*Free Rad. Res.*, Vol. 35, pp. 23–30
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# Geranylgeranylacetone Promotes Induction and Secretion of Thioredoxin in Gastric Mucosal Cells and Peripheral Blood Lymphocytes

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Accepted by Prof. N. Taniguchi

*(Received 29 August 2000; In revised form 17 October 2000)*

Thioredoxin (TRX) is a redox-active protein which is induced by oxidative stresses and shows a variety of biological activities including cytoprotection against oxidative stress. We recently reported that geranylgeranylacetone (GGA), an anti-ulcer drug, induces TRX in rat hepatocytes. In this study, we demonstrate that GGA promotes induction and secretion of TRX in rat gastric mucosal cells and human peripheral blood lymphocytes (PBLs). Western blotting and a sensitive sandwich ELISA showed that TRX was induced by GGA in the cell lysates and culture supernatants of rat gastric mucosal RGM-1 cells and human PBLs. LDH releasing assay showed that GGA protected rat gastric mucosal RGM-1 cells from ethanol-induced cytotoxicity. Moreover, exogenous recombinant wild type TRX decreased $^{51}Cr$ release from primary cultured rat gastric mucosal cells incubated with ethanol or hydrogen peroxide in a dose-dependent manner, whereas recombinant mutant TRX (C32S/C35S), in which the two cysteines were replaced with serines in its active site, did not. These results indicate that GGA promotes the induction and secretion of TRX in a variety of types of cells and suggest that induced or secreted TRX may play an important role in the cytoprotective action of GGA on gastric mucosal cells.

*Keywords*: Geranylgeranylacetone, thioredoxin, gastric mucosal cell, lymphocyte, ethanol, hydrogen peroxide

*Abbreviations*: ATL, adult T cell leukemia; ADF, ATL-derived factor; GGA, geranylgeranylacetone; PBL, peripheral blood lymphocytes; TRX, thioredoxin

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## INTRODUCTION

Geranylgeranylacetone (GGA) is an acyclic polyisoprenoid[1] and widely used as an anti-ulcer drug.[2] GGA reduces the secretion of gastric juice[3] and promotes healing process of gastric ulcer.[4] The cytoprotective effect of GGA is partly explained by induction of prostaglandins[5] and increase of the gastric mucosal hexosamine content.[6] However, other reports showed endogenous prostaglandins are not involved in the action of GGA[7] and GGA induces heat shock proteins (HSPs).[8] Therefore, the molecular mechanism of cytoprotective action of GGA is not yet fully understood.

Thioredoxin (TRX) is a small ubiquitous protein with a redox-active disulfide/dithiol in its conserved active site sequence: -Cys-Gly-Pro-Cys-. TRX was originally idenitified as an electron donor for ribonucleotide reductase in *Escherichia coli*. Reduced TRX catalyzes protein disulfide reduction and oxidized TRX is reduced by thioredoxin reductase and NADPH.[9] Human TRX was cloned as adult T cell leukemia-derived factor (ADF) produced by human T lymphotropic virus type-I-transformed T cells.[10] There is accumulating evidence that TRX is induced by a variety of stresses including viral infection and hydrogen peroxide[11,12] and TRX is involved in the redox-regulation of signal transduction.[12,13] Intracellular TRX suppresses the activation of apoptosis singal-regulating kinase-1 (ASK-1)[14] and MAP kinase p38.[15] Exogenous administration of recombinant TRX can suppress Fas- and hydrogen-peroxide-induced cytotoxicity *in vitro*[16,11] and ischemia-reperfusion injury *in vivo*.[17] TRX-transgenic mice, in which human TRX is systemically overexpressed by $\beta$-actin promoter, are more resistant to focal cerebral ischemia.[18] Overexpression of TRX in pancreatic $\beta$ cells prevents oxidative-stress induced diabetes mellitus.[19] Therefore, TRX is an endogenous cytoprotective and anti-apoptotic factor.

Recently we reported that GGA induces TRX in rat hepatocytes.[20] In this study, we demonstrate that GGA promotes the induction and secretion of TRX in gastric mucosal cells and human peripheral blood lymphocytes (PBLs). As well as the case of hepatocytes, GGA protects gastric mucosal cells from ethanol-induced cytotoxicity. Here we demonstrate that GGA induces the secretion of TRX and exogenous TRX attenuates hydrogen peroxide- or ethanol-induced injury on gastric mucosal cells. These results suggest that GGA is an inducer of TRX in a variety types of cells and induced or secreted TRX may be involved in the cytoprotective action of GGA on gastric mucosal cells.

## MATERIALS AND METHODS

### Reagents

Coon's modified Ham's F-12 medium was from Nissui Pharmaceutical Co., Tokyo, Japan. Collagenase and hyaluronidase were from Sigma Chemical Co., St. Louis, MO. Hydrogen peroxide and ethanol were obtained from Nakalai Tesque Inc., Kyoto, Japan. GGA was provided from Eisai Pharmaceutical Co., Tokyo, Japan. Recombinant wild type TRX and recombinant mutant TRX, in which the two cysteines in the active site were replaced with serines (C32S/C35S), were prepared as described previously[21] and provided by Ajinomoto Co., Inc., Basic Research Laboratory, Kawasaki, Japan. Anti-mouse TRX antibody was prepared as previously described and proved to crossreact to rat thioredoxin.[22]

### Rat Gastric Mucosal Cell Line

Rat gastric mucosal cell line (RGM-1) was provided from Riken Cell Bank, Japan. RGM-1 was incubated in 1:1 mixture of DMEM/Ham's F-12 medium containing 20% fetal calf serum, 100 µg/mL streptomycin and 100 U/mL penicillin. The cells were incubated in culture wells at 37 °C in a humidified atmosphere under 5% $CO_2$.

### Human Peripheral Blood Lymphocytes

Human peripheral blood lymphocytes (PBLs) were obtained from heparinized venous blood by centrifugation over Ficoll-Hypaque (Pharmacia). Human PBLs were incubated in RPMI1640 medium supplemented with 10% fetal calf serum, 100 μg/mL streptomycin and 100 U/mL penicillin. The cells were incubated in culture wells at 37 °C in a humidified atmosphere under 5% $CO_2$.

### Cultured Rat Gastric Mucosal Cells

Gastric mucosal cells were prepared by collagenase and hyaluronidase digestion from 7 to 10-day-old Sprague-Dawley rats as described. Briefly, the fundic area of the stomach was minced into 2–3 $mm^3$ pieces and incubated in Coon's modified Ham's F-12 medium containing 0.1% collagenase and 0.05% hyaluronidase at 37 °C for 60 min. The gastric cells were filtered through a sterile nylon mesh and centrifuged at 600 rpm for 5 min. The cells were next washed twice in F-12 medium and resuspended in F-12 medium supplemented with 15 mmol/L HEPES-Na, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum. The cells were incubated at 37 °C in a humidified atmosphere under 5% $CO_2$ and then inoculated onto culture wells. All experiments were performed at 3–4 days after confluence. Before the cytotoxicity assay was performed, the medium was removed and the mucosal cell monolayer was covered with Dulbecco's phosphate-buffered saline (DPBS).

### Western Blotting

In the case of RGM-1 cells, cells were collected by trypsinization. Cells were washed with PBS twice and then lysed with a solubilizing solution (0.5% NP-40, 10 mM Tris-HCl pH 7.2, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 0.111 U.ml aprotinin) on ice for 30 min. The extracts were cleared by centrifugation. Cell lysates were kept at 95 °C for 3 min and then applied to 12% SDS acrylamide gel, electrophoresed (5 μg protein/lane) and then transferred to a PDVF membrane (Millipore, Bedford, MA). After blocking in 5% skim milk in PBS containing 0.05% Tween 20, the membrane was incubated for 1 h with anti-mouse TRX antibody, followed by peroxidase-conjugated anti-rabbit immunoglobulin antibody for RGM-1 cells. The membrane was incubated with anti-human TRX antibody, followed by peroxidase-conjugated anti-mouse immunoglobulin antibody for human PBLs. Detection by chemiluminascence was performed with an ECL Western blot detection kit (Amersham) according to the manufacturer's instruction. The amount of TRX in each sample was estimated by analysis of the density of each band using a computerized densitometer, NIH image.

### Enzyme-linked Immunosorbent Assay

Human PBL of $1 \times 10^6$ cells were isolated, suspended in 5 ml of culture medium described above, and cultured on 3.5 mm diameter culture dish (Corning) for 4 h, followed by treatment with various concentrations of GGA (final concentrations of 0, 0.1, 1, and 10 μM), and further cultured for 72 h. Then, the concentrations of TRX in each culture medium were estimated by a sandwich ELISA for human TRX (FujiRebio Co. Ltd., Tokyo, Japan) as previously described. As a standard, serial dilution of 1.875–120 ng/ml of recombinant TRX were used. Data were analyzed by a software SOFTmax Version 2.31.

### Lactate Dehydrogenase (LDH) Releasing Assay

Cell viability was assessed by LDH releasing assay. LDH released from damaged cells was determined in aliquots of the culture medium. The remaining cellular LDH was obtained by lysing cells with 0.2% Tween-20 in phosphate buffered saline (PBS). LDH in 50 μl samples of culture medium or cell lysates was measured

using a LDH assay kit (Kyokuto, Tokyo, Japan) according to the manufacturer's procedure. Percentage of cell lysis was determined as the ratio of LDH in the medium/total LDH per well (medium plus cell lysates).

### $^{51}$Cr Release Assay

When rat gastric mucosal cells were > 80% confluent in the culture wells, as confirmed by phase-contrast microscopy, the cells were incubated overnight in a culture medium containing $^{51}$Cr-sodium chromate ($3.7 \times 10^8$ Bq/mL; New England Nuclear, Boston, MA) at 37 °C under 5% $CO_2$. The labeled monolayers were then washed three times with DPBS to remove excess $^{51}$Cr and placed in DPBS for incubation studies. All experiments were performed in triplicates with a final volume of 0.2 ml. After incubation, $^{51}$Cr release into 0.1 mL of the centrifuged cell-free supernatants was measured. Cytotoxicity was expressed as the percentage $^{51}$Cr released, calculated by the formula:

$$\text{Cytotoxicity (\%)} = (A - B)/(C - B) \times 100$$

where A is the cpm released in the supernatant of the wells containing the monolayers plus an injurious agent, B is the cpm released in the supernatant of the wells containing the monolayers alone, and C is the cpm from the monolayers treated with 1% Triton X-100.

## RESULTS

### Induction and Secretion of TRX in Rat Gastric Mucosal Cells by GGA

Western blotting showed that GGA induced TRX in cell lysates of RGM-1 cells in a dose-dependent manner (Figure 1A). Moreover, TRX was detected in the supernatant of RGM-1 cells cultured with 0.1, 1 or 10 μM GGA for 24 h (Figure 1B).

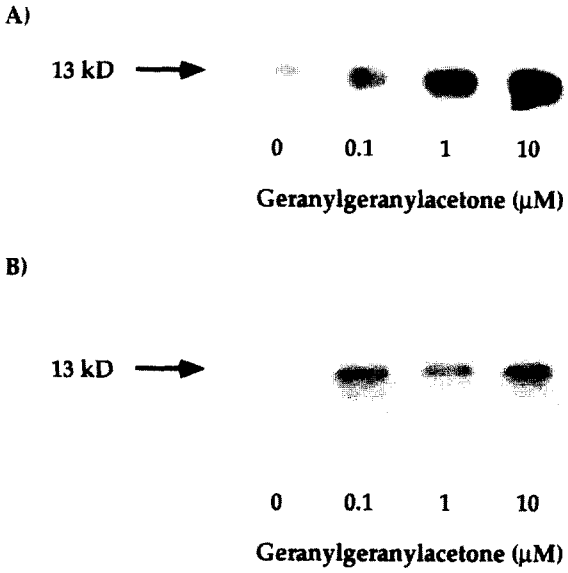


FIGURE 1 $3 \times 10^5$/well rat gastric mucosal RGM-1 cells were inoculated in 6-well plate and allowed to grow overnight. Then, the cells were incubated for 24 h with 0, 0.1, 1 or 10 μM GGA which was dissolved in 0.1% v/v ethanol. The cells were harvested and lysed. (A) TRX was recognized as a band of 13 kD by Western blotting using anti-TRX antibody. (B) Western blotting showed TRX in the 100 fold-concentrated culture supernatant of RGM-1 cells incubated with 0, 0.1, 1 or 10 μM GGA for 24 h without fetal calf serum.

### Induction and Secretion of TRX in Human PBLs by GGA

The induction of TRX in human PBLs by GGA was determined by Western blot. The western blot with anti-human TRX antibody showed that TRX was expressed in human PBLs cultured without GGA and that the expression of TRX in

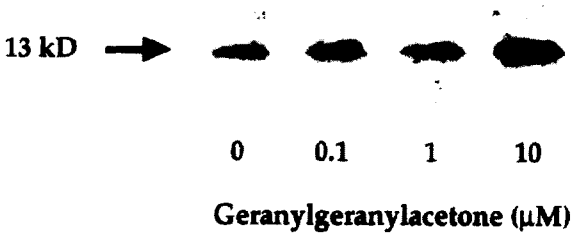


FIGURE 2 $1 \times 10^6$/ml human PBLs were cultured with 0, 0.1, 1 or 10 μM GGA for 72 h and then cell lysates were collected. Western blotting using anti-human TRX antibody (ADF-11) showed that GGA induced TRX as a band of 13 kD in a dose-dependent manner.

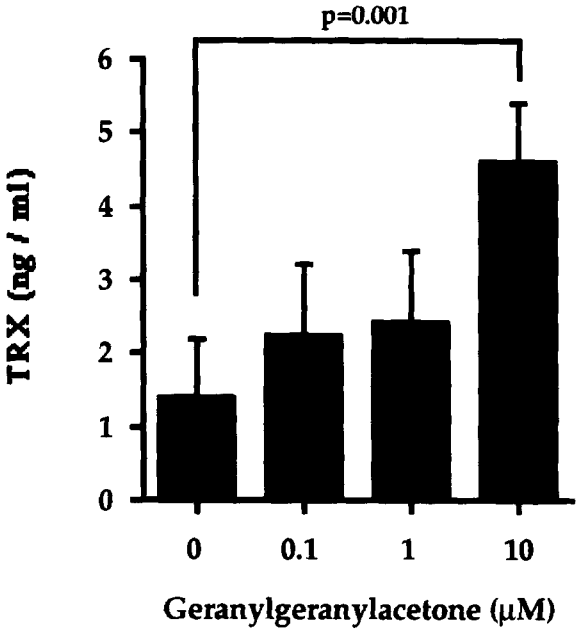


FIGURE 3 A sensitive sandwich ELISA showed human TRX concentrations in the culture medium of human PBLs cultured with 0, 0.1, 1, and 10 μM GGA for 72 h. Each column was expressed as mean ± SD from quadricate analysis. TRX level in the culture medium of human PBLs cultured with 10 μM GGA was statistically significantly higher than without GGA ($p < .001$).

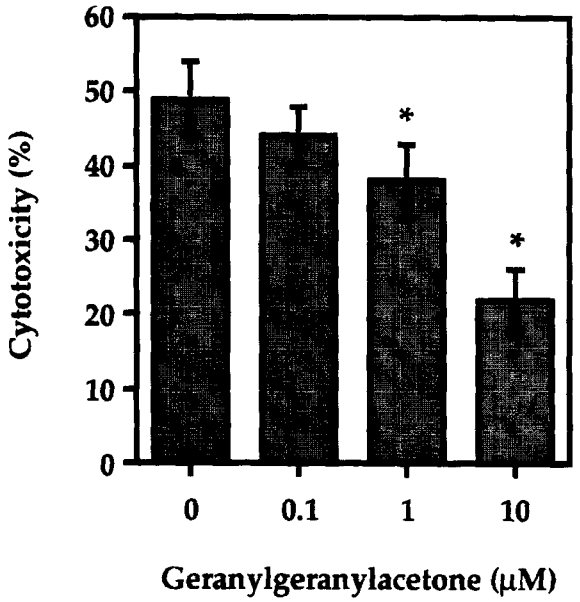


FIGURE 4 RGM-1 cells were cultured with 0, 0.1, 1, or 10 μM GGA for 4 h and then incubated with 15% ethanol for 30 min. The cytotoxicity (%) was measured by LDH releasing assay. *Indicates statistically significant ($p < .05$).

PBLs treated with GGA was upregulated in a dose dependent manner (Figure 2). TRX concentrations in culture medium of human PBLs cultured with GGA for 72 h were determined by a sensitive sandwich ELISA. TRX levels in the medium samples cultured with 10 μM GGA were significantly higher than the medium sample cultured without GGA (Figure 3).

### Effect of GGA on Ethanol-induced Gastric Mucosal Cell Injury

$2 \times 10^4$/ml RGM-1 cells were plated in 96 well microplate for 72 h. After washed with 1:1 mixture of DMEM/Ham's F-12 medium containing 20% fetal calf serum, RGM-1 cells were incubated with 0, 0.1, 1, or 10 μM GGA for 4 h. Then, the cells were incubated with 15% ethanol for 30 min. Percentage of LDH release was measured in the centrifuged supernatant of the medium. GGA suppressed the LDH release caused by ethanol in a dose-depedent manner and the effects of 1 and 10 μM GGA were statistically significant (Figure 4).

### Effect of TRX on Gastric Mucosal Cell Injury

To determine whether TRX could protect gastric mucosal cells from deleterious agents such as ethanol and hydrogen peroxide, the $^{51}$Cr-labeled monolayers of primary cultured rat gastric mucosal cells were incubated with 15% ethanol for 30 min or 1 mM hydrogen peroxide for 4 h, respectively, in the presence of 0, 0.01, 0.1, 1 or 10 μM TRX or mutant TRX. Recombinant wild type TRX decreased ethanol- or hydrogen peroxide-induced $^{51}$Cr release of primary cultured rat gastric mucosal cells in a dose-dependent manner. In contrast, recombinant mutant TRX (C32S/C35S) did not have any protective effect against ethanol or hydrogen peroxide (Figure 5).

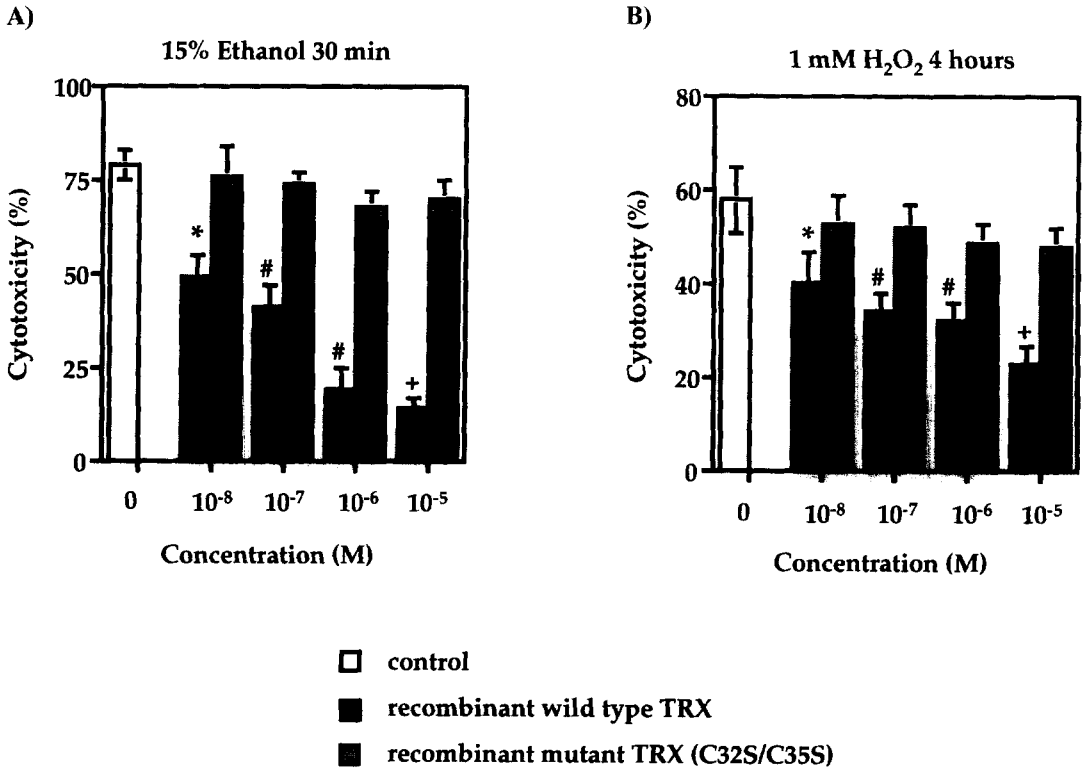


FIGURE 5 Effect of recombinant wild-type TRX and recombinant mutant TRX (C32S/C35S) on (A) 15% ethanol-induced gastric mucosal cell injury after a 30-min incubation and (B) 1 mM hydrogen peroxide-induced gastric mucosal cell injury after a 4-hr incubation. Results are expressed as the means ± SEM of three experiments performed in triplicate. $^{*}p < .05$, $^{\#}p < .01$, $^{+}p < .001$ compared with the control cytotoxicity by 15% ethanol in (A) or by 1 mM hydrogen peroxide in (B), respectively.

## DISCUSSION

Although GGA is a widely used anti-ulcer drug, the molecular mechanism of the cytoprotective effect of GGA is largely to be clarified. GGA induces HSPs in cultured rat gastric mucosa.[7] However, it is difficult to explain all the mechanism of the cytoprotective effect of GGA by induction of HSPs. In fact, GGA protects rat liver from warm ischemic injury without induction of HSPs.[23] We recently reported that GGA induces TRX in rat hepatocytes.[20] Our present data showed that GGA promotes the induction and secretion of TRX in gastric mucosal cells and human PBLs. Moreover, exogenous TRX directly reduced ethanol- or hydrogen peroxide-induced gastric mucosal cell injury. Since recombinant mutant TRX (C32S/C35S), in which cysteines in the active site were replaced with serines, did not protect gastric mucosal cell injury, the dithiol/disulfide exchanging activity is necessary for the protective effect of TRX. Several lines of evidence indicate that TRX is an anti-apoptotic factor and shows cytoprotective effect against various oxidative stresses including hydrogen peroxide and ishcemia-reperfusion.[12] It was previously reported that the expression of TRX in the gastric mucosal tissues is enhanced at hunger time and by vagotomy.[24] TRX in the gastric mucosa may play a role in the cytoprotective function against ulcer formation. Therefore, the present study may add a new direct evidence on the molecular mechanism of the cytoprotective effect of GGA on gastric mucosal cells.

The induction mechanism of TRX by GGA is to be further analyzed. GGA induces the production of prostaglandins and prostaglandins enhances intracellular cyclic AMP levels.[5] The promoter sequence of TRX gene contains cyclic AMP responsive element (CRE) in addition to oxidative responsive element and hemin responsive element.[25,26] We have preliminary data indicating that the induction of TRX by GGA is not totally but at least partly dependent on the CRE (data not shown). The secretion mechanism of TRX is also largely unknown. In *Escherichia coli*, it was reported that TRX is secreted through a mechanosensitive channel.[27] In mammals, previous reports have shown that TRX as well as IL-1$\beta$ is secreted through a leaderless pathway.[28] There is accumulating evidence that TRX shows a variety of extracellular cytokine- or chemokine-like functions. The secretion mechanism of TRX by GGA and other stimuli is to be clarified for better understanding of extracellular functions of TRX.

Our data show that GGA is an inducer of endogenous TRX in a variety of tissues. We have found that GGA can induce TRX not only in hepatocytes[20] but also in gastric mucosal cells, lymphocytes and neuronal cells (Bai *et al.* manuscript in preparation). We also have the data that TRX-transgenic mice are more resistant to oxidative stress and survive longer than C57BL/6 wild type mice (Mitsui *et al.* submitted). Since TRX is an endogenous cytoprotective and redox-regulating factor, GGA may be a leading compound for a new therapeutic strategy for various disorders caused by oxidative stresses. The clinical application of GGA as a TRX inducer will be expanded from just an anti-ulcer drug towards a broad spectrum as an anti-stress drug.

## *Acknowledgments*

We greatly appreciate Prof. Toru Kita at Department of Geriatric Medicine, Graduate School of Medicine, Kyoto University and Prof. William Brown at Denver Veterans Administration Medical Center, Colorado for valuable advice. This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan and by a grant-in-aid of Research for the Future from Japan Society for Promotion of Science, Japan.

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