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Effect of membrane cholesterol on oxaliplatin-induced cell death patterns in HepG2 cells

Graduate School of Chosun University

Department of Bio New Drug Development

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Advisor: Prof. Sung-Chul Lim

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Abbreviation

CRC = Colorectal cancer

PCD = Programmed cell death

DNA= Deoxyribonucleic acid

ATP=Adenosine triphosphate

TNF=Tumor necrosis factor

TNFR= Tumor necrosis factor receptor

FAS=Fas associate signaling

TRAIL= Tumor necrosis factor-related apoptosis inducing ligand

DR4= Death receptor 4

DR5=Death receptor 5

PARP= Poly (ADP-ribose) polymerase

ADP= Adenosine diphosphate

NAD^+= Nicotinamide adenine dinucleotide$^+$

ROS= Reactive oxygen species

NAC= N-acetylcystein

MPT= Mitochondrial permeability transition

GPI= Glycosylphosphatidylinositol

TRAF=Tumor Necrosis Factor Receptor-associate Factor

FADD= Fas-associated death domain

DISC= Death-inducing signaling complex

DFF45= DNA fragmentation factor 45

RIP= Receptor-interacting protein

H_2O_2= Hydrogen peroxide

BHA = butylated hydroxyanisole

DPI = diphenylene iodonium
MBCD= Methylbetacyclodextrin

Z-DEVD-fmk=Benzyloxycarbonyl-asp(ome)-glu(ome)-val-asp(ome)-fluoromethylketone

Z-VAD-fmk= Benzyloxycarbonyl-val-ala-asp (ome) - fluoromethylketone

DMSO=Dimethyl sulfoxide

DMEM=Dulbecco′s modified eagle medium

Abstract

Effect of membrane cholesterol on oxaliplatin-induced cell death patterns in HepG2 cells

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Hepatocellular carcinoma (HCC) is known to be resistant to chemotherapy. However, recently several oxaliplatin-based combinatorial treatments have shown a promising anti-tumor activity in patients with HCC. Previously, our group demonstrated oxaliplatin triggered massive necrosis with some apoptosis in HepG2 HCC cells, while it induced mostly apoptosis in HCT116 and HT29 colon cancer cells. As necrosis is closely associated

with inflammation, causing tumor progression and malignancy, the regulatory mechanisms underlying death mode determination were investigated. In the present study, we observed that the mild and excessive ROS production due to oxaliplatin caused apoptotic and necrotic death and N-acetyl-L-cysteine (NAC) and superoxide scavengers, BHA and Tiron attenuated necrosis, but the simple alleviation of ROS production did not switch necrosis to apoptosis. On the other hand, addition of cholesterol switched oxaliplatin-induced necrosis to apoptosis, which was reverted by methyl beta cyclodextrin (MBCD), a membrane cholesterol depleting agent, suggesting membrane cholesterol level is a critical factor of cell death mode determination. Apoptosis induced by the combined treatment was evident by HO/PI double staining, semi-thin epon sections, and caspase 8, caspase 3 and parp cleavages. Combined treatment-induced apoptosis was prevented by p53 inhibitor. In addition, cholesterol/oxaliplatin-induced caspase 8 activation, and caspase 3 and parp cleavages were suppressed by CD95/Fas interference. Moreover cholesterol/oxaliplatin-induced CD95/Fas colocalization with lipid rafts was detected by immunofluorescence technique and lipid rafts isolation. In conclusion, ROS plays critical roles in oxaliplatin-induced cell death mode and increased membrane cholesterol level switches oxaliplatin-induced necrosis to apoptosis by caspase 8 activation via p53 pathway and triggering CD95/Fas relocalization and death inducing signaling complex (DISC) formation in HepG2 cells. Since necrosis and subsequent inflammation are implicated in tumor progression and malignancy, our results suggest a potential improved efficacy of oxaliplatin-based chemotherapy by shifting cell death mode from necrosis to apoptosis through enhancing membrane rafts formation.

CHAPTER 1: INTRODUCTION

Oxaliplatin is a platinum based chemotherapeutic drug in the same family as cisplatin and carboplatin. Compared to cisplatin the two amine groups are replaced by cyclohexyldiamine for improve antitumor activity. The chlorine ligands are replaced by the oxalate bidentate derived from oxalic acid in order to improve water solubility. Oxaliplatin was initially launched in France in 1996 and subsequently in the rest of Europe in 1999 and, more recently, in the United States in August 2002. It is the first platinum-based drug to demonstrate convincing clinical activity against colorectal carcinoma (CRC).

Apoptosis is the programmed cell death (PCD) that may occur in multicellular organisms. PCD involves a series of biochemical events leading to a characteristics cell morphology and death, in more specific terms, a series of biochemical events that lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation (1-3). Recently, necrosis has been regarded as PCD and controllable. Necrotic cell death is characterized by rapid swelling, a loss of plasma membrane integrity and frequently in connection with dramatic irreversible drops in ATP levels (4, 5). Necrotic cell death triggers an increase in the secretion of proinflammatory cytokines from independently activated macrophage. It is also conceived that the release of cytokines or other factors from the necrotic cells themselves may be crucial for an inflammatory response. Morphologically, necrosis is quite different from “classical” apoptosis. During necrosis cells first swell, and then the

plasma membrane collapses and cells are rapidly lysed. During apoptosis, cells first shrink and their nuclei are condensed, and then they disintegrate into well-enclosed apoptotic bodies. Cell swelling during necrosis is emphasized in the term “oncosis” (derived from “oncos,” meaning swelling), the term for cell death opposite to apoptosis [4].

The members of the TNF receptor family (TNF, FAS, and TRAIL) may initiate not only apoptotic, but also necrotic cell death. Ligation of FAS caused caspase-independent cell death in activated T lymphocytes, a process apparently involved in immune response [6].

Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme containing a Zn-binding domain. Upon activation by DNA breaks it attaches oligomers of ADP-ribose to itself and some other nuclear proteins. Excessive activation of PARP, for example, as a result of profound induction of DNA breaks, is believed to be a cause of cell death due to ATP depletion [7, 8]. This ATP depletion is resulted from use of ATP for synthesis of the PARP substrate NAD^+. PARP inhibition (e.g., by 3-aminobenzamide and nicotinamide) suppressed cell necrosis [7] or switched it to apoptosis, which was associated with a marked increase in caspase activity [9, 10, 11]. During apoptosis, PARP is normally inactivated by caspase-specific cleavage, forming an 89-KD fragment, a biochemical hallmark of apoptosis. If this mechanism of PARP inactivation is not operational, for example, in a PARP mutant resistant to caspase cleavage, cells become more sensitive to necrosis induced by FAS [12, 13].

Oxidative stress refers to an increased level of intracellular reactive oxygen species (ROS) within the cell above the typical levels that the cell normally experiences. Such a

condition may be generated by increased generation of ROS by various mechanisms or by an impaired function of the antioxidant defense system, which is responsible for dealing with basal ROS levels (14). Oxygen free radicals or, more generally, ROS, are the products of normal metabolic and signal-transduction events within a cell but may also play a role in pathologic processes. Hydrogen peroxide, a component of ROS, is often used as a model reagent since it is produced as a factor of immune defense and during various stresses. It can cause both apoptosis and necrosis of cells [15, 9, 10], which can be prevented by the antioxidants glutathione or *N*-acetylcystein (NAC) [16]. Surprisingly, when applied together with antitumor drugs, subtonics doses of H_2O_2 can switch apoptosis to necrosis [10, 17]. This effect was probably associated with specific signaling role of H_2O_2 rather than with inhibition of caspases or PARP activation since much higher concentration of H_2O_2 was necessary for the latter effect [9, 10]. A decrease in cellular content of glutathione can also switch a form of cell death induced by ROS. In most cases, antioxidants suppress both necrotic and apoptotic cell destruction. It seems that oxidative stress induces an apoptotic response when cells can maintain their reducing capacity against ROS, whereas necrosis is triggered when this reducing homeostasis is disturbed (e.g., by excess of ROS or damage of natural antioxidative systems). One such factor may be ROS produced by the mitochondrial respiratory chain, and this ROS generation may trigger a necrotic program [18, 19]. It was hypothesized that when cellular antioxidative defense is limited, ROS caused oxidation of the key molecules and release of executor proteases, lipases, and nucleases from mitochondria [19]. The emergence of such

dangerous mitochondria triggers the cells protective response in the form of autophagia with participation of caspases [19, 20]. This hypothesis may explain why in some cells inhibition of caspases, while inhibiting TNF-induced apoptosis, may trigger necrotic cell death. Indeed, TNF may activate mitochondrial ROS generation, and such dangerous ROS-producing mitochondria are normally eliminated by caspase-dependent autophagia [19]. However, when caspases are inhibited, these mitochondria may trigger necrotic death of a whole cell.

Cholesterol is an important in organizing some types of domains, usually termed lipid rafts. These lipid rafts are thought to be required for cell functions, including directed mobility and capping of membrane proteins, receptor-mediated signaling, entry and exit of pathogens and membrane trafficking. Lipid rafts are dispersed when cell cholesterol is extracted. Hence, an effect of cholesterol depletion on a particular function is usually assumed to show that lipid rafts are required for this function (21). Rafts are specialized membrane microdomains enriched in glycosphingolipids, cholesterol, and glycosylphosphatidylinositol (GPI)-anchored proteins (22, 23). Raft microdomains are most abundant at the plasma membrane, but may also be present in endocytic and secretory pathways. Proteins modified with saturated acyl chain groups, Such as GPI-anchored proteins and double acylated proteins, have been found to be preferentially targeted to rafts.

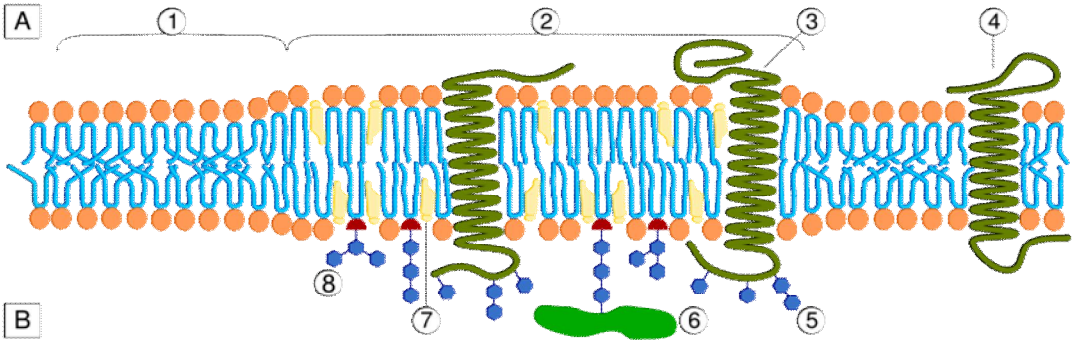

Figure1. Lipid raft organization: 1. Non-raft membrane, 2. Lipid raft, 3. Lipid raft associated transmembrane protein, 4. Non-raft membrane protein, 5. Glycosylation modifications (on glycoproteins and glycolipids), and 6. (Glycosylphosphatidylinositol) GPI-anchored protein, 7. Cholesterol, 8. Glycolipid, (TREND in Cell Biology Vol.11 No.12 Dec 2001 492)

However, certain transmembrane proteins can also be enriched in rafts through a mechanism still unclear. The involvement of rafts has been implicated in many important cellular processes, which include generation and maintenance of cellular polarity, chemotactic migration, and cell surface receptor signaling. For T cell and B cell antigen receptors, raft domains function as signaling platforms where selective signaling molecules are recruited or segregated away (24). Recent report showed that, TRAIL-induced apoptosis occurs through receptor-mediated extrinsic pathways. TRAIL binds to the cell surface death receptor DR4 and DR5, which in turn recruit intracellular Fas-associated death domain (FADD). Through its death effectors domain, FADD recruits caspase-8 to

the receptors for the assembly of a death-inducing signaling complex (DISC; ref. 25). In the DISC, caspase-8 is activated and cleaves caspase-3 directly (26). Once activated, caspase-3 cleaves downstream DNA fragmentation factor 45 (DFF45; ref. 27), leading to apoptotic cell death. TRAIL-DISC is modulated by intracellular adaptor proteins (25).

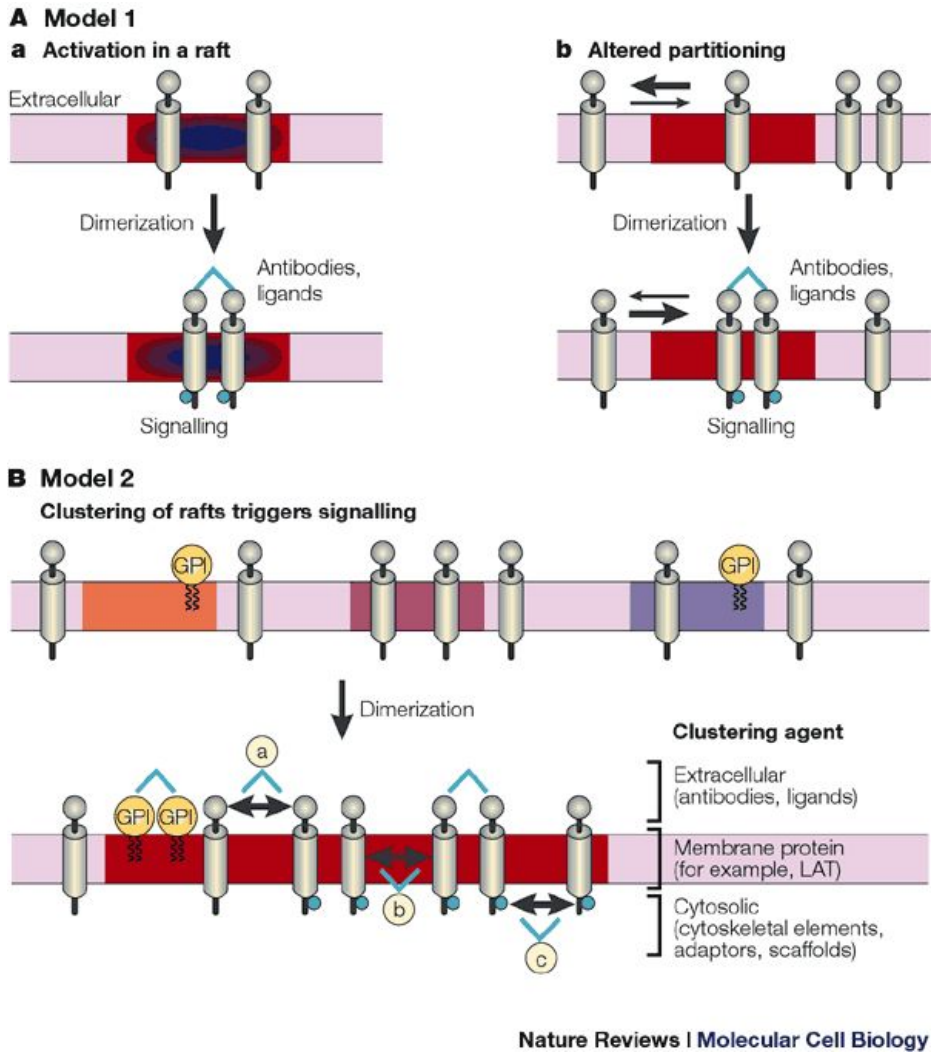

Figure2. Models of how signaling could be initiated through raft

Model 1, signaling occurs in either single raft, clustered rafts (Model 2). Dimerization the protein becomes phosphorylated (blue circle) in rafts. In single rafts this can occur by activation **a.** Within the raft, **b.** By altering the partitioning dynamics of the protein. **B**, several rafts in the membrane, which differ in protein composition (shown in orange, purple or blue). Clustering would coalesce rafts (red). Clustering in the cytosol (a–c, respectively). GPI-anchored proteins (yellow).

In this study, oxaliplatin induced mainly necrosis and a little bit apoptosis in HepG2 hepateocellular carcinoma through the ROS production. The membrane cholesterol modulation has significant effect on necrosis and apoptosis. Here, I studied the role of membrane cholesterol in oxaliplatin–induced cell death mode regulation. I tried to determine how cholesterol with oxaliplatin switches the cell death mode from necrosis to apoptosis.

CHAPTER 2: MATERIALS AND METHODS

2.1. Cell culture and drug treatment

Human hepatocarcinoma HepG2 cells were maintained in DMEM medium and human colon cancer cell lines HCT116 and HT29 were cultured in RPMI 1640 supplemented with heat-inactivated 10% fetal bovine serum, 50 μg/ml penicillin and 50 μg/ml streptomycin at 37°C in a 5% CO_2 -95% air incubator with controlled humidity.

The cells were seeded at a density of 5 x 10^5 cells/6 cm^2 dish/3 ml medium, 2 x 10^5 cells/3.5 cm^2 dish/1 ml medium and 1 x 10^4 cells/well of 96-well plate/100 μl medium then used for the drug exposure experiments after being culture over night. The oxaliplatin was dissolved in H_2O in order to generate 5 mM of stock solution, and this was further diluted with a medium to the desired concentrations. Cholesterol was dissolved with water in order to prepare a 50 mM stock solution and treated in cells to the desired concentration. MBCD dissolved in DMSO and further diluted in media in order to prepare a 200 mM stock solution and treated 1 mM to 5 mM. N-acetyl-L-cysteine (Sigma-Aldrich St, Louis, and 10 mM), DPI, BHA, tiron, deferoxamine and inhibitor of P53 i.e PFT-alpha was pretreated 1 h with oxaliplatin and cholesterol combination.

2.2. Antibodies and reagents

Antibody for p53 (Do-1): SC-126, FAS (M-20): sc-716, FADD (H-181): SC-5559, goat anti-rabbit IgG-horseradish peroxidase-linked and goat anti-mouse IgG-horseradish peroxidase-linked were purchased from Santa Cruz Bio-technology. P-p53 (Ser 15) and

cleaved caspase-3 (Asp 175) antibody: 9661 were obtained from cell signaling. Anti-PARP antibody: 556494, alpha tubulin and RIP1:551041 were obtained from BD pharmingen. The compound MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide) was obtained from sigma (St. Louis, MO, USA).

2.3. Morphological detection of apoptosis and necrosis by Hoechst 33342 and propidium iodide staining.

2 x 10^5 cells were seeded in 3.5 cm^2 dishes per 1 ml of medium. After the overnight incubation, the indicated drugs were treated at various time points. The cells were stained with Hoechst 33342(5 μg/ml) and PI (10 μg/ml) to the culture medium and incubated for 15 minutes at 37°C with 5% CO_2. Cells were collected by trysinization with 0.5x trypsin for 5-10 minutes at 37°C incubator. Some apoptotic and necrotic cells were detached from the cultural substratum into the medium. All cells were collected by centrifugation at 1500 rpm for 5 minutes at 4°C Temperature. The cells pellets were suspended in 500 ul of 3.7% paraformaldehyde and incubated for 10 minutes. The fraction of suspension was centrifuged in cytospinner (Cellspin, Hanil, Korea). Slides were immediately fixed with mounted gel after dried and covered by glass cover slip. Slides were observed under fluorescence microscope (Leica DFC480, Germany). A total of 500 cells were counted from the randomly chosen of field. The number of apoptosis or necrosis cells were counted and calculated into the percentage.

2.4. Cell viability assay

Cell viability was assessed by the MTT assay. In MTT assay, 1 x 10^4 cells were seeded in 96 well plates per 100 μl of DMEM medium. After the overnight of incubation in 37°C at 5% CO_2 incubator, drugs were treated at various time points. After 4 h of incubation with MTT (0.5 μg/ml), plate was centrifuged at 1000 rpm, 5 minutes at room temperature. Then medium was removed and 150 μl of DMSO was added to dissolve formazin crystals. Absorbance was measured at 540 nm using an ELISA microplate reader (Perkin-Elmer). The cell viability was determined relative to the untreated control cells.

2.5. LDH assay

This assay was used for the measurement of cytotoxicity of plasma membrane damage. The lactate dehydrogenase (LDH) is a stable enzyme, present in all cell types, and rapidly released into the cell culture medium upon damage of the plasma membrane. 1 x 10^4 cells were seeded (triplicate) into the 96 well plate per well per 100 μl of DMEM medium. Appropriate drug was treated at various time points. 10 μl of LDH lysis buffer was added into the high control samples before 30 minutes of completed of time points. After the 30 minutes of lysis of cells, plates were centrifuged (600g for 10 minutes) to precipitate the cells. 10 μl of aliquot of supernatant was dispensed into several well of 96-well micro titer plate and combined with LDH reaction mix (mix 200 μl of WST substrate mix with 10.5 ml of LDH assay buffer) to each. Plate was read after 30 minutes of shaking at room temperature using a plate reader (Bio-Rad, Hercules, CA, USA) with primary wavelength 450nm and reference wavelength 650 nm. The percentage of cytotoxicity was calculated on the basis of low control (untreated sample), high control (LHD lysis buffer added

sample) and drug treated samples.

2.6. SDS-PAGE and Western blot analysis

For the detection of various kinds of protein levels, 5 x 10^5 cells were plated into the 6 cm^2 dish in 3 ml DMEM medium and incubated at 37°C in 5% CO_2 contained humidified incubator overnight. At various time after incubated with drugs treatment, HepG2 cells were collected and washed with 1x ice cold PBS. The collected cell pellets were lysed in lysis buffer (50 mM Hepes, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton x-100, 20 mM NaF, 50 mM PMSF, 1 mM Na_3Vo_4, 100 μg/ml leupeptin and 10 μg/ml aprotinin). Cell debris was removed by centrifugation (13000 rpm, 15 minutes at 4°C temperature). Protein concentration was measured by Bio-Rad protein assay and samples were boiled for 10 minutes. The boiled samples were loaded into an 8%, 10%, 12% or 15% SDS-PAGE gel according to the molecular weight of proteins, and electrophoresis was fixed at 50 V up to 30 minutes and increased at 100 V for 2 and half hours. Protein was electrophoretically transferred into 0.22 μM nitrocellulose transfer membrane. The transferred membrane was blocked for 1 hour in 5% nonfat dry milk in TBST and incubated at 4°C overnight with the corresponding antibodies. Immunobilized proteins were incubated with goat anti-mouse IgG and goat anti-rabbit IgG (Santa Cruz biotechnology) and signals were detected using a chemiluminescence kit.

2.7. Transfection

To determine the role of FAS, RIP1, FADD study, and cells were plated into the 6 cm^2

dishes. After the overnight incubation, cells were transfected by small interference RNAs (si FAS, si RIP1, si FADD and si control) to silence gene expression. Polypus reagent was used in the study. After 18 to 24 h of transfection, cells were treated by drugs at 24 h then the cell lysed was subjected to Western blot for detection of specific proteins and performed the caspase-8 activity.

2.8. Caspase-8 activity assay

For the detection of caspase-8 activity, 2 x 10^5 cells were seeded in 3.5 cm^2 dish per 3 ml medium. After the time point of drug treatment, cells were collected by centrifugation. The collected cell pellets were lysed by 50 μl of chilled cell lysis buffer and incubated on ice for 10 minutes. Centrifuged the samples in microcentrifuge at 10,000g, 1 minute at 4°C. Transferred the supernatant to a next tube and kept in ice. Protein concentration was measured by Bio-Rad protein assay methods. Protein samples were diluted at 300 μg to 50 μl by cell lysis buffer for each assay. 50 μl of 2x-reaction buffers (containing 10 mM DTT) and 5 μl of the 4 mM IETD-pNA substrate (200 μM final concentrations) were added to each sample, and then incubated at 37°C for one and half hours. 100 μl of each samples were transferred into the 96 well plates and read at 405 nm wavelength.

2.9. Measurement of intracellular ROS production

ROS generation was measured after staining the cells with DCFH-DA. This dye is the stable nonpolar compound which diffused readily into the cells yields DCFH. Intracellular H_2O_2 or OH^- or O_2^{-} in the presence of peroxidase changes DCFH to the highly

fluorescent compound DCF. Thus, the fluorescent intensity is proportional to the amount of peroxides which are produced by the cells. HepG2 cells were plated in the 48-well plates 2 x 10^4 cells/well. Cells were incubated at 37°C with 5% CO_2 incubator overnight. Next day cells were treated by drugs at various time points and then medium was replaced. Cells were further incubated in 37°C with 5% CO_2 incubator and loaded with 2'-7'-dichlorofluorescein diacetate (DCFH-DA, molecular probes, 50 μM), or dihydroethidium (DHE, molecular probes, 10 μM) for 30 minutes after the indicated times. Fluorescence measurements were obtained by fluorocount plate reader (MQX 200) with excitation at 490 nm and emission at 530 nm for DCFH-DA or excitation at 540 nm and emission at 610 nm.

2.10. Immunofluorescence

Cells were cultures in coverslips in 24 well plates, one night before the experiments. Cells were treated with 100 μM oxaliplatin and 100 μM cholesterol plus oxaliplatin, at 24 h. Cells were fixed with 1% formaldehyde about 15 minutes and rinsed with 1x- PBS and then permeabilized with 0.2% triton x 100/PBS for 10-15 minutes, blocking of cells with 3% BSA/PBS for 1 h at room temperature. Cell was incubated with anti FAS antibody 1:100 dilution in BSA at 4^0C overnight. Cells were then stained with nuclear staining Hoechst 2 μg/ml and cholera toxin B 10 μg/ml (1:100 dilutions in PBS) was treated for 15 minutes. Coverslips were fixed into the slides after washing 2 times with 1x- PBS and one times with water then fixed with mounted gel. Slides were observed under the confocal microscope.

2.11. Lipid raft and non raft fractionation and protein analysis.

Lipid rafts and non rafts soluble fractions were separated by discontinuous sucrose density gradients from treated and untreated cells. Cells from 15 cm^2 cultural dishes ($1x10^7$ cells/dish/20 ml medium) were lysed on ice for 10 minutes in 2 ml of base buffer (20 mM tri-HCl, P^H 7.8, 250 mM sucrose) with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and supplemented with 50 μg/Ml calpain inhibitor (ALLN) and protease inhibitor (Sigma) and then homogenized. The homogenated were mixed with 1 ml of 50% optiprep made in base buffer and placed on the bottom of a centrifuge tube (14 x 89 mm, Backman). The samples were then overlaid with 4 ml of 20% sucrose and 3.5 ml of 2% sucrose and centrifuged at 21,000 rpm using SW-41 rotor in a Backman ultracentrifuge for 1:50 h at 4°C. Eleven fractions of 1 ml was collected from the top to bottom of the gradients and made aliquot.

The protein in the sample was precipitated for concentration and sucrose removal. 800 μl fraction methanol was added into the 200 μl of a protein fraction and 200 μl chloroform was mixed then vortex. 600 μl water was added and then vortex again (appears cloudy white). Immediately centrifuge for 5 minutes at full speed in a microcentrifuge. A white disc of protein was discarded and added 867 μl of methanol to the tube and inverted about 5 times. Again spin for 5 minutes at full speed in a microcentrifuge. All liquid was removed and allowed the pellet to air dry. The precipitated protein pellet was dissolved in 2 x-sample buffers and subjected to western blot for detection of pro-apoptotic proteins (34).

2.12. Protein qualification

As following the Bio-Rad protein assay, the standard curve was set up with BSA dilution in water as final concentration of 0, 1.25, 2.5, 5, 7.5 and 10 μg/μl. 2 μl of each sample and 798 μl sterilized water were added with 200 μl of Bio-Rad protein assay reagent, vortexed and mixed well. And then, take 100 μl of each sample mixture and move into 96 well plate. Light absorbance was read at 595 nm, compared to the standard curve to determine the protein concentration of the samples [28].

2.13. Statistical analysis

All data were analyzed and presented as mean ± SD from the number of independent experiments. Statistical analysis was performed by Student's t-test at a significance level of $p < 0.05$.

CHAPTER 3: RESULTS

3.1. Oxaliplatin-induced mainly necrotic death in HepG2 cell:

Necrotic cell death is characterized by rapid swelling, a loss of plasma membrane integrity and frequently in connection with dramatic irreversible drops in ATP levels. Necrotic cell death trigger an increase in the secretion of proinflammatory cytokines from independently activated macrophage. It is also conceived that the release of cytokines or other factors from the necrotic cells themselves may be crucial for an inflammatory response by which tumor is spreader in different parts of body. To investigate the cell death pattern after the oxaliplatin treatment on hepatocarcinoma and colon cancer cell lines. The HepG2, HCT116 and HT29 cells were plated overnight and treated with various concentration of oxaliplatin (50-200 μM) for 24 h and determined the viable cell number using the MTT assay (Fig. 3A), the cell survival of all three cell lines were inhibited by oxaliplatin in a dose-dependent manner. As the same condition of treatment of all three kinds of cell lines like figure 3A and stained with HO/PI to observe the apoptotic and necrotic cell death. We have found that, necrosis was happened much more than apoptosis in HepG2 cells but HCT116 and HT29 were found as apoptosis (Fig.3B). This result indicates that oxaliplatin induced necrosis in HepG2 cells.

3.2. Oxaliplatin–induced necrosis due to ROS production which is inhibited by ROS scavengers

Necrosis is the accidental type of cell death which can happen due to the oxidative stress.

ROS is the important factor for apoptotic cell death. To determine the role of reactive oxygen species, HepG2 cells were treated 100 μM of oxaliplatin for 1 up to 18 h. The ROS production was dramatically increased up to 12 h as shown in Figure 4A. The production of ROS was suppressed by anti-oxidant agents NAC, BHA, Tiron and DPI. DPI, Tiron and BHA simply suppressed necrosis but did not switch cell death mode. NAC completely prevented oxaliplatin-induced necrosis as well as apoptosis as in Figure 4B. Here we concluded that mild ROS induced apoptosis and excessive ROS production induced necrosis in HepG2 cell. This result indicated that oxaliplatin-induced excessive ROS production caused mainly necrotic cell death.

3.3. Co-administration of cholesterol switched oxaliplatin-induced necrosis to apoptosis which is reverted by MBCD.

Membrane cholesterol is an important component of cells, it plays a significance role for cell homeostasis i.e. cell growth, proliferation and apoptosis. To investigate the effect of membrane cholesterol depletion and addition by membrane cholesterol depleting agent MBCD and cholesterol with oxaliplatin, HepG2 cells were treated by oxaliplatin with and without membrane cholesterol depleting agent MBCD 1 mM, 2.5 mM and 5 mM for 24 h then measure the necrosis and cell viability by LDH and MTT assay respectively. The measurement of percentage of LDH (lactate dehydrogenase) released was increased by the oxaliplatin and MBCD combination but the percentage of cell survival did not altered (Figure 5A and C). Oxaliplatin-induced LDH activity was dramatically decreased by the cholesterol addition with oxaliplatin combination but cell survival rate remained constant

(Fig. 5B and D). These result indicated that membrane cholesterol played a significant role for cell death. Oxaliplatin-induced more necrosis and a little bit apoptosis, when MBCD was treated with oxaliplatin apoptotic cell death switched to necrosis due to the membrane cholesterol depletion. It was recovered by the cholesterol addition.

To further determine the cell death mode, HepG2 cells were treated with cholesterol, oxaliplatin and their combination. After the 24 h of treatment, cells were stained with Hoechst and propidium iodide then examined the apoptotic and necrotic death under the fluorescence microscope. Oxaliplatin-induced necrotic cell death was switched to apoptosis by the cholesterol administration (Fig.6A and B). When the cholesterol concentration was increased 25 μM up to 200 μM with oxaliplatin, the necrotic death was gradually switched to apoptosis as shown in Figure 6C. As previous result showed that, oxaliplatin induced necrosis was switched to apoptosis by cholesterol addition. MBCD is the membrane cholesterol depleting agent, when different concentration of MBCD was treated with cholesterol and oxaliplatin combination, the apoptotic cells were reverted to necrotic cell death (Fig.7). The reverted ratio was drastically happened by the increased concentrations of MBCD i.e.1 mM, 2.5 mM and 5 mM. These results indicate that, membrane cholesterol plays significant role for the modulation of cell death.

3.4. Oxaliplatin/cholesterol-induced apoptosis is mediated by p53 and caspase-8 activation.

p53 is commonly known as the tumor suppressor gene, which helps to suppress the tumor growth and proliferation. It is the pro-apoptotic protein as a mediator for apoptotic

cell death. To determine the role of p53, HepG2 cells were 30 minutes pre-treated by p53 inhibitor i.e. PFT-alpha with cholesterol and oxaliplatin combination for 24 h, then examine the percentage of nuclear fragmentation by Hoechst and propidium iodide double staining. Figure 8A shows that oxaliplatin/cholesterol-induced about 30% of fragmented nuclei which was suppressed by PFT-alpha combination with oxaliplatin/cholesterol. The p53 is activated by both oxaliplatin and oxaliplatin/cholesterol since 6 to 18 h of time points as shown in Western blot result in Figure 8B. The antioxidant agents NAC, BHA, Tiron and DPI were pretreated with oxaliplatin and subjected to western blot for the detection of p53. 10 mM and 15 mM of NAC completely blocked the p53 levels than that of BHA and DPI (Fig.8C). Tiron has no significant effect in p53 level. These result suggested that p53 is the mediated in oxaliplatin/cholesterol-induced apoptosis.

Caspase-8 is the upstream initiator caspase, which has important role for apoptotic cell death both in extrinsic and intrinsic pathways. To determine the role of caspase in the cholesterol plus oxaliplatin-induced apoptosis in HepG2 cells, different caspase inhibitors were treated with and without cholesterol and oxaliplatin combination for 24 h then examine the apoptosis by HO/PI double staining under the fluorescence microscope. The counting of a group of 500 cells of each samples and calculated the percentage of apoptotic cell death. The apoptotic cell death is prevented by the z-IETD-FMK (caspase-8 inhibitor) more effectively. The caspase-3 inhibitor (z-DEVD-FMK), caspase-9 inhibitor (z-LEHD-FMK) and a broad caspase- inhibitor (z-VAD-FMK) blocked the oxaliplatin/cholesterol-induced apoptosis, Figure 9A. The caspase-8 activity is increased in time dependent

manner in cholesterol and oxaliplatin treated cells to compare the oxaliplatin (Fig.9B). The cleaved caspase-3 and PARP is blocked by the caspase-8 inhibitor (z-IETD-FMK). It proved that cholesterol and oxaliplatin combination-induced apoptotic cell death is mediated by caspase-8 (Fig.9C and D). To further conform the role of p53 and caspale-8, HepG2 cells were pre-treated by p53 inhibitor i.e. PFT-alpha and caspase-8 inhibitor z-IETD with oxaliplatin and cholesterol at 24 h. Western blot result in Figure 10A and B, Oxaliplatin/cholesterol treated cell has cleaved caspase-3 and cleaved PARP, which is completely blocked by the p53 inhibitor and caspase-8 inhibitor. The caspase-8 activity result showed that the percentage of Caspase-8 activation by the cholesterol and oxaliplatin is suppressed by p53 inhibitor in Figure 10B. These result indicated that oxaliplatin/cholesterol-induced apoptosis is mediated by p53 and caspase-8 pathway.

3.5. Oxaliplatin/cholesterol-induced apoptosis is mediated by FAS, FADD, and RIP1 and their colocalization in membrane raft.

FAS are the cell surface receptor glycoprotein belonging to the tumor necrosis factor super family, which plays an important role in death of tumor cells. Upon stimulation with ligand, FAS is aggregated into the membrane raft and a death-inducing signaling complex (DISC) is formed by the recruitment of several moieties, including FAS associated death domain (FADD) and pro-caspase-8, then procaspase-8 is activated and subsequently apoptosis is executed. If the cells are stimulated by any specific agents, the pro-apoptotic proteins p53, FAS, FADD and RIP1 are aggregated into the lipid rafts then apoptotic signals are down regulated. To investigate the clustering the FAS from non-raft to

membrane rafts region, HepG2 cells were grown on the coverslips overnight, then treated with 100 μM oxaliplatin and oxaliplatin/cholesterol combination at 24 h. After the time of treatment, cells were fixed and incubated with anti-FAS antibody then coupled by secondary antibody. After the cholera toxin B and nuclear staining, cells were observed under the confocal microscope. The cell which was treated with oxaliplatin and cholesterol combination induced Fas clustering into the membrane raft compare the oxaliplatin treated cells, Figure 11.

To confirm the role of proapoptotic proteins involve in apoptosis, cells were transfected by small interference RNA i.e. siRNA control, siRNA FAS, siRNA FADD and siRNA RIP1. After the 18 h of transfection, cells were treated with oxaliplatin and oxaliplatin/cholesterol combination for 24 h. Cell lysed were used for caspase-8 activity assay. The caspase-8 activity percentage were suppressed in Fas, FADD and RIP1 transfected cells treated with oxaliplatin and oxaliplatin/cholesterol to compare the siRNA control transfected treated cells, Figure 12B and C. the oxaliplatin/cholesterol induced apoptosis is mediated by the proapoptotic proteins. Further the cleaved caspase-3 and PARP is completely blocked in FAS transfected cells. These result indicated that, oxaliplatin/cholesterol-induced apoptosis is mediated by FAS, FADD and RIP1. Membrane raft is considered as the important component for apoptotic cell death. When proapoptotic proteins are redistributed in the membrane raft from non-raft regions, they become activated and transduce the apoptotic signal. HepG2 cells were treated with oxaliplatin, oxaliplatin/cholesterol and cholesterol for 28 h then lipid rafts and non-rafts

were extracted by discontinuous sucrose gradient methods. The western blot result in Figure13 shows that proapoptotic proteins p53, FAS, and RIP1 were redistributed into the raft region. This result indicates that oxaliplatin/cholesterol induced redistribution of membrane receptor domain proteins into the lipid raft. That initiates the DISC formation and induced apoptosis.

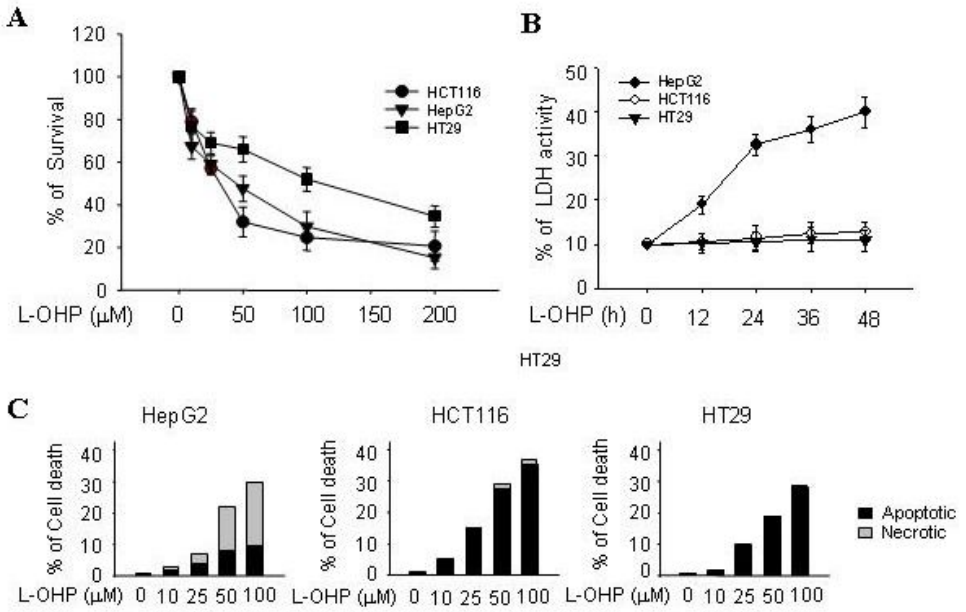

Figure 3. Oxaliplatin induces mainly necrotic death in HepG2 cells. Three kinds of cells HepG2, HCT116 and HT29 were treated with concentrations of 50 μM, 100 μM, 150 μM and 200 μM oxaliplatin for 24 h figure A and B. Oxaliplatin (100μM) was treated upto 48 h and then the cell survival and cell death examined by MTT, LDH and Hoechst/propidium iodide staining.

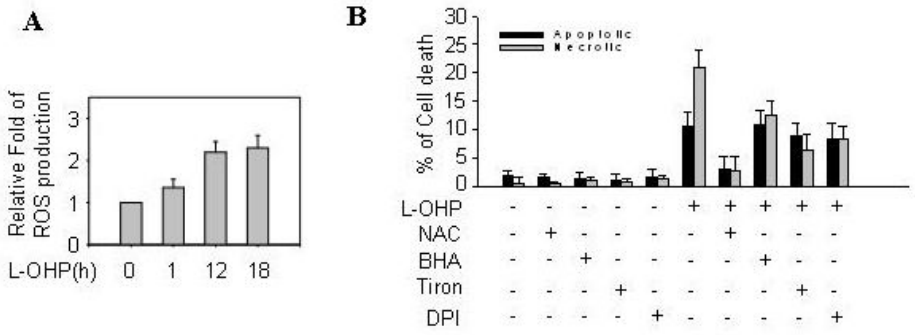

Figure 4. Oxaliplatin-induced ROS production is inhibited by ROS scavengers. A, ROS production upto 18 h after the oxaliplatin treatment, B. Different kinds of ROS scavengers were pre-treated for 1 h with oxaliplatin and then the apoptosis and necrosis were examined by HO/PI staining after counting a group of 500 cells.

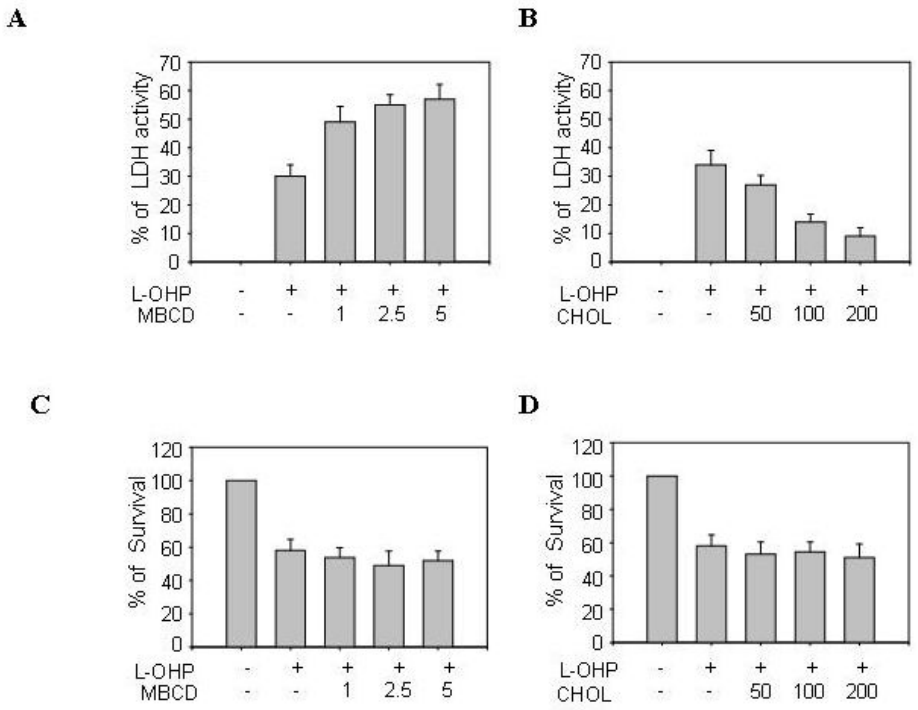

Figure 5. Regulation of necrotic death by membrane cholesterol levels. Different concentrations of MBCD and cholesterol were treated with oxaliplatin for 24 h and then the percentage of cell survival and LDH release were examined.

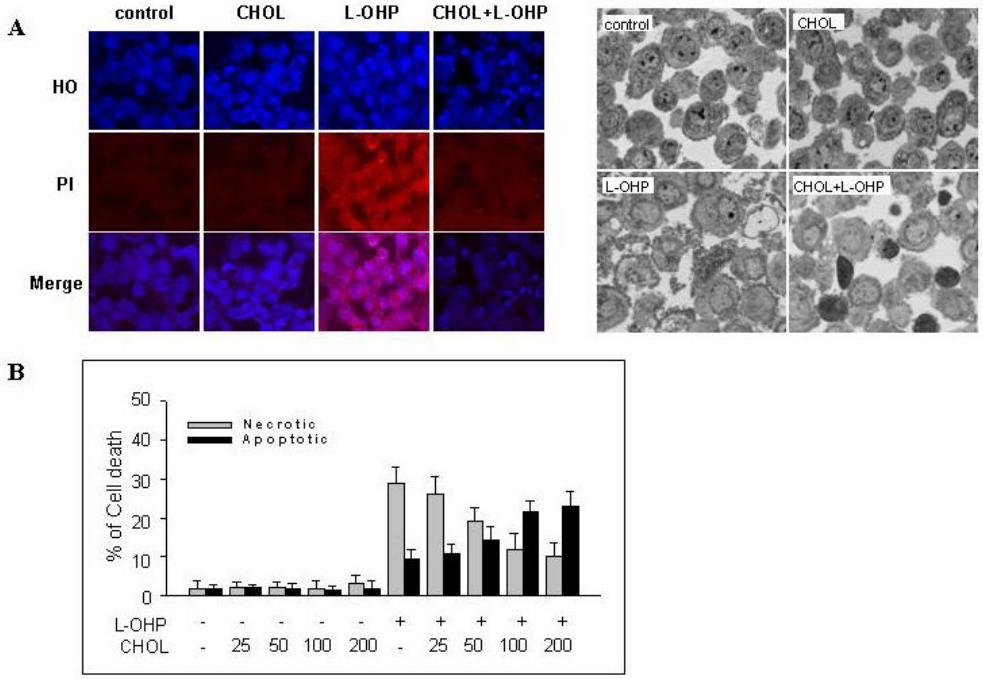

Figure 6. Cholesterol switches oxaliplatin-induced necrotic death to apoptotic death. A, cholesterol (100 μM) was treated with and without oxaliplatin for 24 h then the morphological death pattern were examined by HO/PI staining. B, different concentrations of cholesterol was treated with oxaliplatin combination and then the necrosis and apoptosis were counted in a group of 500 cells.

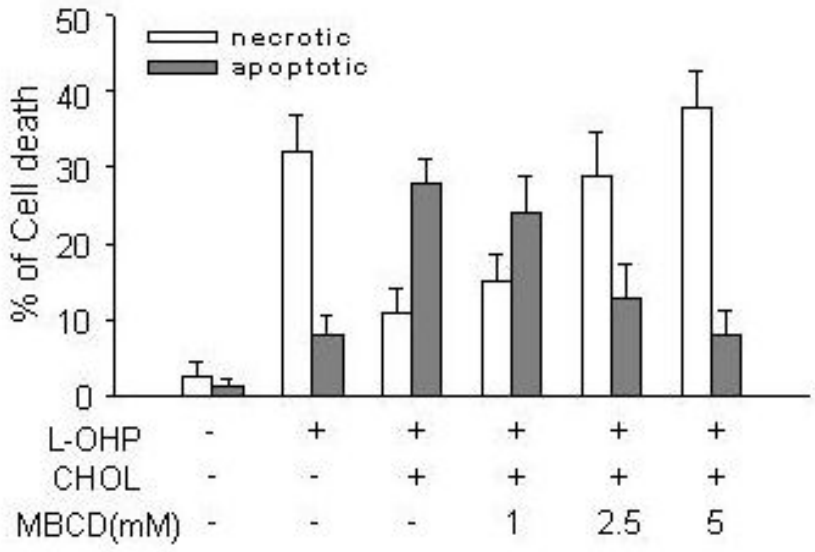

Figure 7. Oxaliplatin/cholesterol-induced apoptotic death is reverted by MBCD. Concentrations of MBCD (1 mM, 2.5 mM and 5 mM) were pre-treated with oxaliplatin/cholesterol and then the necrosis and apoptosis were counted by HO/PI under the fluorescence microscope.

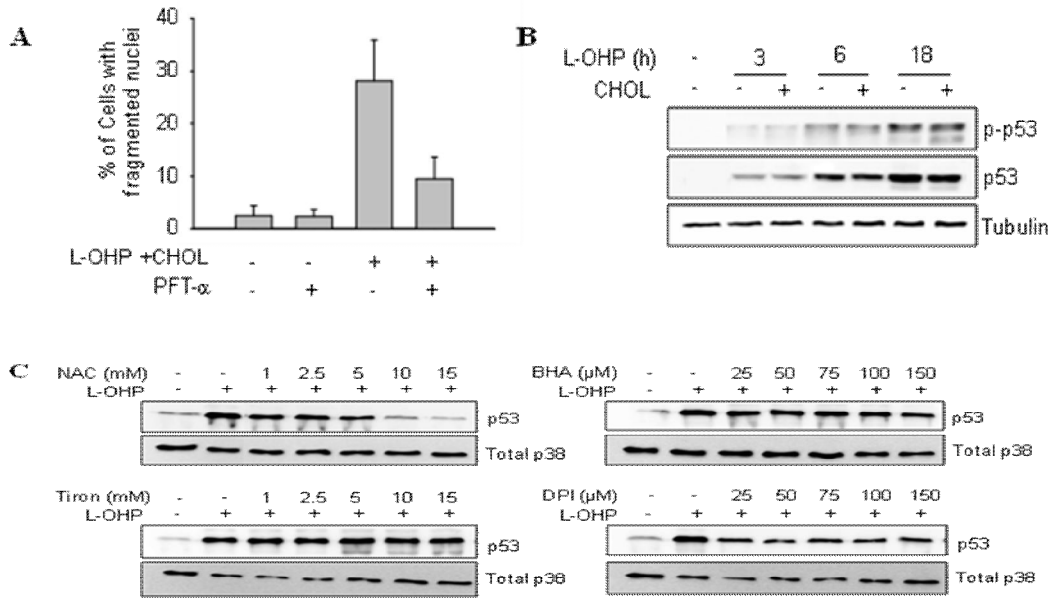

Figure 8. Apoptotic cell death is regulated by p53. A, p53 inhibitor (PFT-alpha) pretreated with oxaliplatin/cholesterol at 24 h. B, Cholesterol treated with or without oxaliplatin for 3 upto 18 h and then the proteins were subjected to Western blot for the detection of p53. Tubulin is the loading control. C, Anti-oxidant agents were pretreated with oxaliplatin at 24 h and samples were subjected to Western blot for the detection of p53 and p38.

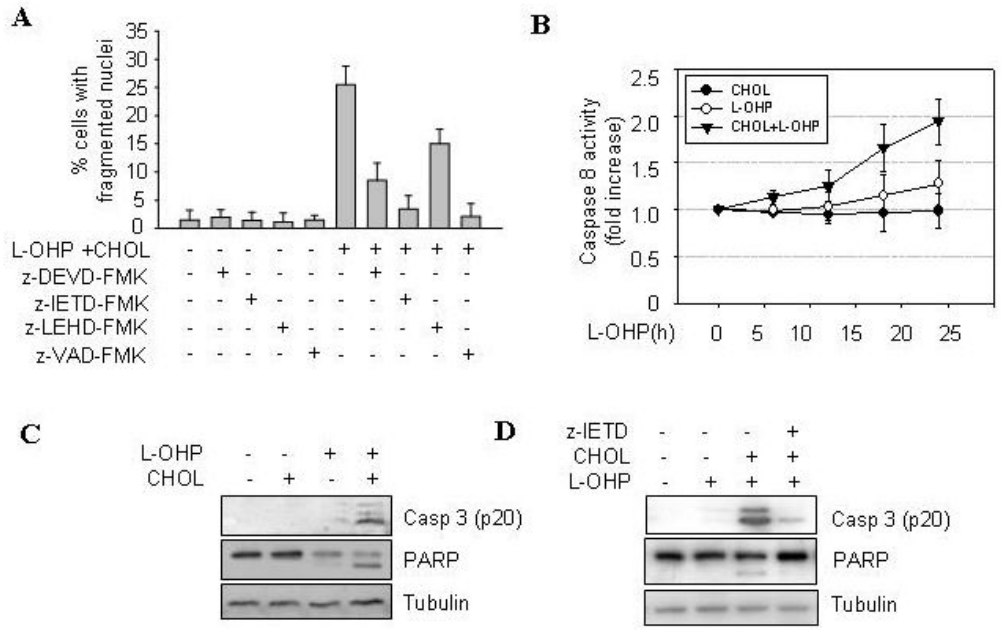

Figure 9. Apoptotic cell death is mediated by caspase-8 activation. Different caspase inhibitors were treated with oxaliplatin/cholesterol for 24 h and then performed HO/PI staining. Proteins were subjected to Western blot for the detection of caspase-3, PARP (Figure A and D). Caspase-8 activity assay and Western blot after the treatment of oxaliplatin and cholesterol at different time points (Figure B and C).

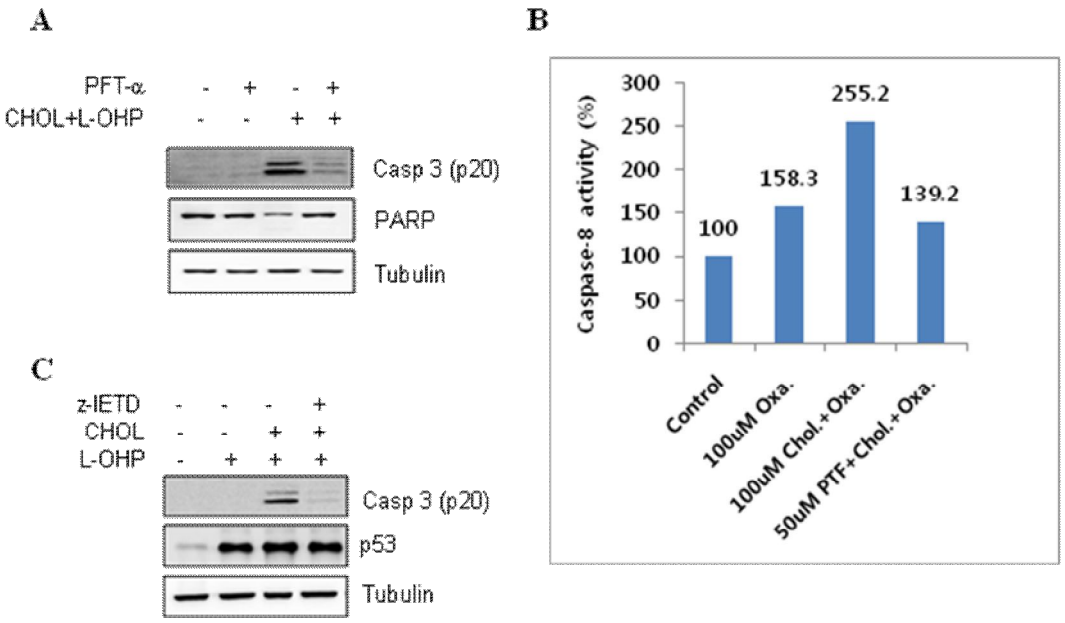

Figure 10. Oxaliplatin/cholesterol-induced apoptotic death is mediated by p53 and caspase-8 pathway. Pre-treatment of p53 inhibitor (PFT-alpha) for 30 minutes and then caspase-8 inhibitor (z-IETD) with oxaliplatin/cholesterol for 24 h and then proteins were subjected to Western blot (Figure A and C). Measurement of caspase-8 activity after the treatment of oxaliplatin with and without cholesterol (Figure B).

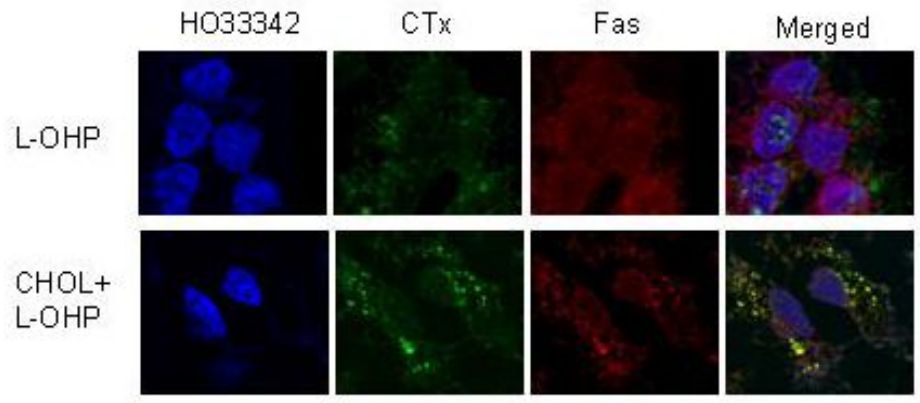

Figure 11. Oxaliplatin/cholesterol induces FAS clustering in lipid rafts. Cells were grown in cover slips overnight and treated with oxaliplatin and oxaliplatin/cholesterol for 24 h. Incubate with anti-FAS coupled to secondary antibody (red emission).Stained by cholera toxin B (CTx-B) for GM1 and Hoechst for nuclear staining, followed by confocal microscopy.

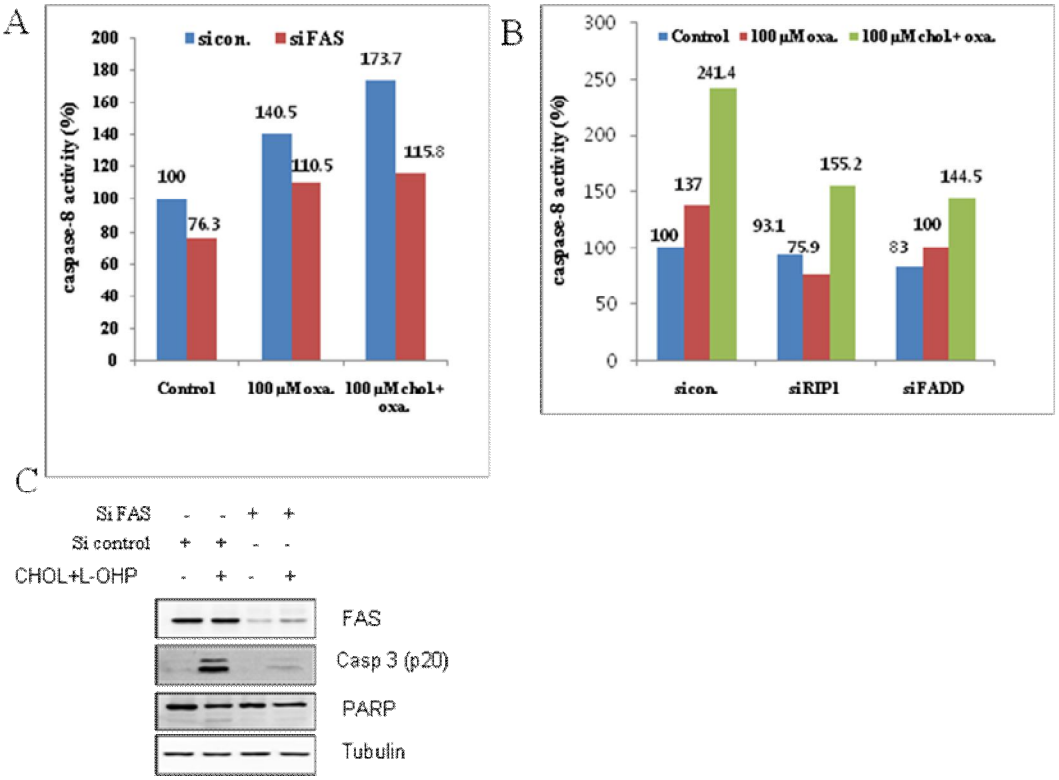

Figure12. Oxaliplatin/cholesterol-induced apoptosis is mediated by FAS, FADD, and RIP1. HepG2 cells were transfected with siRNA control, siRNA for FAS, siRNA for FADD and siRNA for RIP1. Cells were treated with 100 μM oxaliplatin and oxaliplatin/100 μM cholesterol for 24 h. Proteins were subjected for the detection of caspase-8 activity assay (Figure A and B). siRNA control and FAS siRNA samples were also used for detection of cleaved caspase-3, PARP, FAS and tubulin for loading control (Figure C).

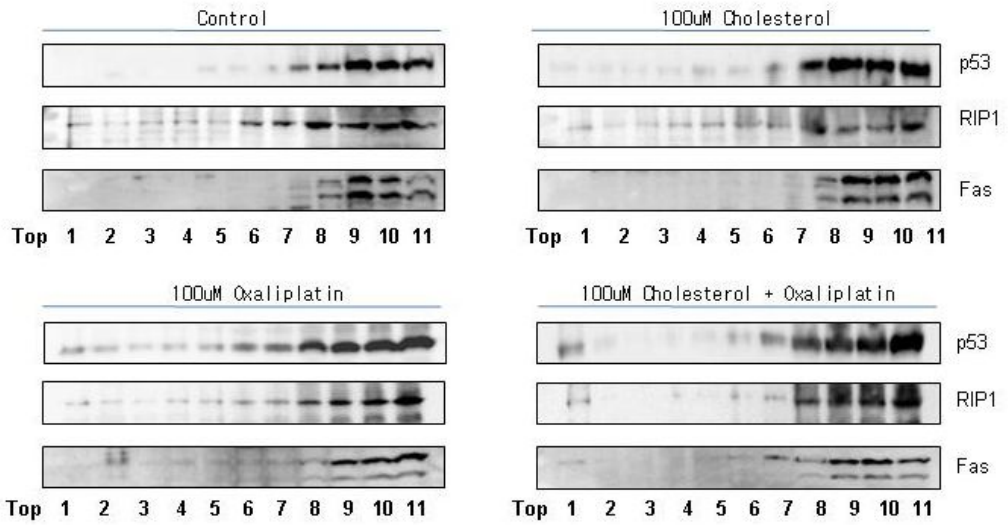

Figure 13. Pro-apoptotic proteins are localized into lipid raft region in response to L-OHP/cholesterol. HepG2 cells were plated in 15 cm^2 dishes overnight and treated with cholesterol (100 μM), oxaliplatin (100 μM) and their combination for 28 h. Raft and non raft fractions were isolated by discontinuous sucrose gradient methods. Eleven fractions of 1 ml gradient were precipitated for sucrose removal and subjected to Western blot for detection of p53, FAS and RIP1.

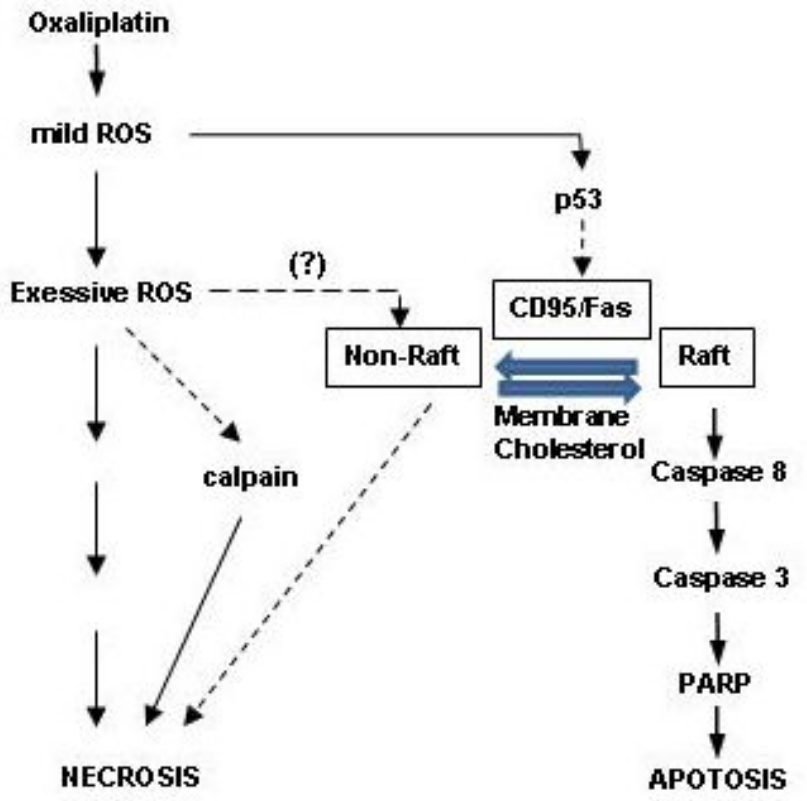

Figure 14. Diagrammatic representation of cell death pattern by the treatment of oxaliplatin alone and In combination with cholesterol. Oxaliplatin induced ROS production caused both necrosis and apoptosis. Excessive ROS induce necrosis but mild ROS activate the p53. Cholesterol administration with oxaliplatin switch the cell death mode from necrosis to apoptosis through the activation of membrane proteins FAS, FADD and caspase-8.

CHAPTER 4: DISCUSSION

Excessive production of ROS is another factor that triggers necrosis, even though its role in the induction of apoptosis has also been reported. Extreme hepatectomy-induced liver failure occurs due to necrosis as a result of production of massive oxidative injury; however, this condition can be recover through IL-6 over expression or administration, or by reducing of oxidative stress and maintaining mitochondrial function (29). In addition suppression of ROS through the blocking of SOD1 released by PKC activation or through the addition of antioxidants switches glucose depletion induced necrosis to apoptosis. In this study, excessive ROS production induced by oxaliplatin caused necrosis in HepG2. The mild production of ROS induced by oxaliplatin caused apoptosis due to the activation of p53 protein. Here ROS production due to oxaliplatin was blocked by the treatment of antioxidant agents NAC, BHA, DPI and in different concentrations. NAC effect was significant to compare the other ROS scavengers; it completely blocked the necrosis by consuming the ROS production due to the oxaliplatin treatment. Other antioxidant agents slightly prevent the necrosis by the decreasing of ROS level after oxaliplatin effect Fig.2B. When oxaliplatin was treated with membrane cholesterol depleting agent MBCD, the necrosis was increased without alteration of cell survival. Oxaliplatin induced excessive ROS production caused necrosis due to the mediator of calpain, which is a family of calcium dependent, non-lysosomal cysteine proteases (proteolytic enzymes). When calpain inhibitor ALLN was pretreated with oxaliplatin at 24 h, the necrotic cell death was suppressed (data not shown….). Some of the non raft components can be the factor for the

necrosis due to the excessive ROS production, that is remain to further study.

In my study, initial ROS production induced apoptosis by the activation of p53. When p53 inhibitor i.e. PFT-alpha was pretreated with cholesterol and oxaliplatin combination, the apoptosis was dramatically reduced. Oxaliplatin induced ROS production activated the p53 protein levels which was suppressed by the 1 hr pretreatment of different antioxidant agents like NAC, DPI, BHA and tiron but did not switch the mode of cell death. When the cholesterol was treated with oxaliplatin the cell death was switched from necrosis to apoptosis. Many studied have revealed that raft/caveolae is abundant in various signaling molecules and that they have been associated with a number of biological functions, Including cell survival, proliferation and migration (30). Cholesterol is a major lipid component of raft/caveola, and these membrane cholesterol levels are an important factor for raft/caveolae stability and organization (31, 32). Cholesterol is required to build and maintain cell membrane. It regulates membrane fluidity over the range of physiological condition. Within the cell membrane cholesterol functions as intracellular transport, cell signaling and nerve conduction. Raft/caveolae disruption by cholesterol depletion from the plasma membrane has been reported to deregulation of intracellular signaling pathways and cross talk between different receptor systems, including that membrane cholesterol integrity is critical for intra cellular signaling triggered by cell surface receptors (33). In this study cholesterol with oxaliplatin combination induced apoptosis by the activation of proapoptotic proteins. These proteins were migrated from non raft and cytosolic regions to membrane raft region. Cholesterol interacts differently with different membrane lipids and,

among naturally occurring lipids, shows a particular affinity for sphingolipids. Here I showed that upstream proteins are aggregated into the raft of plasma membrane. It is assumed that cholesterol acted as a transportation of membrane proteins in to the membrane raft from non raft regions. When those proteins are localized into the raft of plasma membrane, the apoptotic signal is regulated into the downstream proteins. FAS, FADD, RIP1 trigger DISC formation in to the raft. Caspase-8 is activated in the DISC, that activation signal is transduced for apoptosis. In this study, cholesterol and oxaliplatin induced apoptosis is highly suppressed by the caspase-8 inhibitor z-IETD-FMK, caspase-3 inhibitor z-DEVD-FMK, caspase-9 inhibitor z-LEHD-FMK and general caspase inhibitor z-VAD-FMK. Caspase-8 is the upstream initiator caspase, its activation activates the caspase-3 and caspase-9 through extrinsic and intrinsic pathways. The result in Fig.7A shows that, the effects of caspase-8 more significance, when we compare the efficiency of inhibitors of caspase-9, caspase-3 and caspase-8. Caspase-9 inhibitor showed a little bit suppression of apoptosis and caspase-8 and caspase-3 suppressed more apoptosis from this result we can say that cholesterol and oxalipaltin induced apoptosis through the extrinsic pathways and very less through the intrinsic pathways. Cholesterol is the major component of lipid raft, which enhance the aggregation of FAS, FADD and other membrane proteins. The activation of caspase-8 in the DISC of membrane raft initiates the downstream effectors caspase-3. The caspase-8 activity result shows that FAS, FADD and RIP1 proteins are activated by cholesterol and oxaliplatin treatment and apoptotic signals moved into the downstream. The knock down condition of these proteins by the transfection of

small interference siRNA did not oxaliplatin/cholesterol induce apoptosis i.e. shown by the cleaved caspase-3, PARP and caspase-8 activity in Fig.10. The result of lipid raft isolation and confocal microscopy shows that, upstream proapoptotic proteins are localized into the membrane raft in oxaliplatin and cholesterol treated cells to compare the oxaliplatin and control in Figure 11. The immunofluorescence result indicated that membrane raft has the crucial role for the aggregation of FAS and other protein and their activation to apoptosis signal transduction.

Finally, cholesterol and oxaliplatin combination induced apoptosis by the P53 activation and aggregation of other proapoptotic proteins in the lipid raft then DISC was formed. In the DISC, initiator caspase-8 was activated which triggered the activation of cleaved caspase-3 and PARP then apoptosis.

CHAPTER 5: CONCLUSION

In conclusion, Reactive oxygen species is an important factor for cell death. In HepG2 cell, oxaliplatin-induced more necrosis and a little bit apoptosis. The excessive ROS production due to oxaliplatin induced necrosis and mild ROS production induced apoptosis. Membrane cholesterol played an important role to cell death mode. When the membrane cholesterol was depleted by the MBCD with oxaliplatin, increased the necrosis which was decreased by cholesterol addition. Administration of cholesterol switched oxaliplatin-induced necrosis to apoptosis. Cholesterol and oxaliplatin induced apoptosis due to the activation of p53. The proapoptotic proteins were redistributed into the lipid raft from non rafts region of plasma membrane. DISC was formed in the lipid raft Due to the aggregation of FAS, FADD, RIP1 and caspase-8. The activation of caspase-8 in the DISC initiated the downstream effector caspase-3 then apoptosis occured.

CHAPTER 6 : REFERENCES

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저작물 이용 허락서

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논문제목	한글: HepG2 감암세포에서 세포막 콜레스테롤이 oxaliplatin에 의한 세포사멸방식에 미치는 영향				
	영어: Effect of membrane cholesterol on oxaliplatin-induced cell death patterns in HepG2 cells				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(O) 반대( )

2009 년 월 일

저작자: Keshab Raj Parajuli (서명 또는 인)

조선대학교 총장 귀하

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