UVC Mutagenicity Is Suppressed in Japanese Miso-Treated Human RSa Cells, Possibly via GRP78 Expression

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Little is known about the ability of miso, to modulate mutability in human cells. We have observed increased levels of glucose-regulated protein 78 (GRP78) expression in association with suppression of mutation in human RSa cells irradiated with ultraviolet C (UVC). Here we examined to determine whether miso treatment results in increased GRP78 expression and suppression of UVC mutagenicity in RSa cells. Supernatants of water extracts of miso products and their components were tested. In the sample-treated cells, the amount of GRP78, as estimated by RT-PCR and immunoblotting analysis, increased, and the UVC-induced ouabain resistant mutation (Oua⁶) and the K-ras codon 12-base substitution mutation frequency decreased. This decrease was not observed in cells with downregulation of GRP78 by GRP78 siRNA transfection. The results suggest that miso suppresses UVC mutagenicity by increasing GRP78 expression in human cells.

Key words: Japanese miso; human RSa cells; mutation; ultraviolet C (UVC); glucose regulated protein 78 (GRP78)

Miso is a fermented food that has formed an important part of the Japanese diet for over 1300 years.1) It is prepared by microbial fermentation of a mixture of raw materials over a long period until ripe miso is obtained.2) The raw materials used are soybean, wheat, barley, and rice.2) There have been reports on the antimutagenic effects of miso.3) Yamamoto et al. reported that 3-amino-1-methyl-5H-pyrido[4,3-b] indole (Trp-P-2), and 3,4-benzpyrene (B[a]P)-induced mutations were suppressed by the free unsaturated fatty acids and oleic acid in miso in an assay using Salmonella.3) Among more than 200 flavor components confirmed in miso,4,5) 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF) has been reported to have anticarcinogenic activity.5,7) Genetic mutation is thought to be an important process in cancer formation.8-10) Hence, we were intrigued by the possibility that carcinogen-induced mutations are suppressed by Japanese miso.

Recently we found that UVC mutagenicity is suppressed by upregulated expression of chaperones, such as GRP78.11,12) Increased cellular levels result of GRP78 in decreased mutation frequency12) as detected by the ouabain-resistance mutation test and analysis of the base-substitution mutation of the K-ras codon 12 in human RSa cells.13) RSa cells are highly susceptible to mutation as a result of exposure to UVC.14,15) Mutation analysis in RSa cells is used to evaluate modulation of cell mutability by various factors. UVC mutagenicity has been found to be suppressed in RSa cells pretreated with human interferon α (HuIFNα) prior to UVC irradiation.16)

In the present study, cellular amounts of GRP78 were examined by immunoblotting in RSa cells cultured with Japanese miso samples. The mutability of the miso-treated RSa cells was then evaluated using the Oua⁶ mutation test and the differential dot-blot hybridization test for K-ras codon 12 mutation following UVC irradiation. Samples were prepared by water extraction of components from various Japanese miso products, such as rice-koji. HEMF was also examined to monitor its ability to modulate GRP78 levels and the mutability of RSa cells.

Materials and Methods

Miso and reagents. Two kinds of rice-koji miso, Kurasaigetsusujo miso (KU) and Igou-miso (IG), were purchased from Ando Brewery (Kakunodate, Japan). The raw ingredients of miso, boiled soybean and rice-koji, were obtained from Ando Brewery. Miso samples were prepared as follows: Each miso (10 g) was suspended in 20 mL of MilliQ water, and the suspension was warmed at 90 °C for 5 min and then at 70 °C for 10 min. The suspension was centrifuged at 1,780 × g for 10 min, and the supernatant was then filtered through a 0.22-μm membrane. The doses of samples used in the various treatments are shown as percentages of volume per volume (v/v); 1% is equivalent to 5 mg of miso or construction per mL. HEMF was purchased from Tokyo Chemical Industry (Tokyo). It was dissolved in MilliQ water, then filtered through a 0.22-μm membrane. Unless stated

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Abbreviations: Oua⁶, ouabain-resistance; HEMF, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone; MTT, methythiazole tetrazolium
otherwise, the other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cells and culture conditions. RSa cells were established from human embryo-derived fibroblast cells by double infection with Simian virus 40 and Rous sarcoma virus, and were characterized as cells with high UV sensitivity and low DNA-repair activity.17–19) The cells were cultured in Eagle's minimal essential medium (EMEM) (Nissui, Tokyo) containing 10% calf serum (CS, Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere containing 5% CO₂.

UV irradiation. UVC was generated by a 6 W National germicidal lamp (Panasonic, Osaka, Japan). The intensity of UVC was 131 μW/cm² as measured with a UV radiometer, UVR-254 (Topcon Corporation, Tokyo). Irradiation of cells with UVC was performed as described previously.21) Briefly, logarithmically growing cells were seeded in 100-mm dishes (800 cells/dish) and incubated for 20h to allow the cells to attach, and the cells were then treated with UVC before 1h. After treatment, the cells were grown in fresh medium containing 5% CS in 100-mm dishes for about 14 d and then stained with 0.2% methylene blue in 30% aqueous meohanol. Cloning efficiency was defined as the number of colonies. For mutagenicity, the cells were seeded (8 × 10⁵ cells/100 mm dish) in 10 ml EMEM containing 10% CS and 5 × 10⁻⁸ mol ouabain. Every 6 d, the medium was exchanged for 10 ml of fresh EMEM containing 10% CS and 5 × 10⁻⁸ mol ouabain. After 3 weeks, the dishes were stained as described above. The mutation frequency was determined by dividing the total number of cells plated, corrected by the cloning efficiency, and was expressed as mutation frequency per 10⁵ cells.

 Colony survival assay. The survival capacity of cells treated with and without miso samples was measured by colony survival assay, as reported previously.20) Logarithmically growing cells were seeded in 100-mm dishes (800 cells/dish) and incubated for 20 h to allow the cells to attach, and the cells were then treated with miso samples for 1 h. After treatment, the cells were grown in fresh medium containing 5% CS in 100-mm dishes for about 14 d and then stained with 0.2% methylene blue in 30% methanol. The results of the colony survival assay were expressed as percentages of colony numbers relative to those of the untreated cells.

Immunoblotting analysis. Immunoblotting was carried out as described previously.21) Cells were lysed with a lysis buffer containing 20 mmol Tris–HCl (pH 7.4), 1 mmol EDTA, 1 mmol EGTA, 0.5% NP-40, and protease inhibitors, including 1 mmol phenylmethylsulfonyl fluoride, 0.05 mmol leupeptin, 0.05 mmol antipain, and 0.05 mmol pepstatin A. The cell lysates were centrifuged at 15,000 × g at 4 °C for 20 min, and the supernatant was treated with SDS sample buffer. Detection of GRP78 protein was done using mouse monoclonal anti-GRP78 (1:2,000 dilution; SPA-827; Stressgen, Victoria, Canada) antibodies. β-Actin was also analyzed using mouse monoclonal anti-β-actin antibodies (1:30,000 dilution; ab80864; Abcam, Cambridge, UK) as loading control. The antigen-antibody complexes were detected by horseradish peroxidase (HRP) conjugated anti-mouse IgG (Amersham Biosciences, Buckinghamshire, UK) following the ECL system (GE Healthcare, Buckinghamshire, UK). Amounts of GRP78 protein were quantified using Multi Gauge Ver2.2 image analysis software (Fuji Photo Film, Tokyo), and were expressed as values relative to the quantity of β-actin observed.

Detection of the K-ras codon 12 mutation by PCR and differential dot-blot hybridization. Mutations in K-ras codon 12 were detected principally by a method described previously.22) Briefly, logarithmically growing cells were inoculated at near confluency (5 × 10⁵ cells/100 mm dish) to allow cell selection by the lethal effects of UV irradiation, as described elsewhere.23) Six d after UVC or mock irradiation, genomic DNA was extracted by a standard proteinase K/SDS/pooloro/chloroform procedure. The DNA of the SW480 cells carrying the K-ras mutation at codon 12 was used as positive control for genomic DNA. The target sequences of the sample DNA were amplified in vitro by PCR using primers 5'-GACTGAATA-TAACCTGTGG-3' and 5'-CTATTGGTTGGATCATATTCG-3', and the amplified DNA (0.25 μg) was dot-blotted onto nylon membranes. After hybridization with digoxigenin-11-dUTP-3' end-labelled K-ras codon 12 probes, the membranes were reacted with alkaline phosphatase conjugated polyclonal sheep anti-Dig Fab (Boehringer Ingelheim, Mannheim, Germany) and colored with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate solution (Boehringer Ingelheim). As a control probe, oligonucleotide 5'-GGTTGAGCT-GGTTGCCGTAAG-3' was used, and a mixed mutant probe, containing the following oligonucleotides mixed at the same concentration ratios, was used: 5'-GGTGGAAGCTCCTATGGGCCTAGG-3', 5'-GGTGGAAGCT-CGGGCGGTAG-3', 5'-GGTGGAAGCTTCCTGTTGCGTAGG-3', 5'-GGTGGAAGCTTTGGCCGTAAG-3'. Photographs were taken as a permanent record of the results.

Ouabain-resistance mutation test. An assay to detect mutations to ouabain-resistance (Oua²) in the cells, and calculations of the cytotoxicity and mutation frequency, were performed as described previously.26) Cells were seeded in 100-mm dishes (5 × 10⁵ cells/dish), and after 20 h were treated with miso samples for 24 h or left untreated as a control. The dose of miso sample applied for mutation analysis was chosen according to previous reports, miso-treated cells showing about 50-90% the survival rate of the mock-treated cells.22) After miso sample treatment the medium was removed, and the cells were then irradiated with UVC. Immediately after irradiation, the cells were cultured in EMEM containing 5% CS. Forty-eight h after irradiation, the cells were detached from the dish and seeded to determine cytotoxicity and mutagenicity.

Cytotoxicity and mutagenicity were determined as described previously,24) with minor modifications. For cytotoxicity, the cells were grown in fresh medium containing 5% CS (800 cells/100 mm dish) for about 2 weeks, and then stained with 0.2% methylene blue in 30% aqueous meohanol. Cloning efficiency was defined as the number of colonies. For mutagenicity, the cells were seeded (8 × 10⁵ cells/100 mm dish) in 10 ml EMEM containing 10% CS and 5 × 10⁻⁸ mol ouabain. Every 6 d, the medium was exchanged for 10 ml of fresh EMEM containing 10% CS and 5 × 10⁻⁸ mol ouabain. After 3 weeks, the dishes were stained as described above. The mutation frequency was determined by dividing the total number of cells plated, corrected by the cloning efficiency, and was expressed as mutation frequency per 10⁵ cells.

Treatment with GRP78 siRNA. Duplex small interfering RNA (siRNA) with Stealth modification against human GRP78 (GRF78 siRNA) was synthesized based on the protein’s nucleotide sequence (Invitrogen), as described previously.26) The sequence of the duplex was as follows: 5'-UAC CUC UGU CUU CAG CUG UCA UUC G G 3' / 3'-AUG GGA ACA ACA GAA GUC GAC AGU GAG C 5'. Stealth RNAi negative control duplex (NC siRNA), whose GC content is similar to that of the Stealth RNAi described above, was used as negative control.

NC siRNA is designed to minimize sequence homology to any known vertebrate transcript and is ideal for use in RNA interference (RNAi) experiments as a control for sequence-independent effects following Stealth RNAi delivery in any vertebrate cell line. Treatment of cells with siRNA was done as described previously,27) with minor modifications. siRNAs (128 nmol) were transfected for 5 h into Rsa cells using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Forty-eight h after transfection, the cells were detached from the test dish and used in experiments.

RT-PCR. Total RNA was isolated from control, sample-treated Rsa cells, untransfected Rsa cells, and Rsa cells that had been transfected with siRNA duplexes using TRIZOL Reagent (Invitrogen), by a method described previously.26) After treatment with deoxyribonucleic acid (DNA) to eliminate possible DNA contamination, first-strand cDNA synthesis was carried out using 1 μl of RNA and 0.5 μg of oligo (dT). An aliquot (1 μl) of first-strand cDNA was used, together with 200 nmol of each specific primer, PCR buffer (10 mmol Tris–HCl pH 8.3, 50 mmol KCl, and 1.5 mmol MgCl₂) and 1 unit of AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA) to give a total volume of 25 μl. Amplification was performed in a Takara thermal cycler (model TP-400), as follows: initial denaturation at 95 °C for 10 min, followed by 25–30 repeated cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The following sets of primers were used: for GRF78, 5'-GGTC-TTCATCGGAAAGGACTCCT-5' and 5'-CCATCTGTTAGGTT-CTTGAGG-3'; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GACCTCAACATCAGGGTCTGACG-3' and 5'-TGGCCTGTGAAGTCAGGAGGAC-5'. The PCR products were visualized after electrophoresis in 2% agarose gels by ethidium bromide staining. The relative amounts of amplified DNA were semi-quantified using Multi Gauge Ver2.2 image analyzing software (Fuji Photo Film).

Statistical analysis. All experiments were repeated at least 3 times independently, and statistical analysis was performed by one sided Dunnett-Test with SPSS 12.0.23 (SPSS Japan, Tokyo).
Results

Effects of miso samples on RSa cells

To determine the optimal concentration of miso sample in the culture medium for use in the cell mutability tests, colony survival capacity was examined in RSa cells cultured with and without the two kinds of miso sample, KU and IG (Fig. 1). The cytotoxicity of the IG sample was slightly greater than that of the KU sample (Fig. 1). Both miso samples showed decreased cell survival rates at 10% concentration, but no notable decrease was observed at concentrations of less than 1%. Colony survival capacity was over 85% at a concentration of 1% for both miso samples (Fig. 1). The cell lethality of IG was higher than that of KU, possibly due to a higher NaCl concentration. An MTT assay also showed that miso concentrations of less than 1% were not cytotoxic after 48 h of incubation (data not shown), as miso contains a series of Maillard reaction products that contain intensely colored chromophores. In the MTT assay, these colored Maillard reaction products can significantly enhance formazan formation.

Effect of miso samples on GRP78 expression and the mutability of the RSa cells

When RSa cells were cultured with KU and IG samples at a concentration of 1%, the expression levels of GRP78 mRNA and protein were enhanced as compared with the mock-treated cells (Fig. 2A and B). To determine whether miso samples suppress the mutability of RSa cells, K-ras codon 12 mutation assay and OuaR mutation tests were performed. A black dot, indicating a base substitution mutation, was detected after hybridization of PCR products from genomic DNA of SW480 cells containing the K-ras codon 12 mutation, and this was used as a positive control (Fig. 3A). Under the assay conditions, the signal of the black dot was clearly enhanced after UVC irradiation in the mock-treated RSa cells (Fig. 3A). In the miso sample-treated RSa cells, the signal of the black dot was not enhanced after UVC irradiation (Fig. 3A). Furthermore, the miso sample-treated RSa cells showed a reduced incidence of UVC-induced OuaR mutation frequency as compared with the mock-treated RSa cells (Fig. 3B).

Effects of GRP78 siRNA on the suppression of UVC mutagenicity by the miso samples

To determine further whether GRP78 expression levels are involved in the modulation of RSa cell mutability by miso samples, expression was inhibited by GRP78 siRNA transfection (Fig. 4A and B). In the GRP78 siRNA-transfected cells, the cellular amounts of GRP78 mRNA and protein decreased in the KU and IG treated cells as well as in mock-treated cells, while no decrease was observed in the NC siRNA-transfected cells (Fig. 4A and B). The decrease observed was up to 60% of the NC siRNA control (Fig. 4B). In the RSa cells with GRP78 siRNA transfection that had been treated with either miso sample, the dot signal of K-ras codon 12 mutations was enhanced after UVC irradiation (Fig. 4C). The OuaR mutation frequency in the cells with GRP78 siRNA transfection and miso sample treatments also increased after UVC irradiation, to an extent similar to that of the NC siRNA-transfected cells (Fig. 4D).

Fig. 1. Effects of Miso Samples on Cell Survival. RSa cells were treated with the indicated concentrations of miso samples (KU and IG) for 1 h. After treatment, the cells were grown in fresh medium containing 5% CS in 100-mm dishes for about 14 d, and then stained with 0.2% methylene blue in 30% methanol. Data are means ± SD for three experiments. A, Kurasaigetsu-usujiomiso (KU); □, Igou-miso (IG).

Fig. 2. Effects of Miso Samples on GRP78 Expression. Cells were cultured with and without miso sample (KU and IG) at a concentration of 1% for 24 h. A, mRNA levels were analyzed by RT-PCR. The PCR reaction cycles indicated in brackets were predetermined (data not shown) to allow semiquantitative comparisons of the cDNAs to be developed using identical reverse transcriptase reactions. PCR products were separated on 2% agarose gels stained with ethidium bromide.housekeeping gene GAPDH was amplified as an internal control. B, Cell lysates were prepared after miso treatment and subjected to immunoblotting analysis of the GRP78 and β-actin proteins, as described in “Materials and Methods.” Data are means ± SD for three experiments. * indicates p < 0.05 versus untreated cells.
Effects of miso components on UVC mutagenicity

The effects of the miso components, rice-koji and HEMF, were next examined on the mutability of RSa cells. The cell survival rate was measured by colony survival assay (Fig. 5A) and MTT assay (data not shown). The cells treated with rice-koji and HEMF showed more than 80% survival rates at concentrations of less than 10% and 1 mmol, respectively (Fig. 5A and B). On the basis of these results, we selected a 1% concentration of rice-koji and 1 mmol HEMF for further studies, since the dose of rice-koji roughly equaled that used in the experiments with the KU and IG samples. The dose of HEMF was roughly equal to that in soybean sauce.28)

The expression levels of GRP78 mRNA and protein increased slightly, at about 1.5 fold of the mock-treated RSa cells cultured with rice-koji and HEMF (Fig. 6A and B). Moreover, in the rice-koji- and HEMF-treated cells no UVC-induced increases in the K-ras codon 12 mutation were detectable (Fig. 6C), and the UVC-induced OuaR mutation frequency decreased in the HEMF treated cells as compared with the mock-treated cells (Fig. 6D).

We examined to determine whether GRP78 siRNA transfection affects UVC mutagenicity in miso component-treated RSa cells. In the GRP78 siRNA-treated cells, the GRP78 mRNA and protein levels decreased to about 50% of the levels in the NC siRNA treated cells.
Fig. 7A and B). The siRNA-treated cells that had been treated with rice-koji and HEMF showed UVC-induced enhancement of the K-ras codon 12 mutation (Fig. 7C), and the UVC-induced OuaR mutation frequency of the cells increased to an extent similar to that of the NC siRNA-treated cells (Fig. 7D).

**Discussion**

Modulation of cell mutability via chaperone expression in Japanese miso-treated human cells is an intriguing possibility. The present results indicate that the levels of GRP78 mRNA and protein expression were increased by treatment of RSa cells with KU and IG miso, rice-koji, and HEMF (Figs. 2 and 6), and that pretreatment of RSa cells with miso samples resulted in suppression of UVC mutagenicity (Figs. 3 and 6C–D). GRP78 functions as a molecular chaperone, and it can bind to misfolded proteins and unassembled complexes. Expression of it is induced in response to endoplasmic reticulum (ER) stress resulting from factors such as aging, hypoxia, and low carbohydrate levels. The mechanism by which this increased expression was mediated by the miso samples remains unclear, but the intimate relationship between GRP78 upregulation and the suppression of cell mutability is evident in the results of the GRP78 siRNA experiments (Figs. 4 and 7).

In previous studies, dietary supplementation with long-term fermented miso has been found to act as a chemopreventive agent for gastric carcinogenesis and colon carcinogenesis in rats. HEMF is an effective inhibitor of benzo[a]pyrene (BP)-induced mouse forestomach neoplasia. As part of its anticarcinogenic action, HEMF can affect the reactive oxygen species (ROS) concentration and prevent oxidative DNA damage caused by ROS at the post-initiation stage. We
NC siRNA control
GRP78 siRNA
Mock Rice-koji HEMF

GRP78
(GAPDH)

NC siRNA control
GRP78 siRNA
Mock Rice-koji HEMF

β-Actin

UVC − − + + + +
GRP78 siRNA − − + + + +
Components − − − − + + SW480

Fig. 7. Effects of GRP78 siRNA Transfection on the UVC-Induced Mutagenicity of the Miso Component-Treated Cells. Forty-eight h after GRP78 siRNA or NC siRNA transfection, mRNA levels were analyzed by RT-PCR (A). PCR reaction cycles are indicated in brackets. Housekeeping gene GAPDH was amplified as an internal control. B, Cell lysates were separated by SDS–PAGE and analyzed by immunoblotting analysis using anti-GRP78 and anti-β-actin antibodies. β-Actin signals were used as a loading control. C–D, Forty-eight h after siRNA transfection, cells were treated with and without miso components for 24 h and then irradiated with UVC (6 J/m²). The mutability of the RSa cells was determined by K-ras codon 12 mutation assay (C) and the Oua⁶ mutation test (D), as described in “Materials and Methods.” Data are means ± SD for three experiments.

reported recently that downregulation of GRP78 in RSa cells reduces DNA repair capacity in the nucleotide excision repair pathway. Nucleotide excision repair, a highly conserved DNA repair system in human cells, is essential for protection against UV-induced DNA damage, such as the generation of (6-4) photoproducts and cyclobutane thymine dimers. Thus one mechanism that might induce hypo-mutative changes in RSa cells pre-cultured with KU and IG miso, rice-koji, and HEMF, is enhancement of DNA repair functions by upregulation of GRP78 expression.

There are three kinds of miso: rice-koji miso, soybean-koji miso, and barley-koji miso. All KU and IG are rice-koji miso. Rice-koji was also tested, and appeared to have suppressive effects on UVC mutagenicity via GRP78 upregulation. In addition to rice-koji miso, some products of soybean-koji miso were also tested, and were found to have effects similar to those of rice-koji miso products. However, barley-koji miso products do not appear to have the same suppressive effects as the other miso types (unpublished results).

Thus, rice-koji miso and soybean-koji miso might contain compounds that induce suppressive activity as to cell mutability, such as HEMF. The activity might be due to the action of chaperones such as GRP78.

As well as GRP78 expression, we have found that the culture of RSa cells with miso samples results in increased expression of HSP27 (data not shown). Recently we suggested that HSP27 might play a role in DNA repair, regulating the metabolism of nucleotide excision repair enzymes in the nucleus. The molecular mechanisms underlying the suppression of UVC mutagenicity by miso samples remain unknown, but we speculate that changes in the expression of chaperones such as HSP27 and GRP78 are implicated in these mechanisms.

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References