CHAPTER II

MATERIALS AND METHODS

1. Cell lines and culture conditions

A rat normal gastric mucosal cell line (RGM1) and an N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced gastric cancer cell line (RGK1) were cultured in Dulbecco's modified Eagle's medium / Nutrient F-12 (DMEM /F-12; Sigma-Aldrich Inc., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT., USA) at 37 ^{o}C in humidified air containing 5 % CO_2.

2. Mn-SOD cDNA transfection of rat cells

The RGM1 and RGK1 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen Carlsbad, CA., USA) according to the manufacturer's instructions. Briefly, cells were plated 24 hours before transfection at 70 % confluence in a 60-mm dish. The cells were stably transfected with 8 μg of the pCR3.1-Uni plasmids containing a sense human Mn-SOD cDNA with mitochondrial targeting sequence (MTS) insert, linearlized by Sca I, in serum free Dulbecco's modified Eagle medium. The controls were transfected with pCR3.1-Uni plasmids without human Mn-SOD cDNA insert, linearlized by Sca I. Stable clones of both Mn-SOD and control plasmid-transfectants were selected with genicetin (G418 disulfate ; Nakalai Tesque, Inc., Kyoto, Japan) at a final concentration of 500 μg/ml. Selected cellular clones

which expressed Mn-SOD and selectable marker alone were used in all experiments. Selected clones were routinely maintained in DMEM containing 10 % fetal bovine serum and 500 μg/ml genicetin at 37 ^{o}C in humidified air containing 5 % CO_2.

3. SOD activity gel assay

RGM1, RGK1 and ten of stable clones of each Mn-SOD transfected cells were use for detection of SOD activity. Cells were sonicated in 50 mM potassium phosphate buffer (pH 7.8). 20 μg total gramprotein/lane was electrophoresed through a non-dissociating riboflavin gel consisting of 5 % stacking gel (pH 6.8) and 12 % running gel (pH 8.8) at 4 °C. To visualize the SOD activity, gels were first incubated in 2.43 mM nitro blue tetrazolium (NBT; Wako Pure Chemical Industries, Ltd., Osaka; Japan) in de-ionized water for 20 min and then in 0.028 mM riboflavin (Wako Pure Chemical Industries, Ltd., Osaka; Japan), 280 mM N,N,N',N'-Tetramethylethylenediamine (TEMED; Wako Pure Chemical Industries, Ltd., Osaka, Japan) in a 50 mM potassium phosphate buffer (pH 7.8) for 15 min in the dark. Gels were then washed in de-ionized water and illuminated under fluorescent light until clear zones of SOD activity were evident. For stable clones of Mn-SOD transfected cells in RGM1 and RGK1 were determined the SOD activity and selected the positive clones for using in further study. The Mn-SOD transfected RGM1cells (stable clones 5, 6, and 8) and Mn-SOD transfected RGK1 cells (stable clones 1, 2, 3, 9 and 10) were used in all experiments.

4. Cell growth assay

To determine the growth characteristics of the cells, they were plated in 30-mm tissue culture dishes at 5×10^4 cells per dish and cultured for 5 days. They were then trypsinized and the number of cells was counted using a Particle Counter PA-2000 (Erma Inc., Tokyo, Japan) daily. All experiments were repeated three times.

5. Quercetin treatment

The different concentrations of quercetin (Sigma-Aldrich Inc., St. Louis, MO., USA.) (30, 100 and 200 μM) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich Inc., St. Louis, MO., USA.), but the final concentration of DMSO in the culture medium did not exceed 0.1%**.** The quercetin was directly added to cell cultures at the indicated concentrations. As control the cells were incubated with 0.1% DMSO.

6. Determination of reactive oxygen species production

Hydroxyphenyl Fluorescein (2-(6-(4′-hydroxy) phenoxy-3H-xanthen-3-on-9-yl) benzoic acid (HPF) (Sekisui Medical Co., Ltd., Tokyo, Japan), a fluorescent dye for selectively detecting highly reactive oxygen species (hROS), was used in the present study. HPF immediately reacts with hROS such as hydroxyl radical and peroxynitrite and the fluorescence intensity greatly increases (Setsukinai *et al*., 2003). Glass-bottomed (35-mm) dishes with monolayers after quercetin treatment (30, 100 and 200 μM) for two and twenty-four hours, were prepared for staining with HPF. 10 μM HPF was added to the cells before incubation for 15 minutes at 37°C. Bio-images

of HPF were obtained using a CSU-10 confocal laser scanning unit coupled to an IX90 inverted microscope with a UPlanAPO ×20 objective lens and a C5810-01 color chilled 3CCD camera. HPF was excited at 488 nm and the fluorescence emission was filtered using a 515-nm barrier filter. The intensity of the laser beam, the exposure time of the 3CCD camera, and the gain of the amplifier were held at 500 μW, 1.0 sec, and 18 decibels, respectively, to allow quantitative comparisons of the relative fluorescence intensity of the cells between groups. Average fluorescence intensity per cell was determined using IPLab Spectrum version 3.0 software (Scanalystics Inc., Fairfax, VA., USA)

7. Immunofluorescent staining for 4-hydroxynonenal

Glass-bottom (35 mm) dishes with monolayers were prepared for immunofluorescent staining with monoclonal antibody directed against proteins modified with the major membrane lipid peroxidation product, 4-hydroxynonenal (4-HNE). Two and twenty-four hours after quercetin treatment (30, 100 and 200 μM), cells were fixed with 4 % formaldehyde/PBS at room temperature for 30 minute, rinsed twice with PBS. Then membranes were permeabilized by incubation in 95 % ethanol with 5 % acetic acid for 10 minutes. After washing with PBS twice, cells were incubated for 30 minutes in a blocking serum (0.1 % bovine serum albumin in PBS), and for 24 hours in mouse anti 4-HNE monoclonal antibody (NOF Corp., Tokyo, Japan) at 1:200. The cells were rinsed twice with PBS, and incubated with Alexa Fluor 488 goat anti mouse IgG (Invitrogen, Carlsbad, CA., USA) for 24 hours at room temperature. Fluorescence was visualized with CSU-10 confocal laser

scanning unit. Average fluorescence intensity per cell was determined using IPLab Spectrum version 3.0 software (Scanalystics Inc., Fairfax, VA., USA)

8. Immunofluorescent staining for microtubule-associated protein light chain 3 (MAP-LC3)

Microtubule-associated protein light chain 3 (MAP-LC3) is a major constituent of the autophagosome, a double membrane structure that sequesters the target organelle or protein and then fuses with lysosomes where the contents are degraded. The MAP-LC3 was used as a marker for autophagy. Cells were grown on glass bottom dishes and fixed in 4% (w/v) formaldehyde, diluted in phosphate-buffered saline (PBS) for 30 minutes and membranes were permeabilized by incubation in 95 % ethanol with 5 % acetic acid for 10 minutes. After washing with PBS five times, cells were then blocked for 30 minutes with 1% bovine serum albumin (BSA; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at room temperature, followed by an overnight incubation at 4°C with the goat anti-MAP-LC3 IgG at 1:200 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA., USA). The next day, cells were washed three times with PBS and incubated overnight with Alexa Fluor 488 donkey anti goat IgG at 1:200 (Invitrogen, Carlsbad, CA., USA). Fluorescence was visualized with CSU-10 confocal laser scanning unit (Yokogawa Electric Co.,Tokyo, Japan). Average fluorescence intensity per cell was determined using IPLab Spectrum version 3.0 software (Scanalystics Inc., Fairfax, VA., USA)

9. Cell staining procedures with Hoechst 33342

Cells grown on glass-bottomed (35 mm) dishes (MatTek Corp., Ashland, MA.,USA) were stained with the fluorescent dye Hoechst 33342 (Molecular Probes, Eugene, OR., USA). Hoechst 33342 is a DNA stain that binds preferentially to A-T base pair. This dye is often used to distinguish condensed pycnotic nuclei in apoptotic cells. Twenty-four hours after quercetin treatment, the cells were fixed for 30 minutes in a solution containing 4 % formaldehyde in PBS and washed with PBS three times. Then the cells were incubated in PBS with 1μg/ml of the dye for 30 minutes in dark. The cells were washed twice with PBS. Fluorescence was visualized using an IX90 inverted microscope with an UPlanAPO ×20 objective lens (Olympus Optical Co., Tokyo, Japan). The dye was excited at 340 nm and emission was filtered with a 510 nm barrier filter. Photographs of microscope fields were taken using a C5810-01 color chilled 3CCD camera (Hamamatsu Photonics. K. K., Hamamatsu, Japan). The percentage of apoptotic cells in each culture dish was determined.

10. Statistic Analysis

A statistical analysis was performed by descriptive data and *t*-test analyses were performed with SPSS software version 10. A P value of less than 0.05 was considered to be statistically significant.