CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Biological materials

3.1.1.1 Interferon

The interferon used in this study was human lymphoblastoid interferon-α (Wellferon), donated by Wellcome, UK.

3.1.1.2 Cell lines

Thai human hepatoma cell lines designated as S102 and R12 were established by Laohathai (1985)(Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand). The hepatoma cell line, $HepG_2$ was purchased from American Tissue Culture Collection cat.no. HB 8065. The fibroblast cell line, L929, was donated from Nagoya Cancer Institute, Japan.

3.1.1.3 Anti-hepatoma monoclonal antibody (anti-hep MAb)

Hybridomas derived from a fusion of NS-1 cell line (myeloma cell) and splenocytes of BALB/C mice which were

pre-immunized with alive human hepatoma.

3.1.1.4 Fluorescein-labeled $F(ab')_2$ goat antimouse immunoglobulin (FITC-GAM) was purchased from Sigma (cat.no. F. 8264, St.Louis, USA.)

3.1.1.5 Gold probe

Colloidal gold 15 nm in size, conjugated with protein-A (AuroProbe™ EM) was purchased from Amersham International plc (Cat.no. RNP 439, Amersham, UK)

3.1.2 Chemicals

3.1.2.1 Electron microscopy chemicals:

-Glutaraldehyde (16310, EMS, USA)

-Paraformaldehyde (19208, EMS, USA)

-Sodium carcodylate (12300, EMS, USA)

-Uranyl acetate (22400, EMS, USA)

-Lead nitrate (17910, EMS, USA)

-Sodium citrate (21140, EMS, USA)

-Toluidine blue (22050, EMS, USA)

-Osmium tetroxide (19100, EMS, USA)

-Nonenyl succinic anhydride (NSA)(19050, EMS, USA)

-Vinyl cyclohexane dioxide (ERL)(15000, EMS, USA)

-DER resin (13000, EMS, USA)

-Dimethylaminomethan (DMAE)(13300, EMS, USA)

3.1.2.2 Affinity chromatography chemicals:

Protein-A sepharose (Sepharose CL-4B) was purchased from Phamacia Fine Chemical (Cat.no 17-0963-03, Piscataway, Sweden).

3.1.2.3 H&E staining chemicals:

-Hematoxylin (Merck, USA)

-Eosin (Merck, USA)

-Sodium iodate (Fluka, Switzerland)

-Amonium alum (Fluka, Switzerland)

-Citric acid (BDH, UK)

-Chloride hydrate (BDH, UK)

3.1.2.4 Other chemicals:

-Sodium di-hydrogenphosphate (Merck, USA)

-Disodium monohydrogenphosphate (Riedel de Haen)

-Potassium di-hydrogenphosphate (Fluka, Switzerland)

-Potassium chloride (Merck, USA)

-Sodium hydroxide (Merck, USA)

-Sodium m-periodate (Sigma, USA)

-Sodium acetate (Merck, USA)

-EDTA (Merck, USA)

-Sodium barbital (Merck, USA)

-Sodium borate (Merck, UAS)

-Calcium chloride (BDH, UK)

-Potassium ferrocyanate (Merck, USA)

-Trypan blue (BDH, UK)

-Sodium chloride (Famitalia Carro Erba)

-Hydrochloric acid (Famitalia Carro Erba)

-Tris-HCl (Sigma, USA)

-Tris base (Sigma, USA)

-Sodium bicarbonate (Merck, USA)

-O-phenylenediamine (Sigma, USA)

3.2 METHODS

3.2.1 Maintaining and preparation of cell lines

All hepatoma cell lines and fibroblast cell line were maintained in RPMI 1640 supplemented with 5 % heat-inactivated fetal calf serum. For the experiments, the cells were trypsinized with 0.05 % trypsin/EDTA. Each samples were started at $3\text{-}4\times10^{5}$ cells in the 50 ml of cultured flask (Nunc, Denmark) and maintained at 37°C in a humidified atmosphere containing 5 % CO_2 in air (Yamato, Japan).

3.2.2 Anti-hepatoma monoclonal antibody

Massive volume of monoclonal antibody (MAb) mostly produced by 2 methods. First, is the *in vitro* culture, the MAb was harvested from the cultured medium. Second, is

to grow the hybridomas intra-abdominal cavity of the mouse and large quantities of MAb were collected from the ascitic fluid. For better method of producing ascitic fluid, mouse has to be pre-injected pristane (2, 6, 10, 14-tetramethyl-pentadecane) intraperitoneally for 2 week before injection of hybridoma cells. The ascitic fluid was collected 2 to 3 weeks after hybridoma injection.

3.2.3 Purification of anti-hepatoma monoclonal antibody

Monoclonal antibodies were purified using protein-A sepharose affinity chromatography as described by Ey, Prowse, and Jarkin (1978). Briefly, 4 ml of ascitic fluid were applied to Protein-A sepharose column. Before started the elution, the column was washed with 0.1 M phosphate buffer, pH 8.0. Monoclonal antibodies were eluted by different pH of 0.1 M citrate buffer, such pH 6.0, 5.5, 4.5, 3.5 according to its subclass. The fractions were collected and neutralized immediately with 1 M Tris-HCl buffer, pH 8.0. After the elution, the column was regenerated by washing with 0.1 M citrate buffer, pH 3.0 and reequilibrate in 0.1 M PB, pH 8.0. Monoclonal antibody was dialyzed in PBS overnight and determined for protein concentration by UV absorption (Shimadzu, Japan).

3.2.4 Immunoreactivity of anti-hepatoma monoclonal antibody

Before and after purification, immunoreactivity of anti-hep MAbs was done in order to confirm and recheck the activity of MAbs.

Preparation of the antigen: hepatoma cells were used as antigen. After the cells grew confluently on 96 well microplate (Nunc, Denmark), cells were fixed in 2% formalin for 2 hours at room temperature. Cells were stored in PBS pH 7.4 with 0.02% thimerosal at 4°C until use.

The ELISA method: Anti-hep MAb used in this study was immunoglobulin IgG2a subclass. The MAb was diluted with 0.5% BSA in PBS. The volume of 100 μl (50 ng/well) of diluted monoclonal antibody was added into each well. Each sample was run in duplication and incubated overnight at 4°C. One hundred microliter of the secondary antibody, rat anti-mouse immunoglobulin IgG_{2a} (Zymed Cat.no. 04-6120) in dilution 1:1000, was added and incubated for 2 hours at 37°C. The O-phenylenediamine was used as substrate by adding 150μl per well. The reaction was stopped with 100 μl of 2.5 M sulfuric acid. The color reaction was read at 492 nm by MICROELISA spectrophotometer (Titertek, Finland).

3.2.5. Selection of IFN-α sensitive hepatoma cell line

The S102, HepG2, R12 hepatoma cell line as well as L929 fibroblast cell line (as cell control) were tested for IFN sensitivity. The antitumor assay was used to detect the viability of cells by trypan blue exclusion method. Cells were seeded in 30-mm tissue culture dishes (Nunc, Denmark) together with 2 ml of culture medium. The initial cell density was $5x10^4$ cells/dish. After 24 hr incubation, the medium was changed with 2 ml of fresh medium with or without IFN in different concentration. One condition was prepared triplicately and determined every 2 day for 8 days. Cell counting was done by gentle trypsinization with 0.05% trypsin/EDTA and washing with RPMI. After staining with 0.4% trypan blue for 3 min, cells were counted in hemocytometer. The unstained cells were demonstrated as viable cells and blue stained as dead cells. The accuracy count can be provided by dispersing of cells and carely taking to prevent sedimentation of cells.

3.2.6 Selection of IFN-α concentration

From previous experiment, S102 hepatoma cell was selected as a cell model for study. In this experiment, different concentration such as 200, 500, 1000 IU/ml of IFN-α was tested on S102 hepatoma cell in order to evaluate which concentration gives lethal effect of IFN. The antitumor assay, viability and cell growth inhibition

determined by trypan blue exclusion as described above, were used in this experiment.

The percentage of viable cells or viability was calculated using the following formula (Paul, 1975):

$$\% \text{ Viability} = \frac{\text{number of viable cells}}{\text{number of viable + number of dead cell}} \times 100$$

The cell growth inhibition represented as relative percentage to the control cell growth (without IFN) was calculated using the following formula (Motoo, 1986):

$$\% \text{ Cell growth inhibition (\% CGI)} = \frac{Cc - Ce}{Cc - Cs} \times 100$$

Where Cc is the control cell count, Ce is the experiment cell count and Cs is the starting cell count.

3.2.8 The effect of IFN-α on hepatoma cell line

The hepatoma cell, S102 cell, and IFN-α concentration at 1000 IU/ml were selected to study the effect of IFN-α. In this study, the experiment was divided into 2 parts:

3.2.8.1 Antitumor and reversibility effect

The S102 hepatoma cells were treated with 1,000 IU/ml of IFN-α for 2, 4, 6, and 8 days. Thereafter, the medium was changed with fresh IFN-free medium with additional incubation for 2, 4 and 6 days. The reversibility effect of IFN treated cells was evaluated. Floating cells were tested for viability by trypan blue exclusion and recultured with IFN-free medium in order to reconfirmed the viability. The adherent cells were tested for viability, cell growth inhibition and cell multiplication. In addition, IFN-resisted cells were also evaluated for reversibility effect. The experiment was divided into 2 parts: One was cultured with IFN-free medium and the other was continuously cultured with IFN for 20 days. The medium was changed every 3 days. Cells were counted and tested for viability using trypan blue exclusion method as described above.

The percentage of viability and cell growth inhibition were calculated using the formula as mentioned above.

The cell multiplicaton was calculated according to the following formula (Paul, 1975):

$$\log N = \log N_o + n \log 2$$

where N is the cell number at days after treatment

N_o is the initial cell number

n is cell multiplication

3.2.8.2 The process of lethal effect and the influence of to the cell surface antigen

1. Light microscopic examination

This experiment was performed with H&E staining method to evaluate the time related to the morphological changes caused by IFN-α treatment including inclusion body and segmented nucleus. The results from this part will be used as an information schedule the preparation of specimens. Cell organelles and surface antigens of IFN-α treated hepatoma cells were observed using an electron microscope.

The S102 hepatoma cells were cultured at inoculum of $2x10^5$ cells/well of 24-multiwell culture plate (Nunc, Denmark) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. After 24 hr incubation, the medium with 1000 IU/ml of IFN and without IFN as control was changed. The samples were harvested sequentially after 6, 12, 18, 24 hr, 2, 3, 4 and 5 days. Mainly each observing treated cells were divided into two groups, the floating and the adherent cells. They were centrifuged to poly-L-lysine coated slide using cytospin centrifuge (Cytospin 2, Shandon, USA.) at 600 rpm for 2 min. Cells were fixed with 2% paraformaldehyde for 1 hr at room temperature and stained with H&E staining and mounted by

glycerine permount (Sigma, USA.).

2. Electron microscopic examination

The major aims of this study were to find out the origin of inclusion bodies and the outcome parallel to the changes of cell surface antigens. Firstly, several attempts were tried by following conventional methods. It was found that cell surface antigen was failed to show up together with the distorted of cell membrane and organelles. The sample preparation has to start back to the fixative, the concentration of anti-hep MAb for antigen detection, the kind and dilution of gold conjugated as secondary antibody.

2.1 Fixative for hepatoma cell

The experiment was expected to evaluate fixatives for preserving surface antigens on S102 and $HepG_2$ hepatoma cells by indirect immunofluorescence technique. The fixatives tested in this experiment were 2% paraformaldehyde, 2.5% glutaraldyhyde, 1% glutaraldehyde, and 2% Periodic-Lysine-Paraformaldehyde (PLP).

Immunofluoresence technique was as followed.

1. S102 and HepG2 hepatoma cell were cultured on glass slide in culture dish which full with culture medium.

2. After the cells grew confluently, the slides were washed with phosphate buffer saline (PBS). They were

fixed with fixatives mentioned above at 4 °C for 1 hr.

3. At the first started, the concentration of 20 μg of anti-hepatoma monoclonal antibody (anti-hep MAb) namely #27 was used and incubated at 37 °C for 2 hr. The control, non-specific immunoglobulin, NS-1 (myeloma cells) in ascitic form was used at the same concentration as anti-hep MAb.

4. The second antibody, FITC-labelled goat anti-mouse immunoglobulin (FITC-GAM) at dilution of 1:250 in PBS was added 100 μl each and incubated for an additional 1 hr at 4 °C.

5. After washing, slides were mounted with glycerol and observed under fluorescence microscope (Olympus, Japan)

2.2 The concentration and incubation time of anti-hep MAb

The appropriate concentration and incubation time of anti-hep MAb was selected by showing the optimum expression of surface antigen. The result from this experiment was used for further immunoelectron microscopy technique. Immunofluorescence method used in this experiment was described as follow:

1. Preparation of the antigens, hepatoma cells, S102 cell and HepG2 were seeded in amount of 1×10^5 cells per slide and cultured in the condition described above.

2. The cultured slides were washed with PBS and fixed in 1% glutaraldehyde at 4°C for 1 hr.

3. Different concentration of anti-hep MAb: 5, 20, 40 μg were used for selecting and incubated in various conditions: 37 °C for 2 hr; 37 °C for 3 hr; 4 °C for overnight.

4. The FITC-GAM at dilution 1:250 was added in 100 μl each and incubated at 4 °C for 1 hr.

5. Expression of surface antigen on hepatoma cell was scored as percentage of all countable cells (at least 500 cells).

2.3 The immunogold staining method

The anti-hep MAb used in this study was only immunoglobulin subclass type IgG_{2a}. This had given a hard time in established the staining method with conventional secondary Ab which mostly was whole immunoglobulin. Finally, it was found that protein-A conjugated gold was the most effective agent.

Two procedures, fixed and unfixed cells before immunolabelling were compared. The following staining was as follows .

1. Fixed and unfixed S102 cells were added with 200 μg of anti-hep MAb#27 per $1x10^6$ cells per experiment and incubated at 4 °C overnight.

2. 400 μl of protein-A conjugated gold particle was added with dilution 1:10 or 1:50 in gold buffer. This step was incubated for 6 hr at 4 °C.

3. After the immunostaining, the cells were all went through the process in transmission electron microscopy sample preparation.

The protocol in sample preparation for transmission electron microscopy was described below:

Preparation of specimen blocks

1. The cell pellets were fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 1 hr at 4°C.

2. The pellets were rinsed in 0.1 M sodium cacodylate buffer, pH 7.4 for 3 times, each for 5 mins.

3. The pellets were embedded in 1 % agar (warmed agar about 37°C)

4. Cells embedded in agar were cut into 1-mm^3 pieces.

5. Pieces of specimens in agar were post fixed in 2% reduced osmium tetroxide for 1 hr at room temperature.

6. After washing with sodium cacodylate buffer for 5 min. The specimens were subsequent washed in 0.11 M veronal acetate buffer 2 times, each for 5 mins.

7. For increasing the contrast, cells were incubated in 1% uranyl acetate for 1 hr at room temperature.

8. The specimens were washed in veronal acetate

buffer 3 times.

9. Dehydration was performed in a graded series of ethanol such 35%, 65%, 85% and absolute alcohol. Every steps took 15 min incubation, except the absolute alcohol which needed two changes.

10. The infiltration with spurr resin started with 1:3 mixture of spurr resin: absolute ethanol for 2 hr.

11. Then incubated with 1:1 mixture of spurr resin: absolute ethanol for 2 hr or overnight.

12. Then incubated with 3:1 mixture of spurr resin: absolute ethanol for 2 hr.

13. The final was incubated with pure spurr resin for 2 hr, two changes.

14. The specimens were embedded in embedding mold with spurr resin.

15. Soon after, the specimens were incubated at 70 °C for 8 hr for polymerization of spurr resin.

Preparation of the sections

This step was divided into 2 parts.

1. The thick sections: These sections were good for rough localization the area which was important for investigation. Sections were cut about 1 μm thickness with the ultramicrotome (Ultrotome V, LKB, Sweden) and stained with toluidine blue. Under a light microscope, thick section was selected and trimmed for the appropriate areas.

2. The ultra thin sections: These sections were cut from the blocks which the important areas were already selected. The ultra sections were picked up on uncoated 300-mesh copper grids. The sections were stained with uranyl acetate first and then followed by lead citrate.

Ultra thin sections were examined with JEOL-JEM model 100 sx or 200 cx electron microscope at 60 kV or 80 kV, respectively.

The method for EM sample preparation, there were some problems in immunoelectron microscopic specimens. The reaction with antibody for gold labelling must perform on unfixed or alive cells. After several hours of anti-hep MAb and protein-A conjugated gold particle incubation step, the cells were fixed and passed through conventional process for EM. The result from this process showed that viable cells did not show normal ultrastructure. Thus, the sample preparation in this study was divided in two parts: sample preparation for ultrastructural study was performed by immediately fixing of sample and routinely processed for EM. For antigenic study was performed by completely immunogold staining. Subsequent, cells were fixed and general EM preparation.

For studying the effect of IFN-α on the hepatoma cells, the result from light microscopic examination was

used to evaluate time related to morphological changes including inclusion body and segmented nucleus. The result from light microscopy showed that IFN-α treated cells began cellular changes after 6 hours treatment and subsequent showed the pattern of cytoplasmic and nuclear changes. Thus, the EM preparation was performed at the same time as light microscopic results in order to evaulate ultra-structure level.

The method of sample preparation for ultra-structural examination was performed as follow:

1. S102 cells were cultured in media with 1000 IU/ml of IFN-α and without IFN-α as control. Culture media were changed every 3 days thereafter until the end of the experiment.

2. After IFN treatment for 3 hr, 6 hr, 1 day, 3 days, 4 days and 8 days, the cells were scraped from culture dishs and divided in 2 parts in a number of $1x10^6$ cells/experiment. The cells were spinned into the pellet.

For the antigenic alteration, the cells were added with 200 μg of anti-hep MAb and incubated at 4°C overnight. 400 μl of protein-A conjugated gold particle at 1:10 dilution was added and then incubated at 4 °C for 6 hr. After the immunogold staining, the cells were fixed with 1% glutaraldehyde at 4 °C for 1 hr and processed for EM preparation.

For morphological alteration, the cells were fixed in 1% glutaraldehyde at 4 °C for 1 hr and then processed for transmission electron microscpoic preparation as described above.