Hamada (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research) for providing the plasmid SKhTNF- α including the hTNF- α gene. This study was partially funded by a Grant-in-Aid for Scientific Research on Priority Areas (No. 10145104) from the Ministry of Education, Science, Sports and Culture of Japan.

References

1) Old L.J.: Tumor necrosis factor (TNF). Science 230:630-632, 1985.
2) Bellomo G., Perotti M., Taddei F., Mirabelli F., Finardi G., Nicotera P. & Orrenius S.: Tumor necrosis factor α induces apoptosis in mammary adenocarcinoma cells by an increase in intranuclear free Ca^{2+} concentration and DNA fragmentation. Cancer Res. 52:1342-1346, 1992.
3) Palladino M.A., Shalaby M.R., Kramer S.M., Ferraiolo B.L., Baughman R.A., Deleo A.B., Crase D., Marafino B., Aggarwal B.B., Figari I.S., Liggitt D. & Patton J.S.: Characterization of the antitumor activities of human tumor necrosis factor- α and the comparison with other cytokines: Induction of tumor specific immunity. J.Immunol. 138: 4023-4032, 1987.
4) Shinkai M.,Yanase M., Honda H., Wakabayashi T., Yoshida J. & Kobayashi T.: Intracellular hyperthermia for cancer using magnetite cationic liposomes: *in vitro* study. Jpn. J. Cancer Res. 87: 1179-1183, 1996.
5) Yanase M., Shinkai M., Honda H., Wakabayashi T., Yoshida J. & Kobayashi T.: Intracellular hyperthermia for cancer using magnetite cationic liposomes: An *in vivo* study. Jpn. J. Cancer Res. 89: 463-469, 1998.
6) Luethy J.D. & Holbrook N.K.: Activation of the *gadd*153 promoter by genotoxic agents: a rapid and specific response to DNA damage. Cancer Res. 52: 5-10, 1992.
7) Luethy J.D., Fargnoli J., Park J.S., Fornace A.J. & Holbrook N.J.: Isolation and characterization of the hamster *gadd*153 gene. J. Biol. Chem. 265: 16521-16526, 1990.
8) Delmastro D.A., Li J., Vaisman A., Solle M. & Chaney S.G.: DNA damage inducible-gene expression following platinum treatment in human ovarian carcinoma cell lines. Cancer Chemother. Pharmacol. 39: 245-253, 1997.
9) Bouhon I.A., Ito A., Shinkai M., Honda H. & Kobayashi T.: Heat induction of reporter gene expression via the *gadd*153 promoter and its possible application to hyperthermia treatment of cancer. Cytotechnology, in press.
10) Yoshida J., Wakabayashi T., Mizuno M., Sugita K., Yoshida T., Hori S., Mori T., Sato T., Karashima A., Kurisu K., Kiya K. & Uozumi T.: Clinical effectiveness of tumor necrosis factor- α for malignant glioma. J Neurosurg. 77: 78-83, 1992.
11) Jäättelä M., Saksela K. & Saksela E.: Heat shock protects WEHI-164 target cells from the cytolysis by tumor necrosis factors alpha and beta. Eur J Immunol. 19: 1413-1417, 1989.
12) Mizuno M., Yoshida J., Oyama H. & Sugita K.: Growth inhibition of glioma cells by liposome-mediated cell transfection with tumor necrosis factor- α gene -its enhancement by prior γ -interferon treatment-. Neurol Med Chir. 32: 873-876, 1992.
13) Tos A.G., Cignetti A., Rovera G. & Foa R.: Retroviral vector-mediated transfer of the tumor necrosis factor α gene into human cancer cells restores an apoptotic cell death program and induces a bystander-killing effect. Blood. 87: 2486-2495, 1996.

温熱誘導型プロモーター*gadd* 153による*TNF-α*遺伝子発現誘導

井藤　彰・新海政重・イザベル アンネ ブホン・本多裕之・小林　猛

名古屋大学大学院工学研究科生物機能工学専攻

要　旨: *TNF-α*遺伝子発現誘導の有効性を示した．ヒトグリオーマ細胞株U251-SPに熱誘導型プロモーター*gadd* 153下*TNF-α*遺伝子を導入した．温熱を加えない場合，遺伝子導入細胞において殺細胞効果は示さなかった．遺伝子導入細胞を43℃ 1時間加温，あるいは45℃ 1時間加温した場合，TNF-αの発現誘導がみられた．遺伝子発現のパターンはそれぞれの温度条件で異なった．43℃処理において，加温1日後に最大4.3倍のTNF-α発現誘導，45℃処理においては4日後に発現誘導が最大になった．遺伝子導入細胞の殺細胞効果は，43℃処理においては加温3日後に81%，45℃処理では加温4日後に99.9%だった．