CHAPTER III

MATERIALS AND METHODS

Cultures and treatments of microglial cells

To elucidate the role of iron on the expression of microglial matrix metalloproteinases, I have selected the HAPI cells, which are an immortalized rat microglial cell line possessing the characteristics of microglia/brain macrophages as previously described (Cheepsunthorn *et al.*, 2001a), as an in vitro model for these studies. To begin the experiments, HAPI cells were plated at density $5x10^4$ cells/well in 24-well plates and grown in 5% fetal bovine serum (FBS) supplemented Dulbecco's modified Eagle's medium (DMEM). Both were purchased from HyClone. When cells reached 80% confluence, the serum concentration was decreased to 0%. Cells were then allowed to grow in this serum-free medium for 24 h before the experiments. To begin the treatments, the medium in each well was completely removed. Cells in each well were gently washed with serum-free DMEM once before adding freshly prepared serum-free DMEM containing either 1 μg/ml lipopolysaccharide (LPS) (Sigma); 50 μg/ml ferric ammonium citrate (FAC) (Sigma), or LPS in the presence of FAC. Cells in serum-free DMEM served as the untreated control. Times for treatments were indicated in each experiment (see below).

RNA Isolation

After 6 h treatments, total RNA from each condition was isolated using Trizol reagent (Invitrogen). Briefly, the medium in each well was completely removed. The cells were washed once with phosphate-buffered saline (PBS), pH 7.4. Then, Trizol reagent (300 μl) was added into each well. The homogenates were placed on a low speed rotator for 5 min at room temperature. This is to allow the complete dissociation of nucleoprotein complexes. Next, the homogenates were transferred into steriled 1.5

ml tubes. Then, chloroform (60 µl) was added into each tube followed by vortexing vigorously for 15 seconds. The mixture was allowed to stay at room temperature for 5 min before centrifugation at 12,000 g for 15 min at 4°c. RNA in the colorless upper aqueous phase was carefully transferred to a fresh tube. Then, isopropanol (150 µl) was added into each tube. Each sample was stored at room temperature for 10 min and centrifuged at 12,000 g for 10 min at 4°c. RNA will precipitate and form a white pellet at the bottom of the tube. The supernatant was carefully removed before adding iced-cold 75% ethanol (300 µl) into each tube to wash RNA pellet for 5 min. Subsequently, the pellet was centrifuged at 10,000 g at 4°c for 5 min. At the end of procedure, the RNA pellets were allowed to air dry briefly. It is important not to let the RNA pellet dry completely as it will greatly decrease its solubility. Dissolve the RNA pellets in RNase free water. The amount of RNA obtained by spectophotometry was calculated using the formular below.

RNA (µg) = Absorbance at 260 nm x 40 x dilution factor

The solution containing RNA sample can be stored at -80°c until use.

Revers transcription (cDNA synthesis)

A volume of 2 µg total RNA from each sample was added into the reaction mixture containing 10x reaction buffer, 25mM $MgCl_2$, dNTPs, random primer, Ribonuclease inhibitor, AMV Reverse transcriptase and RNase free water. All reagents were purchased from Promega. Each sample was kept at room temperature for 10 min incubated at 42°c for 60 min followed by inactivation at 99°c for 5 min. Then, distilled water was added into each sample to a final volume of 100 µl. At this point, each sample can be kept at -20°c until use. See appendix A for preparing the reaction mix.

Polymerase Chain Reaction (PCR)

The PCR reaction was performed in a total volume of 25 μl. A 5 μl of cDNA (as described above) was use as templates for subsequent PCR reaction in the perkin Elmer Thermocycle. PCR reaction was composed of distilled water, PCR buffer, dNTP mix, Taq DNA polymerase (Promega) and primer pairs (see Table 4). To ensure there was no genomic DNA contamination in the RNA samples, the PCR reaction was performed without reverse transcription. As a control reaction, the RNase free water was replaced the RNA samples in the PCR protocol. Subsequently, amplified PCR products were visualized by gel electrophoresis. Briefly, a 10 μl of each PCR product was mixed with 2 μl of loading buffer, vortexed, spined, and then loaded on to 1.5 % agarose gel containing ethidium bromide. The gel was run at 95 volts until front dye reaches the end of gel. Estimate size of PCR products was compared to the DNA marker (100 bp ladder, Fermentas). No PCR products were observed in any control reactions (data not shown). See appendix A for preparing the reaction mix.

Table 4 Specific primer for iNOS, TNF-α, IL-1β, MMP-1, MMP-10, and GAPDH

Gene	Primer sequences (5′ ⟶ 3′)	bp.	PCR Protocols
iNOS	ATC CCG AAA CGC TAC ACT TCC	311	1
	GGC GAA GAA CAA TCC ACA ACT C		
TNF-α	ATC CGA GAT GTG GAA CTG GCA	659	2
	CCG GAC TCC GTG ATG TCT AAG T		
IL-1β	GCT ATG GCA ACT GTC CCT GAA C	200	3
	TGA GTG ACA CTG CCT TCC TGA A		
GAPDH	AAG CTC ACT GGC ATG GCC TTC C	300	4
	TTG GAG GCC ATG TAG GCC ATG AG		
MMP-1	CTT GCG GGA ATC CTG AAG AAG TCT A	521	5
	GCC AAG CTC ATG GGC AGC AAC AAT		
MMP-10	GGA GAT GAC AGG GAA GCT GGA CT	870	6
	TCG GAC TGC CCA GAA CTG ACT TC		

Protocol 1 Pre denature at 94°c for 4 min followed by 35 cycles of 94°c for 1 min, 58°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 2 Pre denature at 94°c for 4 min followed by 35 cycles of 94°c for 1 min, 60°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 3 Pre denature at 94°c for 4 min followed by 32 cycles of 94°c for 1 min, 60°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 4 Pre denature at 94°c for 4 min followed by 35 cycles of 94°c for 1 min, 61°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 5 Pre denature at 94°c for 4 min followed by 32 cycles of 94°c for 1 min, 62°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 6 Pre denature at 94°c for 4 min followed by 32 cycles of 94°c for 1 min, 61°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Western blot analysis

An immunoblot analysis of an iron storage protein, ferritin was included in these studies to demonstrate that iron added into the cell culture medium could enter the cells and was sufficient to induce cellular response in microglia. The expression of ferritin in the presence of iron, LPS, or LPS plus iron was determined using rabbit anti rH-ferritin antiserum (a gift from proffessor James R Connor, College of Medicine, The Pennsylvania State University, USA). The specificity of this antiserum was previously determined by western blot analysis (Cheepsunthorn *et al.*, 1998). Briefly, following 6 h treatments, total cells from each sample were collected and lysed in lysis buffer containing 1% Triton-X 100. Total protein content in each sample was quantified using the Bio-Rad protein determination assay (see appendix B). Then, equal amounts (40 μg) of total protein from each sample was mixed with sample buffer containing beta-mercaptoethanol, boiled for 10 min, and separated on 10% SDS-PAGE. Then, proteins were transferred to PVDF membranes, blocked in 5% dry milk for 1 h

at room temperature, rinsed with TBS, and incubated overnight at 4°c in the primary antibody (1 : 200). Subsequently, the membranes were washed with TBS containing 1% Tween 20 and incubated in the secondary antibody (1 : 500) goat anti-rabbit IgG peroxidase conjugate (Sigma) for 1 h at room temperature. Bound antibodies were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma). Briefly, the immunoblots were incubated for 5 min at room temperature in DAB solution prepared by dissolving DAB (6 mg) in TBS (10 ml) in the presence of 30% H_2O_2 (100 µl) and $NiCl_2$ (50 µl). The reaction can be stopped by rinsing the blot with TBS.

For the detection of MMP-1, the serum free medium from each condition was collected at 48 h by separating from the cell monolayer for concentration. Briefly, the medium was centrifuged at 1,500 g for 15 min to remove cellular debris. The protein in the supernatant was concentrated approximately 25 folds by centrifugation at 12,000 g in centriprep-10 concentrators. Concurrently, the cell monolayer was washed with serum free-DMEM. The cell pellet from each condition was collected by centrifugation at 200 g for 4 min at 25°c. The supernatant was carefully removed. The pellets were resuspended in iced-cold lysis buffer (see appendix B). Total Protein concentration in both concentrated media and cell lysates were determined using the Bio-Rad protein determination assay (see appendix B).

Equal amounts (60 µg) of total protein from each sample were boiled for 10 min and separated on 10% SDS-PAGE. Then, proteins were transferred to PVDF membranes, blocked in 5% dry milk for 1 h at room temperature, rinsed with TBS, and incubated overnight at 4°c in the primary antibodies; a mouse monoclonal anti-rat MMP1 (1 : 200, Calbiochem) or in a mouse monoclonal anti-human beta actin (1 : 1000, Sigma). Subsequently, the membranes were washed with TBS containing 1% Tween-20 and incubated in appropriated secondary antibody a goat anti-mouse IgG (H+L) peroxidase conjugate (1 : 2000; Sigma) for 1 h at room temperature. Bound antibodies were visualized with the chemiluminescent substrate for the detection of

HRP purchased from Pierce. See appendix B for assembly of apparatus, preparation of slab gel and buffer preparations.

Zymography

MMP-9 and MMP-2 releases were measured by zymography in the serum-free supernatant collected from cultures of either rat microglial or human fibroblast according to the protocol previously described by Pattamapun *et al.*, 2003; Chauvet *et al.*, 2001. The serum free medium from each treatment was collected at 48 h by separating from the cell monolayer and centrifuged at 1,500 g for 15 min to remove cellular debris. The protein in the supernatant was concentrated approximately 25 folds by centrifugation at 12,000 g in centriprep-10 concentrators. Total Protein concentration in the concentrated media was determined using the Bio-Rad protein determination assay. Samples (300 μg/lane for MMP-9 and 120 μg/lane for MMP-2) were mixed with 2x non-reducing sample buffer and incubated for 10 min at room temperature. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis containing 1 mg/ml gelatin (Sigma). After eletrophoresis, gels were washed in renaturing buffer for 45 min to remove SDS and restore enzymatic activity. The gels were then incubated overnight at 37°c in developing buffer The gelatinolytic activity, after staining with Coomessie blue, appeared as a clear band against a dark blue background of gelatin gel. The relative molecular weights of the clear bands were determined using prestained protein markers (Pierce). See appendix C for assembly of apparatus, preparation of slab gel and buffer preparations.

Phagocytosis

To assay phagocytic activity of rat microglial cells under different cell culture conditions, cells were plated at $5x10^4$ cells/well in 96-well plates in the presence of 5% FBS and allowed to grow for 24 h. To begin the experiment, the medium was

removed and fresh serum-free DMEM containing either 1 μg/ml LPS, 50 μg/ml ferric ammonium citrate (FAC), or LPS plus FAC was added to the cells. Untreated cells in serum-free DMEM were served as a control group. At 24 h later, the medium was removed. The cell monolayers were washed twice with freshly prepared serum-free DMEM before adding a assay mixture containing p-Nitro blue tetrazolium chloride (NBT) (Sigma) and zymosan A suspension (final concentration 300 μg/ml) (Sigma). Serum-free DMEM containing only NBT was served as negative control previously described by Schopf *et al.*, 1984. (see appendix D). Cells were incubated at 37°c in the atmosphere containing 5% CO_2 for 1 h. All assays were set up in duplicate. Then, NBT/zymosan solution mixture was removed. Cells in each well were washed with absolute methanol 3 times and left air dry. KOH (2 M, 120 μl) was added to each well followed by DMSO (140 μl). The contents in each well were mixed vigorously and the plates were read the absorbency at 570 nm.

Statistical analysis

All data were presented as means and standard error of means (mean ± SEM). One way analysis of variance (one-way ANOVA) was used to compare the significance between the control and the treatment groups. The p-value of less than 0.05 was set for the significant difference.