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Inhibitory Effects of Heparin Derivatives on A549-derived Exosomes

Graduate School, Chosun University

Department of Biomedical Sciences

Sumeet Manandhar

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지도교수 황 승 림

이 논문을 약학 석사학위신청 논문으로 제출함

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Graduate School, Chosun University

Department of Biomedical Sciences

Sumeet Manandhar

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위원장 조선대학교 교 수 조 승 주 (인)

위 원 조선대학교 교 수 온 탁 범 (인)

위 원 조선대학교 교 수 황 승 림 (인)

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조선대학교 대학원

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ABBREVIATIONS

TEM	**Transmission electron microscopy**
NTA	**Nanoparticle tracking analysis**
DLS	**Dynamic light scattering**
DC	**Dendritic cells**
MHC	**Major histocompatibility complex**
CD	**Cluster of differentiation**
ILVs	**Intraluminar vesicles**
MVBs	**Multivesicular bodies**
ESCRT	**Endosomal sorting complex required for transport**
NSCLC	**Non-small-cell lung carcinoma**
LMWH	**Low molecular weight heparin**
UFH	**Unfractionated heparin**
HSPG	**Heparan sulfate proteoglycan**
EVs	**Extracellular vesicles**
FBS	**Fetal bovine serum**
PBS	**Phosphate-buffered saline**
BSA	**Bovine serum albumin**

ABSTRACT

Inhibitory Effects of Heparin Derivatives on A549-derived Exosomes

Sumeet Manandhar

Advisor : Prof. Hwang Seung Rim, Ph.D.

Department of Biomedical Sciences

Graduate School of Chosun University

엑소좀은 세포에서 분비되는 나노 크기의 입자로, 특정 조직에 상주하는 세포와 융합하여 단백질, 핵산 및 지질을 전달할 수 있는 매개체 역할을 한다. 종양세포로부터 분비된 엑소좀은 그 유래세포에 대한 중요한 정보를 담고 있어, 암전이 억제를 위한 진단 및 치료 표적으로서의 가능성을 지닌다. 헤파란 설페이트 프로테오글리칸은 엑소좀이 그 유래세포로부터 수용세포 안으로 이동할 때 수용체로서 작용하는 것으로 알려져 있다. 따라서, 헤파란 설페이트와 구조적으로 유사한 헤파린이 헤파란 설페이트와 엑소좀의 상호작용을 경쟁적으로 저해 또는 유도할 수 있을 것으로 가정하여, 본 연구에서는 암세포 유래 엑소좀에 의해 촉진된 암전이에 대한 헤파린 유도체들의 효과를 비교 연구하였다.

폐암세포주인 A549 세포로부터 분비되는 엑소좀을 분리하기 위해 세포배양액의 순차적 원심분리 후 초원심분리기법을 적용하였다. 분리한 입자의 물리적 특성을 투과전자현미경, 나노입자 추적 분석 및 동적 광산란을 이용하여 분석하였다. 투과전자현미경 사진을 통해 엑소좀의 소낭성 막 구조를 확인하였다. 나노입자 추적 분석에 의해 측정한 유체역학적 입자 직경의 평균값은 170.8±21.7 nm, 최빈값은

141.0±13.5 nm 였으며, 일정 부피 당 입자 개수도 추정 분석하였다. 또한, 분리한 엑소좀 시료의 총단백질을 소혈청 알부민 표준액과 비교하여 정량하였고, 엑소좀 표면에 있는 tetraspanins 중 CD63 및 CD81 을 ExoELISA 키트로 검출 정량하였다.

세포 이동 측정 실험에서, 저분자량 헤파린 및 periodate 산화를 통한 저분자량 헤파린 유도체 (0.4 mg/mL)는 A549 유래 엑소좀 (10 μg/well)으로 촉진된 암세포 이동을 완전히 억제하였다. 반면, 비분획 헤파린 및 periodate 산화를 통한 비분획 헤파린 유도체는 동일한 농도에서 저분자량 헤파린 및 periodate 산화를 통한 저분자량 헤파린 유도체에 비해 효과적으로 세포 이동을 억제하지 못했다.

이상에서와 같이, 종양세포에서 분비된 엑소좀은 암전이에 있어 중요한 역할을 하며, 저분자량 헤파린 유도체가 이러한 종양세포 유래 엑소좀의 작용을 억제할 수 있는 가능성을 본 연구를 통해 제시하였다.

I. INTRODUCTION

A. Exosome

Exosomes are small vesicles derived from cells that are present in cell culture media, urine and blood, whose sizes are in the range of 30 to 200 nm. They are released to the extracellular environment when the multicellular bodies are fused with the plasma membrane [1]. Many types of cells such as mast cells (Raposo et al., 1997), dendritic cells (DC) (Thery et al., 1999), tumour cells (Mears et al., 2004), reticulocytes (Johnstone et al., 1987), epithelial cells (Van Niel et al., 2001), B-cells (Raposo et al., 1996) and neural cells (Faure et al., 2006) release exosomes.

In 1986, Pan and Johnstone became the first scientists to discover the exosome while studying maturation process of sheep reticulocytes. They found that 50-nm-sized vesicles containing the transferrin receptor were released as a consequence of fusion of larger vesicles with the plasma membrane. The released vesicles were named exosomes [2-5]. In those days of their discovery, exosomes were believed to only function as shedding unwanted proteins from cells.

After 13 years, scientists found that exosomes had immunological functions [6]. As demonstrated by Raposo et al., exosomes secreted by B lymphocytes consisted of major histocompatibility complex (MHC) class II on their surface, recognized by cluster of differentiation (CD)4+ T cells. These immunological functions have increased a great interest in the research field of exosomes. Recently, exosomes have been discovered in various body fluids including blood plasma, breast milk, bronchoalveolar lavage fluid and malignant effusion under both disease and healthy conditions [7-10].

1. Biogenesis

Life of exosomes begins with proteins protruding inward at the surface of cell. It may be initiated by either clathrin-dependent (e.g. transferrin receptor) or clathrin-independent (glycosylphosphatidylinositol-anchored proteins) manners. Exosomes are transferred to the early endosome, where housekeeping receptors are detached and sent back to the plasma membrane or transferred to the late endosome. In the late endosome, there is formation of intraluminal vesicles (ILVs) by inward budding into the endosomal lumen, resulting in formation of multivesicular bodies (MVBs). The MVBs either fuse with the plasma membrane which consequently releases exosomes, or degrade protein by fusing with lysosomes [11, 12]. Due to the inward budding processes, exosomes retain the similar orientation to their original cells, cytosol found inside and membrane protein found outside.

The way of arrangements of lipids and proteins at the endosomal membrane of ILVs and functions of MVBs are not yet clear, even though there are some suggested mechanisms. The component or machinery that is involved in transporting and sorting the ILVs at the endosomal membrane has been recently found. This machinery is named as Endosomal Sorting Complex Required for Transport (ESCRT), like ESCRT-0, I, II or III. ESCRT-0 is the firstly identified protein complex binding Tsg101, the ubiquitinated protein. ESCRT-I acts as a bridge between ESCRT-0 and ESCRT-II. And through ESCRT-II, Tsg101 then recruits ESCRT-III, which leads to sequester proteins into the budding vesicles during MVB formation. ESCRT-III has an important role in recycling the ESCRT machinery via interaction with Vps4 and the AAA-ATPase spastin. In addition, ESCRT complexes showed sorting of non-ubiquitinated proteins, which could be directly interfering with ESCRT-II and III [12-15]. Sorting of proteins can also be seen independently of ESCRT. As reference to the latest publication, sphingolipid ceramide is involved in sorting mechanisms in spite of ESCRT complex. Sorting of heat shock protein, one of the cytoplasmic proteins, can be disclosed by random engulfment of portion of cytoplasm as formation of vesicles.

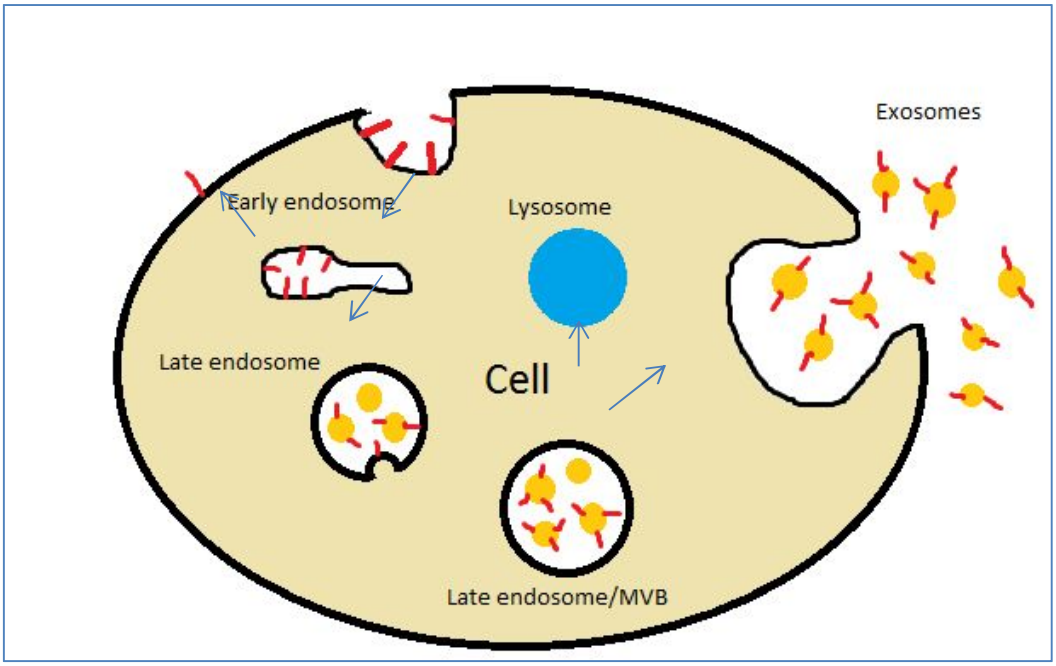


Figure 1. Biogenesis of exosomes. Membrane proteins are delivered to the early endosome. In the early endosome, proteins may be delivered to the plasma membrane or sent back to the late endosome. Multivesicular bodies are formed from intraluminal vesicles (ILVs) by budding of endosomal membrane into the endosomal lumen. Consecutively, ILVs are released to the external compartment. The released ILVs are known as exosomes.

2. Composition

Some common characteristics in density, size and total protein composition are found in exosomes secreted by various cells. Lipid bilayer membrane of exosomes is enriched with sphingomyelin, and rapid flip-flop occurs between the inside and outside of the membrane [16, 17]. As determined by electron microscopy, exosomes range from 30 to 200 nm in their size, and from 1.13 to 1.21 $g \cdot ml^{-1}$ in their density [18]. Exosomes are rich in proteins which are originated from the endosome, cytosol and plasma membrane. The most commonly identified proteins in exosomes are membrane transporter proteins or fusion proteins (i.e., GTPase and annexins), tetraspanins (i.e., CD9, CD63, and CD81), heat shock proteins (i.e., Hsp70), MVB proteins (i.e., Alix and TSG101), lipid-related proteins and phospholipase [19]. Among these proteins, tetraspanins such as CD63 and CD81, are mostly used for characterization of exosomes. Specific cell protein markers can make identification of exosomes derived from the specific cells with their own functions. In addition, different subpopulations of exosomes are produced from a single cell line. Laulagnier et al. revealed that subpopulations of vesicles enriched with various phospholipids and proteins are produced from a basophil leukemia cell (RBL-2H3). One population of exosomes containing MHC class II was enriched with phospholipids from granules, whereas tetraspanin-containing exosomes were enriched with phospholipids from golgi. CD63, CD81 and MHC class II were contained 47%, 32%, and 21%, respectively in the total exosomes [20].

3. Isolation

Various methods can be used for the isolation of exosomes based upon properties like size, density and biochemical properties. Among all of the methods, serial centrifugation is one of the common methods in purifying exosomes, subsequently high speed ultracentrifugation for pelleting the exosomes [1]. Filtration process can also be used for removing cells and debris. Further Purification of the pelleted exosomes gained by the ultracentrifugation step can be necessary depending upon the downstream application to remove contamination of proteins. Furthermore, specific density of exosomes can be one of the key factors for purification of exosome by density gradient or by sucrose deuterium oxide (D_2O) cushions [21]. In addition, chromatography can be used as another method which is based on size exclusion. Exosomes can also be isolated using their membrane properties. Specific antibody coated on the beads against a known target protein which is enriched on the surface of exosomes can be mixed with cell culture supernatant after cell depletion [22]. But it has a drawback that only a little part of the exosomes will be selected unless all the exosomes contain the target protein used for the isolation. For clinical applications, a good manufacturing practice method has been developed for the isolation of exosomes, which is based on diafiltration and ultrafiltration subsequently followed by ultracentifugation on sucrose cushions, resulting in a highly pure and sterile exosome pellet. It is the best method for the large-scale isolation of exosome which requires certain equipments. Nonetheless, there is a high demand for more reliable and faster techniques that yield highly purified exosome fraction.

4. Function

Function of exosomes is most dependent upon the cell type and conditions under which exosomes are produced, giving them their unique composition. When the exosomes from reticulocytes were discovered, they presented function as a method to eliminate proteins at transferrin receptor during maturation process of reticulocytes into erythrocytes [3]. In reticulocytes, it looks as if lysosomes are absent because lysosomes are degraded by the release of exosomes. On later research, exosomes were found to have an important role in transferring of messages from one cell to another. This type of mechanism may be seen either in microenvironment or over distance. Since blood plasma also consists of exosomes [23], they may be transferred between organs via systemic circulation.

Mechanism of the interaction between cells and exosomes is not fully cleared. Still, there have been many hypotheses about the mechanism. Receptor-ligand interaction is seen when exosomes bind to cells, similar to cell-cell communication, and it can mediate antigen presentation [24]. B cell-derived exosomes express integrins, which are equipped for adhesion to extracellular components and activation of fibroblasts. The powerful adhesion also causes an elevation in intracellular calcium level. In addition, exosomes also transport the exosomal surface proteins and cytoplasm to the recipient cell when they fuse with the target cell membrane [25]. Exosomes with MHC class II have been seen attached to follicular DCs in which MHC class II is not expressed. It gives them unique properties [25]. Ultimately, endocytosis is one of the mechanisms by which the recipient cell can internalize exosomes. The internalization of exosomes and antigen presentation to CD4+ T cells provide immature DCs with an important role [26].

B. Lung Cancer

Lung cancer is a tumor characterized by uncontrolled cell growth in lung tissues. Like other cancers, there is an acquired abnormality in the lung cancer cell. Alteration in the homeostasis for cell growth results in unstable division and cell proliferation that finally form a mass known as tumor. About 90% of lung cancers proceeds from epithelial cells, bronchioles and bronchi [27]. Occasionally, supporting tissues like blood vessels within lungs can be a factor proceeding to lung cancer. Lung cancers are developed through the various genetic changes in main cell-cycle genes. The variations may collect in bronchial epithelium leading to formation of daughter cells from a parent cell.

In some patients with lung cancer, tumor suppressor genes are damaged by the chromosomal abnormalities. Most commonly mutated chromosome regions are 3p, 9q, 13q and 17p.

Lung cancer can be distinguished into the following two types:

1. Small-cell Lung Carcinoma:

Small-cell lung carcinoma occupies 20 % of overall lung cancers. Aggressive and rapid growth is seen in this type of cancer [27]. Smoking of cigarettes is one of the main reasons that cause small-cell lung cancer. This type of cancer is found in late stages after the rapid metastasis to many sites. Small-cell lung cancer cells are smaller than non-small-cell lung cancer cells in their size [27].

2. Non–small-cell Lung Carcinoma:

Lung cancer has a very high fatality rate around the world, and non-small-cell lung carcinoma (NSCLC) is seen as the most prevalent form of lung cancers [28]. The poor prognosis caused by the lack of effective lung cancer screening method results in the poor survival rate, which is attributed to metastasis at the time of diagnosis. NSCLC is relatively insensitive to conventional chemotherapy, and the role of low-molecular-weight heparin (LMWH) in NSCLC has increased the attention of researchers for improving patient's survival rate [29]. It led to several clinical trials of LMWH in lung cancer [30]. NSCLC can be classified into the following three types:

(1) Adenocarcinoma: Adenocarcinoma is commonly diagnosed in Women and non-smokers. This type of cancer is seen in lung's gland, which causes production of mucus. NSCLC is a type of epithelial lung cancer, and A549 cells are known as adenocarcinomic human alveolar basal epithelial cells.

(2) Squamous Cell Carcinoma: Central areas mainly around bronchi in stratified or pseudo ductal arrangement are seen grown in this type of cancer.

(3) Large cell Carcinoma: This cancer is one of the rare type of Non Small Cell Lung Cancer. This type of cancer shows no specific morphological features.

C. Heparin

Heparin is widely known as an anticoagulant till today. It is a glycosaminoglycan formed by uronic acid residues and N-acetyl and O-sulfo-linked glucosamine residues. It is highly negatively charged molecule due to its many sulfate groups. Heparin has repeated polysaccharide chains, and heparin's molecular weight differs from 4-40 kDa. It has also a mean weight of 2.7 sulfate groups for each disaccharide [31].

For pharmaceutical use, starting proteoglycan form of heparin is split away from the protein core, leaving behind polysaccharide unfractionated chain. Heparatinase, heparin lyase, and heparinase are the three enzymes that act on heparin. There are various ways of heparin depolymerization such as treating with hydrochloric acid or oxidation of glycosidic bond, which results in the formation of LMWH. Since LMWH has smaller size (3-12 kDa) which decreases the nonspecific interaction with positively charged groups, making it active and circulate longer. LMWH can easily pass through the membranes and has higher plasma half-life, resulting in the elevation of bioavailability for pharmacological action. .

Heparin was firstly synthesized in 1916, and there has been a long debate on its inner structure and also on its anticoagulant properties. Even though heparin is well-known as an anticoagulant, it also has anticancer activities [32, 33].

The anticancer activity was first noted in 1930s, where heparin inhibited the growth of tumors implanted in rats [34]. Another research during the mid-90's also showed that the survival rate was increased with decrease in metastasis in heparinized rats [35]. Besides preclinical research, there were some ongoing clinical trials showing the effect of LMWH on NSCLC patients on its outcome and survival. Even though these clinical studies showed the prolonged survival, the exact antitumor mechanisms of LMWH is still unclear in lung cancer [36]. A study done on fraxiparine (LMWH) showed antimotility effect on lung cancer cells (A549), which also revealed that LMWH is

clinically useful in lung cancer therapy [37]. Another study on dalteparin (LMWH) showed the inhibition against A549 cell viability dose- and time-dependently as well as the induced early apoptosis [38].

Heparan sulfate proteoglycans (HSPGs) are receptors on the cell surface which have similarities in structure to heparin and play a role in biological activities. Heparin has characteristic to competitively block the ligand that binds to the HSPGs. Maguire CA et al. showed that incubation of heparin with the labeled extracellular vesicles (EVs) derived from 293T cells almost completely blocked the exosome uptake by the recipient 293T cells (unlabeled), and they also showed that transfer of tumor-derived EVs was inhibited by heparin [39]. In addition, HSPG was seen as an important part on the surface of the recipient cell for the uptake of tumor–derived EVs. Furthermore, in recent study, fibronectin present on the exosome surface showed the interaction with heparan sulfate on target cells. Fibronectin has heparin-binding site known as Hep II, found in the 12-14 position within C–terminal repeat units [40]. It shows a higher bonding to the heparin/heparan sulfate. In addition, exosome derived from myeloma showed fibronectin on their surface, and fibronectin of exosome interacted with the heparan sulfate chains found on the surface of target cells through Hep II domain [40]. These findings provide the evidence that heparin has the inhibitory effect for the uptake of exosomes, via blocking the interaction between the HSPGs found on the target cell and the exosomes delivered to the receipt cell.

II. Experimental Methods

A. Cell Culture

A549 cells, adenocarcinomic human alveolar basal epithelial cells, were purchased from Korean cell line bank. A549 cells were cultured in RPMI supplemented with 10% of exosome-depleted fetal bovine serum (FBS) and 1% of 10 mg/ml streptomycin with 25 μg/ml amphotericin B, incubated at the temperature of 37 °C in 5% CO_2. Cells were cultured in T-75 flask. Media was changed every 3-4 days. After the cell reached the confluency of about 70 to 80%, the cells were divided into 6-7 plates. Firstly, the cells were washed with 7 ml of phosphate-buffered saline (PBS) twice. After 1 ml of trypsin was added in the culture dish the dish was incubated for 3 min in the incubator. After observing whether the cells have detached from the culture dish or not, the detached cell was mixed with 5 ml of media and centrifuged for 3 min in 1400 rpm. The cell debris was mixed with about 5-6 ml of media and divided into 6-7 plates. Again after the confluency has reached until 70-80%, the media was changed with FBS-free media with 1% of above mentioned antibiotics for 48 h.

B. Isolation of Exosomes

Ultracentrifugation is used for the purification of exosome. Clarified culture media was harvested from A549 cells, and then centrifuged at 300 x g for 10 min at the temperature of 4 °C (VS-30000i, Vision Scientific, Korea) in order to eliminate the large dead cells and large cell debris followed by a series of centrifugation process. Then, the supernatant was collected and centrifuged for 15 min in 2500 x g, followed by another collection of supernatant and centrifugation for 1000 x g for 15 min. Then the supernatant collected from the centrifugation is filtered through a 0.22-μm bottle-top filter. And the final ultracentrifugation was done in 100000 x g for 3 h to get the pellet of exosomes. The pellet was then suspended with 100 μl of PBS.

C. Transmission Electron Microscopy (TEM)

Firstly, the grid was placed in the plasma cleaner. Then 5 μl of the resuspended pellets were deposited on grid for a while. Blotting paper was used to dry the grid, and 20 μl of 2% uranyl acetate was deposited on grid for 30 sec. The grids were removed from stainless steel loops, and excess fluid was blotted by gently pressing the edge of the grid onto Whatman no. 1 filter paper. The grid was left to air dry for at least 5-10 min.

D. Nanoparticle Tracking Analysis (NTA)

To measure the concentration and distribution of particles in liquid suspension, exosome samples were analyzed by Malvern Nano Sight N300 using both light scattering and Brownian motion. Approximately, 0.3 ml of the sample was diluted to 1ml in PBS. A laser beam was passed through the sample chamber, and the suspended particles in the path of the beam scattered light in such a manner that they could be easily visualized via 20x magnification microscope onto which a camera was mounted. The camera recorded video files of particles individually moving under Brownian motion at 30 frames/sec. After recording movements of particles, the NTA software calculated hydrodynamic diameters of particles using the Stokes-Einstein equation.

E. Dynamic Light Scattering (DLS)

DLS analysis for the size of exosome particles was performed with Zetasizer Nano ZS instrument (Malvern Instruments,Worcestershire, UK) at 22°C. It was equipped with 633 nm laser beam operated at an angle of 173. Exosome samples were diluted in 1ml of PBS and placed in solvent-resistant microcuvettes. Results were obtained from the Malvern software. For each sample, the relative size distribution curve was created.

F. Total Protein Quantification

10x RIPA buffer was added to A549-derived exosome sample, which was vortexed and incubated on the ice. The concentration of protein in the exosome lysate was then quantitatively analyzed through the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Each bovine serum albumin (BSA) standard and exosome sample solution were pipetted into each well in the microplate. Coomassie Brilliant Blue (G-250 dye; Bio-Rad Laboratories) was added to each well, which was mixed using a microplate mixer. After incubation of the microplate for 5 min at room temperature, absorbance values of standards and samples were measured with a microplate reader at 595 nm. The exosomal protein concentration was determined using the standard curve.

G. ExoELISA

The exosomal protein markers CD63 and CD81 were detected using ExoELISA-63 and ExoELISA-81 kits, respectively (System Biosciences Inc., Mountain View, CA). A549-derived exosome pellets isolated with ultracentrifugation were resuspended in 200 µl of exosome binding buffer (System Biosciences Inc.) followed by vortexing for 15 sec. The vortexed pellets were incubated at room temperature for 10 min. Prepared ExoELISA protein standards for each marker and A549-derived exosome protein samples (50 µl/well) were transferred to pre-coated wells of the microtiter plate. After the plate was incubated overnight at 37°C, it was washed 3 times with the washing buffer included in the kit. 50 µl of primary antibodies specific to CD63 and CD81 (1:100) were added into the wells, which was incubated for 1 h with shaking. The plate was again washed and then incubated with 50 µl of horseradish peroxidase-linked secondary antibody (goat anti-rabbit, 1:5000) for 1 hr at room temperature. After a colorimetric substrate and stop buffer were added, the absorbance of standards and samples was measured with a microplate reader at 450 nm. The results were calibrated by the standard curve. The samples were tested in triplicate.

H. Cell Migration Assay

After the cells have reached the confluency of 70-80%, trypsin was used to detach the cells, and the detached cells were collected by centrifugation. Cell pellets were resuspended in the media at a final concentration of $1x10^5$ cells/100 μl. Then the upper chamber of the transwell plate was filled with 100 μl of the cell suspensions and 100 μl of 2 x serum-free medium containing treatment reagent. 10 μg/well exosome was treated on the upper chamber, and lower chamber was filled with 600 μl of 1x 10% FBS medium containing treatment reagent. Then the plate were incubated for 24 h at 37°C. For staining of the invading cells, Eosine-hematoxylin were used. After 24 h, the remaining media in the upper chamber was removed, and the cells from the upper portion were removed using cotton swab. Then the new empty wells were filled with 1 ml of MeOH, and the invaded cells were fixed for 5 min. 1 ml of hematoxylin was added in new 24 well plates, and the transwell inserts were marked. After the fixed transwells were inserted into hematoxylin and incubated for 10 min, the inserts were submerged into a beaker filled with deionized water for washing. The transwell inserts were allowed to air dry.

III. RESULTS AND DISCUSSION

A. Physical Properties of Exosomes

Exosomes are difficult to be quantitated and visualized due to their small size. Isolation of exosome was done by ultracentrifugation technique. To characterize and address A549 cell-derived exosomes, we firstly used TEM analysis. Figure 2 shows the round-shaped vesicular membrane structure of exosomes isolated from A549 cells. From TEM images, the approximate diameter of exosomes also could be estimated. For determining the size range of exosomes secreted from A549 cells, NTA and DLS instruments were then used. According to the results from NTA, the hydrodynamic diameter of our exosome sample was 170.8 ± 21.7 nm (Figure 3). According to the result of DLS measurement, the mean diameter of our exosome sample was 158.3 nm (Figure 4). The Brownian motion of exosomes is shown in NTA because of charge-charge repulsion between exosome particles. The phosphatidylserine exposed on the surface membrane of exosomes provides negative charges on the exosomal surface.

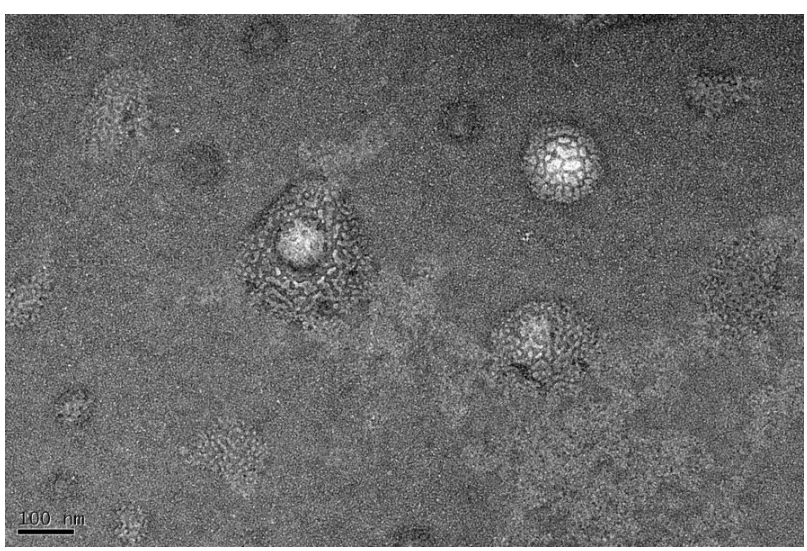
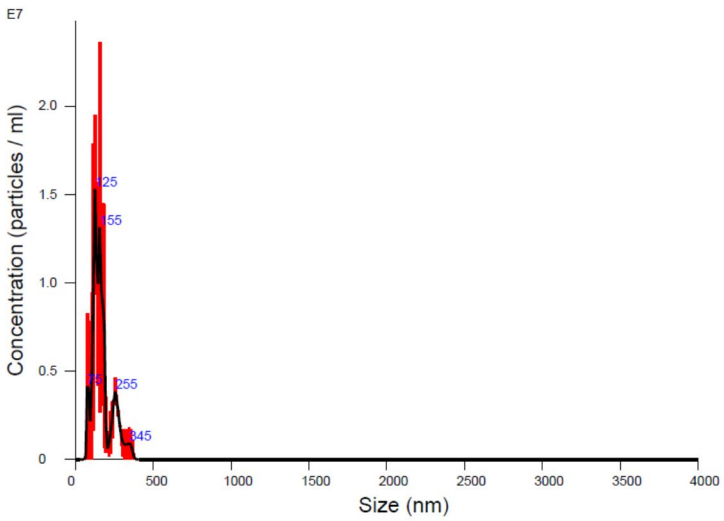


Figure 2. Detection of A549-derived exosomes by transmission electron microscopy (TEM)

(A)



(B)

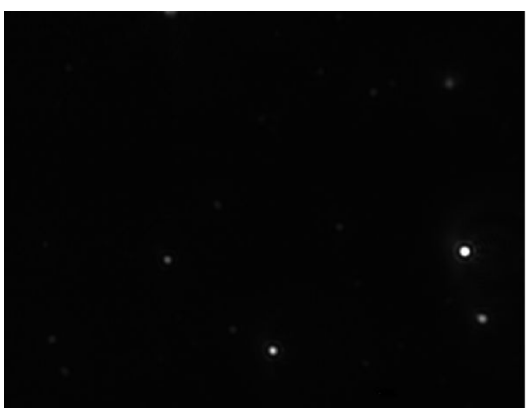


Figure 3. Nanoparticles tracking analysis (NTA) of A549-derived exosomes. (A) The concentration of particles per ml and the size distribution of sample. (B) Picture of exosomes taken from NTA.

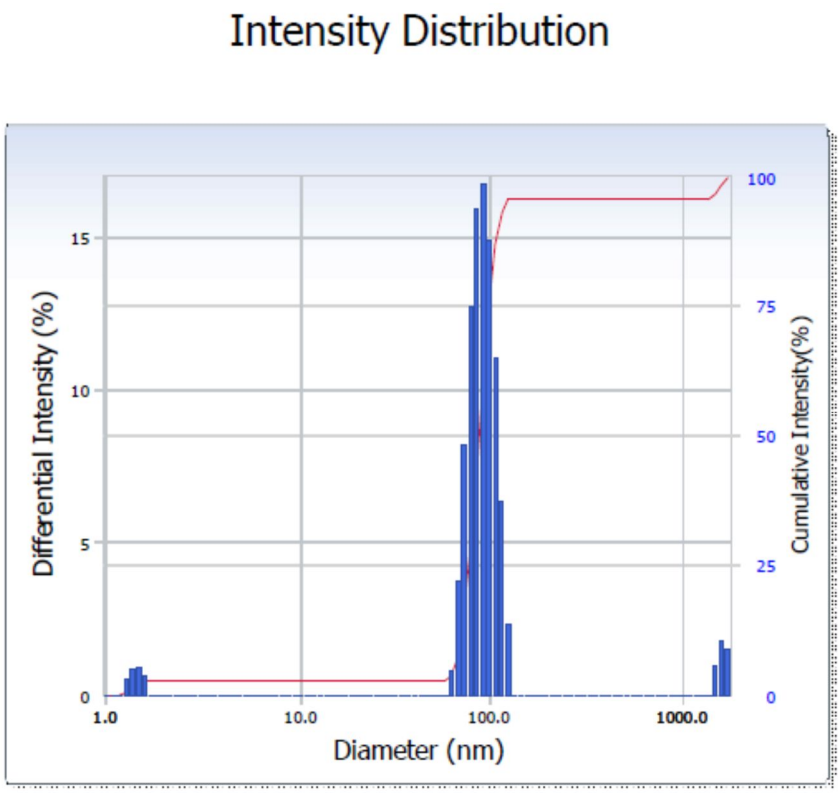


Figure 4. Size distribution profile of A549-derived exosomes measured by dynamic light scattering (DLS)

B. Protein Quantification

1. Total protein quantification

Total protein quantification is one of the methods to quantifying the amount of exosomes. For the protein quantification, we isolated the vesicles from conditioned cell culture medium. Proteins were extracted from the isolated exosome samples. After the protein extraction, we analyzed the protein amount in our sample. The mean value was 579.6154 ug/ml .

2. ExoELISA

For the quantification of exosome concentration with the specific protein in the EV samples, ExoELISA kits were used to detect tetraspanin proteins CD63 and CD81. The assay results showed that CD63 and CD81 were detected on A549-derived exosomes (Figure 5 and 6). The absorbance values of our exosome samples were shown within the standard range and above the detection limit of ExoELISA kits for both CD63 and CD81. The particle numbers in our exosome samples were estimated at $2.75 \times 10^9 \pm 3.07 \times 10^9$ and $3 \times 10^8 \pm 1.90 \times 10^8$, respectively from CD63 and CD81 ELISA standard curves.

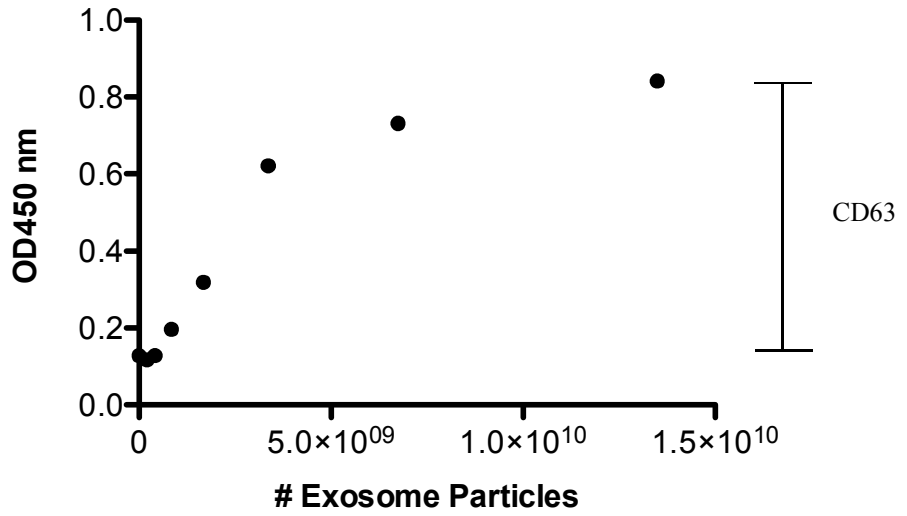


Figure 5. Quantification of CD63 protein on A549-derived exosomes.

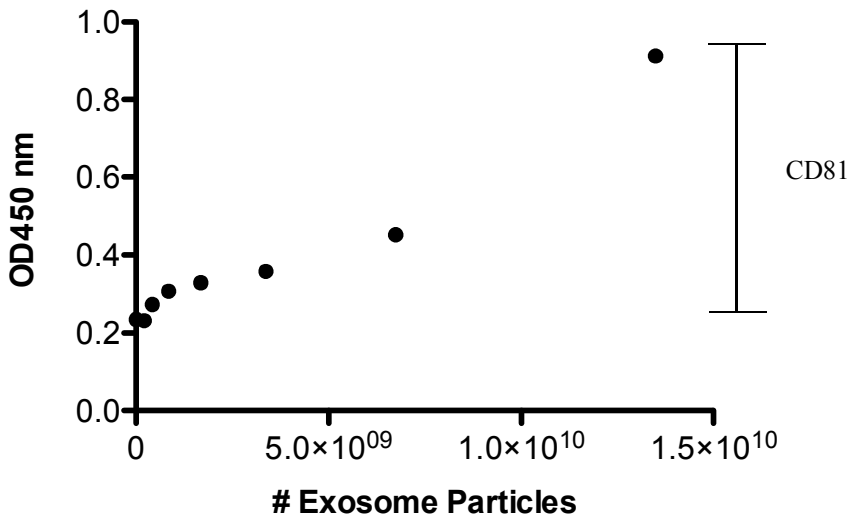


Figure 6. Quantification of CD81 protein on A549-derived exosomes.

C. Effects of Heparin Derivatives on Cell Migration with Exosomes

As exosomes derived from lung cancer cell elevates migration of cancer cells and endothelial cells, the present study is to find out the level of effects of various heparin derivative in the migration characteristics of exosome derived from A549 cell line. Both UFH and LMWH have an efficient inhibition effect of uptake of exosome to the receipt cell [41]. A549-derived exosomes (10 μg/well) dramatically increased the migration of A549 cell. Interestingly, among the four groups of heparin derivative, 0.2 mg/ml of LMWH or periodated LMWH effectively inhibited migration of lung cancer cell stimulated by A549–derived exosome, as compared to UFH or periodated UFH which showed less inhibitory effect on cell migration in the same concentration (Figure 7 and 8). As the concentration of all the heparin derivative was increased to 0.4 mg/ml, there was complete inhibition of the cell migration stimulated by A549–derived exosome in LMWH or periodated LMWH group, and less migration in UFH or periodated UFH group compared to lower concentration (data not shown). It shows that all heparin derivatives have dose-dependent effects on the migration of lung cancer cell stimulated by A549–derived exosome. HSPGs were reported to function as receptors of glioblastoma cell-derived exosomes. It is hypothesized that the uptake pathway of exosomes is dependent on the activation of the receptors on the recipient cells [41]. Thus, stronger interaction of the ligand/exosome with LMWH than the interaction with HSPG might be the reason that LMWH inhibited migration of cancer cells compared to the exosome-treated control group. For finding how heparin inhibits the uptake of exosome by the recipient cell and NSCLC metastasis, further studies should be performed. The phosphorylation of intracellular signaling pathways also should be checked for the detailed mechanism study. Proteomics can be applied for analysis of protein contents in exosomes. In addition, a panel of miRNAs (30b, 30c, 103, 122, 195, 203, 221 and 222) correlating with NSCLC can be analyzed, taking advantage of Real-Time PCR for exosomal RNAs from various types of cells.

As there is concern for bleeding side effects in therapeutic doses of heparins as anticancer agent due to their potent anticoagulant effects, some reports support the use of non-

anticoagulant/less anticoagulant heparin derivatives as a potential way for prevention of the tumor metastasis [42]. As we could not observe a direct correlation between anti-metastatic and anticoagulant activities of heparin derivatives in our cell migration assay, further screening for various derivatives should be needed to check the chances of splitting the anti-metastatic and anticoagulant activities of heparins. Antimetastatic heparin derivatives without bleeding side effects could be safely given to cancer patients, particularly in higher doses (if needed), thereby fully making use of the therapeutic efficacy of heparin [43].

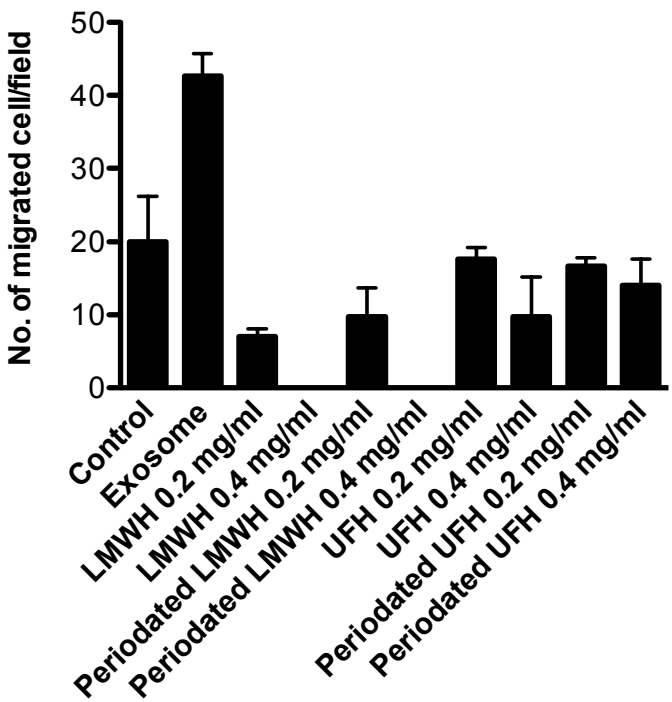


Figure 7. Cell migration assay regulated by A549-derived exosomes with the treatment of heparin derivatives. $1x10^5$ A549 cells/well were added to the upper chamber and RPMI with 10 μg/ml exosomes where added to lower chamber and different heparin derivatives where treated on the upper chamber. Cells were allowed to migrate at 37°C for 24 hr in the medium.

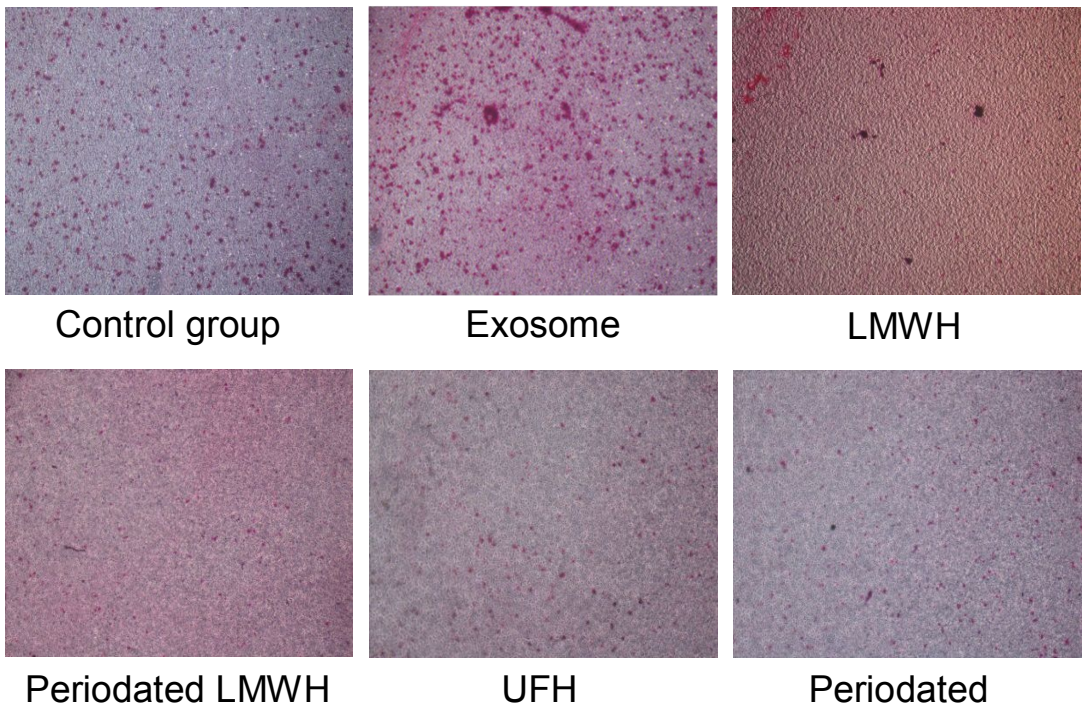


Figure 8. Microscopic images of cell migration regulated by A549-derived exosomes with the treatment of heparin derivatives (at 40-fold magnification)

IV. Conclusion

In summary, heparin derivatives have been identified as inhibitors against metastatic activities generated by cancer-derived exosomes. Among various heparin derivatives, LMWH and periodated LMWH showed higher inhibitory effects on cancer cell migration. Heparin derivatives might block exosomes that bind to HSPG on the receipt cell. Therefore, LMWH derivatives represent a promising class of antimetastatic agents.

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