Heat induction of reporter gene expression via the *gadd153* promoter and its possible application to hyperthermia treatment of cancer

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Abstract

The use of the *gadd153* promoter to induce expression of a reporter gene under heat stress conditions was investigated, since the results of previous studies have suggested that the *gadd153* promoter is likely to be activated by the indirect effects of hyperthermia, that is, by DNA damage that occurs when reactive oxygen species are produced by heat stress. The optimum temperature for a significant induction was found to be between 41 and 43 °C and increased expression of the reporter gene was observed at about 24 h after the heat treatment. Under these conditions, the cell integrity was not altered morphologically and the growth stopped temporarily, while the viability was maintained. A second increase in expression occurred at a later stage when the cells were severely damaged at 43–45 °C. At these temperatures, the cellular morphology showed significant alteration and the growth was strongly arrested. This is likely to be due to a different mechanism which could involve DNA repair processes. It is expected that this method of induction can be exploited to drive the production of a protein of interest in a cancer treatment program that includes hyperthermia.

Introduction

Hyperthermia has been considered as a treatment for cancer based on the differential sensitivity of cancer cells to heat compared with normal cells (Suit et al., 1974). Technical improvements to various methods and clinical protocols have enabled physicians to obtain significant better results. In most cases, hyperthermia is used as part of a multi-modality therapy since it is shown to greatly augment the sensitivity of tumor cells to chemotherapy (Raaphorst et al., 1996), radiotherapy (Montes et al., 1995) and immunotherapy (Watanabe et al., 1988; Lin et al., 1996). Brain cancer, i.e. glioma and glioblastoma, are typical candidates for a multi-modality treatment based on hyperthermia because these types of tumors are especially difficult to treat and thus a method is needed to make their cells more susceptible than normal cells to treatment. In this case, a local heating method is needed because it will be less toxic and more effective.

In our laboratory, hyperthermia generated by heating magnetite nanoparticles incorporated into the cells was found to be highly effective in cancer therapy because of the nanoparticles' ability to create local heating (Yanase et al., 1997, 1998).

The recurrent forms of brain cancer have extremely poor prognoses. This is largely due to the resistance of tumor cells to most chemotherapy and immunotherapy treatments (Gosland et al., 1996; Reynolds, 1998). However, in the case of immunotherapy, the intracellular production of a cytokine in tumor cells is often much more effective in enhancing the immunotherapy than the exogenous addition of the same cytokine (Mizuno et al., 1990). Expression of a cytokine by the tumor itself and the suicide gene concept in general have enlarged the scope of the classical immunotherapy approaches.

These developments prompted us to consider a combination of gene therapy and hyperthermia. The efficiency and usefulness of intracellular expression

of a gene of interest, such as a cytokine, depends on its efficiency and relevance on diverse factors including the transfection method, the level and duration of production, the proper excretion, and the expression regulation. Among these factors, the choice of a promoter to drive the expression in a heat stress environment is an important component.

Apart from heat shock protein promoters which have been extensively studied, several families of promoter are potentially exploitable to drive local expression in a high temperature environment. Indeed, heat stress has been shown to induce expression of some genes as part of the defense mechanisms of the cell (Wahl et al., 1997; Mirochnitchenko et al., 1995). However, the enhancement of expression driven by the heat shock protein 70B promoter was only 3-fold increase (unpublished result).

The *gadd153* gene (the Growth Arrest and DNA Damage gene) of the *gadd* transcription factor family was originally identified from the experiments using DNA damaging reagents in hamster cell lines (Luethy et al., 1990). Its promoter is one of the candidates for a heat stress-inducible promoter as it was shown to be inducible under stress conditions that damage DNA integrity, for example, in the case that alkylating or cross-linking agent is added in the medium or in the case that radical producers or ionizing radiation is given (Luethy et al., 1992; Delmastro et al., 1997). Because *gadd153* has been shown to inhibit transcription, its role may be to help stop cell growth in order to provide the cell with time for the repair processes. Its expression actually coincides with growth arrest but its mechanism of action is not clear yet.

In this article, we report the optimum conditions for induction of the *gadd153* promoter by heat treatment based on expression of a reporter gene.

Materials and methods

Plasmid construction

The *gadd153* promoter fragment (799 bp) was isolated by restriction at *Cla* I and *Hind* III sites from the plasmid Jym CAT0 (kindly provided by Prof. Nikki Holbrook) and inserted into the multiple cloning site of pGL3-Basic Vector (Promega, Madison, WI, U.S.A.) containing a firefly luciferase reporter gene. The promoter controls the transcription of the luciferase gene and the plasmid has no other promoter or enhancer elements.

Cell culture

The NIH/3T3 human fibroblast cell line was grown in Dubbelco's modified essential medium, supplemented with 10% fetal calf serum, 10 mM non essential amino acids, 0.1 mg ml⁻¹ streptomycin sulfate and 100 U ml⁻¹ potassium penicillin G as antibiotics. The cells in the log phase were plated one day before transfection at 2×10^5 cells per 35 mm diameter dish (Iwaki, Tokyo, Japan) and incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

Transfection method

The cells were transfected by mean of cationic liposomes as follows. DLPC (dilauroyl phosphatidylcholine) and DOPE (dioleoyl phosphatidylethanolamine) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and TMAG (*N*-(α -trimethylammonio-acetyl)-didodecyl-D-glutamate chloride) was provided by Sogo Pharmaceutical Co. (Tokyo, Japan). These lipids were used at a molar ratio of 1:2:2 as TMAG:DLPC:DOPE as described by Koshizaka et al. (1989). After formation of a lipidic membrane in a conical tube, 20 μ g of plasmid was added in PBS (pH 7.4), and then the lipids were dispersed by vortexing. The suspension was further diluted to a final DNA concentration of 40 μ g ml⁻¹. The plasmid-liposome solution was added to the medium to reach 0.2 μ g ml⁻¹ in DNA concentration.

Heat stress treatment

Preliminary experiments have revealed that gene expression mediated by the *gadd153* promoter was heat inducible (Bouhon et al., 1999). The experimental conditions were optimized using luciferase expression. Heat stress was applied at 60 h after transfection, as this was shown to result in the maximum heat induction. To simulate hyperthermia, the culture dishes were wrapped individually with hermetic film and immersed directly in a water bath at a given temperature. The temperature of the medium increased quickly and reached equilibrium within 5 min. Temperature increase in the medium was measured with a fiber optic thermometer probe (FX-9020, Anritsu Meter Co, Tokyo, Japan).

Evaluation of cell viability and luciferase expression

The toxicity of the transfection and the heat stress was evaluated by daily cell counting by trypan blue exclusion in a hemocytometer. At regular time intervals,

Figure 1. Time course of induction of *gadd153* promoter-mediated luciferase gene expression by different heat treatments. Luciferase activity was evaluated per dish. (A) 39 °C, (B) 41 °C, (C) 43 °C, (D) 45 °C. RLU means relative luciferase unit. One unit of RLU corresponds to the basal luciferase activity expressed in the cells without heat treatment.

cells were harvested until the fourth day after transfection and luciferase activity was determined with a luciferase assay kit (Promega).

Results

Early phase induction

Figures 1 and 2 show the luciferase gene expression obtained by heat stress at different temperatures and

for different durations and the growth curves in the corresponding conditions, respectively. The induction of *gadd153* promoter-mediated luciferase gene expression by various heat treatments was studied at first between 20 and 30 h after the stress period. As shown in Figure 1, a slight induction was obtained at 39 °C (a 5-fold increase in the case of heat treatment for 6 h). Higher induction levels were observed at 41 °C for 4 h (a 10-fold increase) and at 43 °C for 1 h (a 18-fold increase). However, the maximum induction decreased to 9 fold at 45 °C for 15 min. The induction

Figure 2. Growth curve of cells after different heat treatments. A) 39 °C, B) 41 °C, C) 43 °C, D) 45 °C. Cell viability was evaluated by trypan blue exclusion in a hemocytometer. At 43 °C for 4 h and at 45 °C for 1 h, the cells died and the cell counting could not be performed.

peaks appeared at about 24–28 h after the heat treatment. Later, the expression gradually decreased and approached the basal level. The induction seemed to be related to the heat stress dose, i.e. both temperature and duration, instead of to a temperature threshold only.

It is known that mild hyperthermia induces a cascade of cellular events that coordinate a temporary cell cycle arrest (Roti Roti et al., 1992) and, if the stress is

maintained, eventual death by apoptosis. On the other hand, several groups have shown that *gadd153* protein plays a role in cell growth arrest (Beard et al., 1996; Jeong et al., 1997; Kaeck et al., 1997) and that the induction peak due to several agents appears around 24 h after the stress. Therefore, our results in early phase after the heat stress seem to correspond to the participation of *gadd153* in cell cycle arrest due to mild hyperthermic conditions.

Figure 3. Cell morphology at 24 h after severe heat treatment. (A) control (37 °C), (B) 43 °C for 1 h, (C) 43 °C for 2 h, (D) 45 °C for 15 min, (E) 45 °C for 30 min, (F) 45 °C for 1 h.

However, after the more severe heat treatments, the *gadd153* promoter activity was clearly suppressed as shown in Figure 1 and the cell morphology was significantly affected (Figure 3). The proper spindle shape of the control cells is shown in Figure 3A. Indeed, the cells were severely damaged at 43 °C for 2 h or at 45 °C for 30 min. The cell growth was inhibited as shown in Figure 2 and the cells changed to spherical shape (Figure 3C and E). Although a suppression of expression was seen in early phase under these severe conditions, a gradual increase of expression was observed typically in the cells treated at 45 °C for 20 min. Therefore, we investigated the expression beyond 30 h in the cases of 43 and 45 °C.

Late phase induction

As depicted also in Figure 1, in the cells treated at 43 °C for 1.5 and 2 h and at 45 °C for 20 and 30 min, the expression increased gradually after an initial low stage and passed beyond the basal level of the control cells. In the case of the cells treated at 45 °C for 20 min particularly, the expression increased 16 fold at

60 h after the heat treatment. When severer conditions were applied, however, i.e., at 43 °C for 4 h and 45 °C for 1 h, the cells died and consequently, no expression could be detected. This late phase induction of the promoter activity did not coincide with the growth arrest phenomenon since on the contrary, the cells regained their original spindle shape and were ready to resume their proliferation at a later stage.

This particular induction was obtained after cell recovery from severe heat treatment, i.e. at 43 °C for 1.5 or 2 h and at 45 °C for 20 or 30 min. Under these conditions, the cell morphology changed at first to a spherical shape. In this early phase, the expression was suppressed. Then, the cells finally recovered two days after the treatment and they regained their initial state. Therefore, the early and late phase inductions are likely due to different mechanisms.

Discussion

Our results suggested that *gadd153* promoter-mediated gene expression can be exploited in hyperthermia

treatment combined with gene therapy for two reasons.

First, the range of temperatures for induction corresponds to the sublethal temperature used in hyperthermia. Hyperthermia at these temperatures is usually called mild hyperthermia. The temperature needed for completion of necrosis depends on the evenness and density of the distribution of the magnetite particles incorporated at the tumor site (Yanase et al., 1997). Consequently, invaginate parts and peripheral parts of tumorous tissue may not reach lethal temperature. However, the sublethal temperatures that these regions are exposed to will be sufficient to induce gene expression and are thus feasible for gene therapy.

Second, it is expected that the burst of expression driven by the *gadd153* promoter in the late phase in cells recovering from severe damage will complete the cell killing effect that is needed for cancer treatment. In addition to *gadd153*'s role in inhibiting transcription, our results suggest that *gadd153* product also participates in the DNA-repair process after severe damage. However, further studies at the molecular level will be needed to determine whether *gadd153* product has such a second role.

In the future, we will attempt to use gene therapy to support local hyperthermia treatment. We are considering expressing the TNF- α gene under the control of the *gadd153* promoter. Promising results are expected from this approach since TNF- α is known for its synergism with elevated temperatures (Watanabe et al., 1988; Tomasovic et al., 1989). In addition, it has been reported that TNF- α generates oxidative stress (Oberley et al., 1997), i.e., TNF- α produces oxygen-reactive intermediates which in turn damage DNA. Therefore, we can assume that a *gadd153*-TNF- α system would result in a positive feedback on the promoter activity.

Conclusions

We have investigated the induction of *gadd153* promoter-mediated gene expression by heat stress and found that under mild hyperthermic conditions, expression of a reporter gene increased up to 18 fold compared with the basal level in the early phase after the heat treatment. This correlates with previous reports that *gadd153* participates in cell cycle arrest by inhibition of transcription. The maximum induction was seen at 43 °C for 1 h. When higher heat stress was applied, *gadd153* promoter activity was dramatically suppressed as long as the cells showed severe morpho-

logical damage. However, when the cells recovered from this damages, the expression was clearly observed one day after the heat treatment and continued at least up to 60 h.

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