CHAPTER III

MATERIALS AND METHODS

Animals

Male Wistar rats were obtained from the National Laboratory Animal Center of Salaya Campus, Mahidol University. Each weighted 90-100 g

Cells

Mouse fibroblasts (Balb/c 3T3; ATCC Number: CCL-163) were grown on coverslips in Dulbecco's Modified Eagle's (DME) medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37° C with 5% carbon dioxide (CO_2)

Human cervical cancer cells (HeLa cells; ATCC Number: CCL-2) were grown on coverslips in Minimum Essential Medium supplemented with 10% fetal calf serum 100 units/ml penicillin and 100 μg/ml streptomycin at 37° C with 5% carbon dioxide (CO_2)

Chemicals and Reagents

Sodium arsenite and other chemicals were obtained from Sigma unless indicated otherwise. Dulbecco's Modified Eagle's (DME) medium (Cellgro ®), Minimum Essential Medium (MEM) (Life Technologies ®), fetal calf serum (FCS) (HyClone ® Laboratories, Inc.), penicillin-strptomycin (10,000 units/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate in 0.85% saline (Gibco ®), trypsin-EDTA 10x (0.5% trypsin and 5.3 mM EDTA-4Na) (Life Technologies ®), phosphate buffer saline (PBS) pH 7.4 (Gibco ®), Fluoromount-G (Southern Biotechnology Associates; Inc.), sodium borohydride (Fisher Scientific), dimethyl sulfoxide ultrapure (DMSO) (Johnson Matthey Electronics), 10% formaldehyde ultrapure EM grade (methanol-free) (Polyscience, Inc.), rubbing alcohol (70%) (Pharmaco), microscope immersion oil low viscosity (150cs) (Stephens Scientific), monoclonal anti-α-actinin (Sigma), monoclonal anti-vinculin (Sigma), Alexa Fluor 488 phalloidin (Molecular Probes), anti-mitochondrial HSP70 (Affinity Bioreagents, Inc), Aleaxa Fluor 568 (Molecular Probles), YLI/2 Rat

immunoglobulin and YOL/1 rat immunoglobulin (Murray Lab.), anti-rat Texas Red (Molecular Probes), anti-PAK, anti-SAPK/JNK, anti-phospho-SAPK/JNK, anti-p38 MAP kinase and anti-phospho-p38 MAP kinase antibodies were purchased from Cell Signaling Technology, in situ cell death detection kit (Roche Diagnostics), fluorescein, anti-rat interleukin-6 kit (Endogen).

Equipment

Fluorescence microscope model Olympus T041, laser scanning confocal microscope model LSM 510 (Carl Zeiss Microscopy), orbital shaker (Belcol Biotechnology), incubator model 3158 (Forma Scientific, Inc.), microscope (Carl Zeiss Microscopy), biological safety cabinet model U1276 (Forma Scientific), clinical centrifuge (Diamond/IEC Division),UV/visible spectrometer, autoclave and hot air oven model SV-120 (Amsco Scientific), micro slide glass thickness 0.8-1.0 mm (Matsunami), coverslip 18x18 mm. thickness 0.12-0.17mm. (Matsunami), slide boxes (Labscientific, Inc.), polystyrene dishes 100x20 mm. (Fisher Scientific), 6-well tray (Fisher Scientific), serological pipets (Fisher Scientific), transfer pipet (A. Daigger& Company, Inc.), balance Sartorius model 1712 MP8 (Brinkmann Instruments Co.), balance Ohaus model CT 200-S, magnetic stirrer (Bellco Class, Inc.), pH meter (Orion Research).

In Vitro Assays

Immunofluorescence Microscopy

Immunofluorescence microscopy was used to observe the formation of stress fiber (F-actin), focal adhesions (vinculin, alpha-actinin), microtuble, mitochondrial localization, nucleus, filopodia and lamellipodia formation.

Formaldehyde Fixation (Allan, 2000)

1. Assemble all equipment and solutions before taking the cells on coverslips out of the incubator.

2. Dilute stock formaldehyde to 3% in phosphate buffer saline (PBS) pH 7.4 and transfer to 6-well trays.

3. Remove coverslips from the culture dish, dip in PBS (in a beaker) to wash off excess culture medium, then place in the formaldehyde well. For this, and all subsequent steps in the protocol, the cells must be uppermost. Fix for 3 minutes on orbital shaker at room temperature.

4. Aspirate off the 3% formaldehyde in PBS by vacuum suction and wash the coverslips with PBS 3 times, for 1 minute each, on orbital shaker at room temperature.

5. Permeabilize cells, in 0.25 % Triton X-100 in PBS for 30 minutes on orbital shaker at room temperature

6. Aspirate off the 0.25 % Triton X-100 in PBS by vacuum suction and wash the coverslips with PBS 3 times, for 1 minute each, on orbital shaker at room temperature.

7. Blocking, in 2% bovine serum albumin (BSA) in PBS and 1:50 dilution of normal goat serum (NGS) for 30 minutes on orbital shaker at room temperature

8. Aspirate off the blocking agent by vacuum suction and wash the coverslips with PBS 3 times, for 1 minute each, on orbital shaker at room temperature.

9. Antibody labeling, dilute the primary antibody in 1% BSA in PBS and 1:2000 dilution of Tween 20, and put a drop (200 μl) of each antibody onto a sheet of Parafilm that has been taped to the bench surface.

10. Take a coverslip out of PBS using fine forceps, and blot the edge of the coverslip on towel paper. Wipe excess PBS from the forceps and the back of the coverslip using the torn edge of a piece of paper towel, without allowing the cells to dry out. Carefully place the coverslip, cells downwards, onto the drop of antibody.

11. Incubate at room temperature for 30 minutes.

12. Raise the coverslip off the Parafilm by pipetting PBS at the edge of the coverslip, which serves to raise the coverslip from the Parafilm surface. Then lift coverslip using forceps, and place in PBS in a 6-well tray.

13. Wash the coverslips with PBS 3 times, for 1 minute each, on orbital shaker at room temperature, take care to prevent the coverslip drying out.

14. Repeat steps 9-13, but using diluted fluorescently-labeled secondary antibody. Coverslips are then ready for mounting.

15. Take coverslips after their final PBS wash and dip them briefly in water. Wipe off excess water from the back and edges of the coverslip, and from between the forcep

blades, using paper towel. Too much drying will cause the cells to flatten; too little will result in poor antifade performance, because it will have been diluted.

16. Place the coverslip (cells downwards) onto a drop (10 μl) of the mounting medium of choice (Fluoromount-G) on the microscope slide.

17. Allow the mounting medium to set (5 minutes)

18. Seal around the edges of the coverslip using nail varnish

19. Keep the coverslips in the dark at 4°C (cold room).

To visualize F-actin, cells were incubated with the Alexa Fluor 488 phalloidin. The F-actin will be visualized in green color.

To visualize alpha-actinin, cells were incubated with anti-alpha-actinin mouse immunoglobulin as a primary antibody and then were incubated with Alexa Fluor 568 as a secondary antibody. The alpha-actinin will be visualized in red color.

To visualize mitochondria, cells were incubated with anti-mitochondrial HSP70 mouse immunoglobulin as a primary antibody and then were incubated with Alexa Fluor 568 as a secondary antibody. The mitochondria will be visualized in red color.

To visualize nucleus, cells were incubated with DAPI (4',6-diamidino-2-phenylindole). The nucleus will be visualized in blue color.

Fluorescence photomicroscopy was carried out with appropriate filters for fluorescence detection.

Microtubule Staining (Geuens *et al.*, 1986)

1. Remove cells from incubator, rinse once quickly with Buffer A (Appendix 1) at 37°C, then partially fix and simultaneously partially by shaking for 1 minute in Buffer A + 1% Triton X-100 + 0.5% glutaraldehyde.

2. Complete fixation by shaking in Buffer A + 0.5% glutaraldehyde for 10 minutes at room temperature.

3. Finish permeabilization with Buffer A + 1% Triton X-100 for 30 minutes at room temperature.

4. Rinse with MgPBS (Appendix A) briefly 3 times

5. Reduce and residual free aldehyde groups by incubating in MgPBS + 1 mg/ml $NaBH_4$ for 5 minutes. Repeat twice more with freshly dissolved $NaBH_4$ each time.

6. Rinse with MgPBS briefly 3 times

7. Block with Buffer A + 1% BSA + 1:20 dilution of normal goat serum in MgPBS for 30 minutes at room temperature

8. Rinse with MgPBS briefly 3 times

9. Incubate with 1:2000 dilution of YL1/2 (YLI/2 + YOL/1; 1:1 volume) in MgPBS + 1% BSA for 2 hours at room temperature.

10. Rinse with MgPBS briefly 3 times, then rinse with MgPBS 2 times for 5 minutes each, then rinse with MgPBS 1 time for 30 minutes (= "standard wash").

11. Incubate 1 hour room temperature with Anti-Rat Texas Red as a secondary antibody, followed by standard wash.

12. Place the coverslip (cells downwards) onto a drop (10 µl) of the mounting medium of choice (Fluoromount-G) on the microscope slide.

13. Allow the mounting medium to set (5 minutes).

14. Seal around the edges of the coverslip using nail varnish.

15. Keep the coverslips in the dark at 4°C (cold room).

Laser Scanning Confocal Microscopy

A laser scanning confocal microscope was used for recording the immunofluorescence images including cell area and cell fluorescence intensity measurement.

Study on the Dose-Response Relationship of Sodium Arsenite Effects on the Cytoskeleton, Focal Adhesions and Mitochondrial Localization

Sodium arsenite was added to Balb/c 3T3 cells which were grown on coverslips in culture medium. The final concentrations of sodium arsenite were 0.25, 2.5, 5, 10, and 25 µM. The cultures were incubated at 37°C with 5% CO_2 for 16 hours. Cells were investigated by immunofluorescence microscopy coupled with laser scanning confocal microscopy in order to observe the formation of stress fiber (F-actin), focal adhesions (vinculin, alpha-actinin), microtubule, mitochondrial localization, nuclei, filopodia and lamellipodia formation.

Study on the Non-Specific Tyrosine Kinase Inhibitor (Genistein) or Non-Specific Serine/Threonine Kinase Inhibitor (Staurosporine) Blockage of the Toxic Effects Induced by Sodium Arsenite

Sodium arsenite (final concentration 25 µM) coupled with genistein (final concentration 10, 40, 80, and 100 µg/ml) or staurosporine (final concentration 1, 5, 10, and 20 nM) was added to Balb/c 3T3 cells which were grown on coverslips in culture medium. The cultures were incubated at 37°C with 5% CO_2 for 16 hours. Cells were investigated by immunofluorescence microscopy coupled with laser scanning confocal microscopy in order to observe the formation of stress fiber (F-actin), focal adhesion (vinculin), mitochondrial localization, nuclei, filopodia and lamellipodia formation.

Study on the Epidermal Growth Factor Receptor (EGFR) Inhibitor (4,5-Dianilinophthalimide) or Phosphatidylinositol 3-Kinase (PI3K) Inhibitor (Wortmannin) Blockage of the Toxic Effects Induced by Sodium Arsenite

Sodium arsenite (final concentration 25 µM) coupled with 4,5-dianilinophthalimide (final concentration 0.1, 1, 10, and 100 µM) or wortmannin (final concentration 25, 50, 100 and 200 nM) was added to Balb/c 3T3 cells which were grown on coverslips in culture medium. The cultures were incubated at 37°C with 5% CO_2 for 16 hours. Cells were investigated by immunofluorescence microscopy coupled with laser scanning confocal microscopy in order to observe the formation of stress fiber (F-actin), focal adhesion (vinculin), mitochondrial localization, nuclei, filopodia and lamellipodia formation.

Study on the RNA Synthesis Inhibitor (Actinomycin D) or Protein Synthesis Inhibitor (Cycloheximide) Blockage of the Toxic Effects Induced by Sodium Arsenite

Sodium arsenite (final concentration 25 µM) coupled with actinomycin D (final concentration 0.1, 1, 5, and 10 µg/ml) or cycloheximide (final concentration 5, 10, 20 and 40 µg/ml) was added to Balb/c 3T3 cells which were grown on coverslips in culture medium. The cultures were incubated at 37°C with 5% CO_2 for 16 hours. Cells were investigated by immunofluorescence microscopy coupled with laser scanning confocal

microscopy in order to observe the formation of stress fiber (F-actin), focal adhesion (vinculin), mitochondrial localization, nuclei, filopodia and lamellipodia formation.

Study on the Phosphatidylinositol 3-Kinase (PI3K) Inhibitor and MAP Kinase Inhibitor (Apigenin) Blockage of the Toxic Effects Induced by Sodium Arsenite

Sodium arsenite (final concentration 25 µM) coupled with apigenin (final concentration 25, 50, and 100 µM) was added to Balb/c 3T3 cells which were grown on coverslips in culture medium. The cultures were incubated at 37°C with 5% CO_2 for 16 hours. Cells were investigated by immunofluorescence microscopy coupled with laser scanning confocal microscopy in order to observe the formation of stress fiber (F-actin), focal adhesion (vinculin), mitochondrial localization, nuclei, filopodia and lamellipodia formation.

Detection of Apoptosis in Sodium Arsenite-Exposed Mouse Fibroblasts (In Situ Cell Death Detection Kit, Fluorescein)

Sodium arsenite was added to Balb/c 3T3 cells which were grown on coverslips in culture medium. The final concentrations of sodium arsenite were 0.25, 2.5, 5, 10, and 25 µM. The cultures were incubated at 37°C with 5% CO_2 for 16 hours.

1. Remove coverslip from the culture dish, dip in PBS (in a beaker) to wash off excess culture medium.

2. Fix cell samples with a freshly 4% formaldehyde in PBS, pH 7.4 for 1 hour at room temperature (25°C).

3. Rinse coverslip 2 times with PBS and incubate in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 minutes on ice (4°C).

4. Rinse coverslip 2 times with PBS and dry area around sample with paper towel

5. Add 50 µl TUNEL reaction mixture on sample. To ensure a homogeneous spread of TUNEL reaction mixture across cell monolayer and to avoid evaporative loss, samples should be covered with parafilm.

6. Incubate slide in a humidified chamber for 60 minutes at 37°C in the dark

7. Rinse coverslip 3 times with PBS

8. Mount the coverslip with mounting medium

9. Samples can directly be analyzed under a fluorescence microscope using an excitation wavelength in the range of 450-500 nm and detection in the range of 515-565 nm.

Study on Cervical Cancer Cell (HeLa) Morphology Change Induced by Sodium Arsenite

Sodium arsenite was added to HeLa cells which were grown on coverslips in culture medium. The final concentrations of sodium arsenite were 0.25, 2.5, 5, 10, and 25 µM. The cultures were incubated at 37°C with 5% CO_2 for 16 hours. Cells were investigated by immunofluorescence microscopy coupled with laser scanning confocal microscopy in order to observe the formation of stress fiber (F-actin), focal adhesions (vinculin, alpha-actinin), microtubule, mitochondrial localization, nuclei, filopodia and lamellipodia formation.

Immunoblotting (Appendix B)

PAK, SAPK/JNK, Phospho-SAPK/JNK, p38 MAP Kinase and Phospho-p38 MAP Kinase expression were investigated by immunobloting assay.

Mouse fibroblasts (Balb/c 3T3) were grown on coverslips in Dulbecco's Modified Eagle's (DME) medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37° C with 5% carbon dioxide (CO_2) and cell lines were treated with the following conditions:

1. Sodium arsenite at 0 µM (Control) for 16 hours
2. Sodium arsenite at 25 µM for 16 hours
3. Sodium arsenite at 25 µM + Wortmannin 200 nM for 16 hours
4. Sodium arsenite at 25 µM + Genistein 40 µg/ml for 16 hours
5. Sodium arsenite at 25 µM + 4,5-Dianilinophthalimide 1 µM for 16 hours
6. Sodium arsenite at 25 µM + PP1 (Src-family tyrosine kinase inhibitor) 10 µM for 16 hours
7. Sodium arsenite at 25 µM + Cycloheximide 5 µg/ml for 16 hours
8. Sodium arsenite at 25 µM + Actinomycin D 10 µg/ml for 16 hours

Protein Blotting

1. Aspirate media from cultures; wash cells with 1XPBS

2. Lyse cells by adding 1XSDS sample buffer (500 μl per plate of 10 cm plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.

3. Sonicate for 10-15 seconds to shear DNA and reduce sample viscosity.

4. Heat a 20 μl sample to 95-100°C for 5 minutes; cool on ice.

5. Microcentrifuge for 5 minutes.

6. Load 20 μl onto SDS-PAGE gel (10 cm x 10 cm).

Note: Loading prestained molecular weight markers to verify electrotransfer and to determine molecular weights.

Membrane Blocking and Antibody Incubations

1. After electrotransfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.

2. Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.

3. Wash three times for 5 minutes each with 15 ml of TBS/T.

4. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.

5. Wash three times for 5 minutes each with 15 ml of TBS/T.

6. Incubate membrane with HRP-conjugated secondary antibody (anti-rabbitHRP) at a dilution of 1:2000 in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.

7. Wash three times for 5 minutes each with 15 ml of TBS/T.

Note: anti-PAK, anti-SAPK/JNK, anti-phospho-SAPK/JNK, anti-p38 MAP kinase and anti-phospho-p38 MAP kinase antibodies as primary antibodies.

Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO ®.

2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10-second exposure should indicate the proper exposure time.

In Vivo Assay

Detection of Interleukin-6 Level in Sodium Arsenite-Exposed Rats

Treatments

Rats were divided into 6 groups (n=8 for control group and n=6 for each treatment group) treated with the following conditions:

Group 1: Control

Group 2: Sodium arsenite 2.5 mg/kg body weight (single dose, p.o.)

Group 3: Sodium arsenite 5 mg/kg body weight (single dose, p.o.)

Group 4: Sodium arsenite 10 mg/kg body weight (single dose, p.o.)

Group 5: Pretreatment with genistein 30 mg/kg body weight (single dose, p.o.) 1 hour and then sodium arsenite 10 mg/kg body weight (single dose, p.o.)

Group 6: Pretreatment with apigenin 30 mg/kg body weight (single dose, p.o.) 1 hour and then sodium arsenite 10 mg/kg body weight (single dose, p.o.)

After 24 hours, blood was collected using disodium EDTA 1 mg/ml as an anti-clotting agent.

Rat IL-6 Level Detection (Appendix C)

Whole blood was centrifuged at 1500 rpm for 10 minutes. Plasma was separated for interleukin-6 level determination. Rat IL-6 levels will be detected by Rat IL-6 ELISA Kit.

Data Analysis

Control group and treatment groups were compared by using Independent-Samples T Test (SPSS 11.0 for Windows) for *in vitro* assay, p-value of < 0.05 was regarded as significant.

Control group and treatment groups were compared by using Nonparametric Tests coupled with Wald-Wolfowitz Test (SPSS 11.0 for Windows) for IL-6 level determination, p-value of < 0.05 was regarded as significant.