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The development of the new anticancer agents using synthetic peptides capable of binding to cancer specific markers

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The development of the new anticancer agents using synthetic peptides capable of binding to cancer specific markers

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ABSTRACT

The development of the new anticancer agents using synthetic peptides capable of binding to cancer specific markers

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인간의 몸을 구성하는 가장 작은 단위는 세포이다. 몸을 구성하는 다양한 세포들이 모여 장기를 이루고, 장기는 다른 장기와 연결되어 하나의 생명체가 되는 것이다. 생명체는 삶을 유지하기 위해 장기의 크기, 호르몬 수치, 체액의 밸런스와 같은 몸의 항상성을 유지하여야 한다. 항상성을 유지하기 위해 세포는 각자의 기능을 수행하고, 적절한 시기에 노화되어 없어지기도 하고, 새롭게 만들어 지기도 한다. 세포가 새롭게 만들어 지려면 원래 존재하던 하나의 세포가 분열을 하면서 새로운 세포를 만들어 낸다. 그 과정 중 자신의 유전정보(chromosomal DNA)와 동일한 정보를 딸 세포에게 주는데, 이 유전정보에 이상이 생기면 질병이나 암이 발생하는 것이다. 이상이 생긴 세포가 자신의 기능을 잃어버리고 증식하여 새로운 조직을 만들거나 떠돌아 다니며 다른 장기들로 침입하는 현상을 암이라 한다. 비정상적 증식을 하는 세포는 주변

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정상세포를 잠식시켜 기관이 제 기능을 하지 못 할 때까지 끝없이 자라나기 때문에 몸의 항상성이 망가져 결국 죽음에 이르게 되는 것이다. 강제 100세 시대에 접어든 현 인류는 생존 기간에 비례하여 세포의 분열 횟수가 증가하였고, 발암물질에 노출되는 시간도 길어져 DNA에 오류가 발생 할 수 있는 확률이 높아졌다. 통계에 따르면 인간의 평균 수명이 80세이면 암을 경험 할 확률이 34%에 달한다 [1]. 3~4인 가족 중 1명은 암을 경험하는 매우 높은 수치인 것이다. 암은 세포로 구성된 우리 몸 어디에나 발생 할 수 있기 때문에, 발생하는 위치에 따라 증상이 매우 다양하고, 진단 및 치료방법 또한 다양하다. 암을 치료하는 가장 좋은 치료법은 외과적 수술을 통해 암 조직을 절제하는 것이다. 하지만 장기의 기능이나 위치에 따라 수술이 불가할 경우 다른 항암요법을 적용하게 된다. 항암요법은 크게 물질적 요법인 방사선치료와, 약물을 이용한 화학적 요법으로 나뉜다. 방사선 요법은 고 에너지의 방사선을 쬐어 암 조직이 죽어 없어지도록 하는 방법인데, 과한 방사선의 노출은 오히려 정상세포에 유전정보를 변형시키는 mutagen으로 작용하기 때문에 장기적 치료가 어렵다. 화학적 요법은 독성이 있는 약물을 처리하여 암세포를 죽이는 방법이다. 본 논문에서는 화학적 요법에 초점을 두어 연구를 진행하였다. 세포의 삶과 죽음은 세포 내부에서도 결정되지만 외부로부터 전달되는 화학적 신호에 의해서 더 크게 조절된다. 외부로부터 전달되는 신호 중 세포사를 유발하는 기전은 크게 세 가지로 나뉘게 된다. 먼저 apoptosis는 세포가 주변 조직에 영향을 주지 않으면서 조용히 자살하는 시스템으로, 발달 과정에서 필요 없어진 조직, 노화된 조직, 손상된 조직을 제거하는 방식이다. 두 번째는 세포가 물리적, 화학적으로 매우 극한에 환경에

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빠지면 세포가 터져 죽는 현상을 necrosis라한다. 최근 연구를 통해 necrosis 또한 programing 된 세포사가 존재한다는 것이 밝혀졌다. 마지막 세 번째는 세포가 에너지가 없는 환경에 놓이거나, 세포 내의 기관에 이상이 발생하여 제거되어야 하는 경우 세포가 살아남기 위해 자신의 소 기관을 소화시키게 되는데 그 과정에서 세포가 더 이상 생존이 불가해지면 죽게 되는 현상이다. 기존의 항암제는 주변 세포에 영향을 주지 않으면서 세포사가 유발되는 apoptotic agent의 사용이 높았다. 하지만 암을 유발하는 유전적 변이는 성장에 관련된 유전자의 획득뿐만 아니라 죽음과 관련된 유전자의 손실이 크게 작용하는데, 그 중 세포의 자살시스템을 관리하는 apoptosis 관련 유전자의 손실이 쉽게 발견된다. 그 외에도 한번 감시체계를 벗어난 암세포는 쉽게 변이를 일으킬 수 있어 결과적으로 apoptotic agent에 대한 저항성을 쉽게 획득한다. 최근 항암제 연구 추이는 Apoptotic agent에 저항성이 생긴 세포를 죽이기 위해 다른 세포사 기전인 necrosis 또는 autophagy를 유발하는 약물을 개발하는 방향으로 조금씩 바뀌고 있다. 그 중 Necrosis를 유발하는 물질의 장점은 기존의 항암제에 내성이 생긴 세포를 죽일 수 있다는 것 외에도 세포사 경로가 매우 짧아 약물에 대한 내성이 생기기가 어렵다는 장점을 가진다. 본 연구에서도 apoptotic agent에 저항을 보이는 암을 제거하기 위해 necrosis를 유발하는 peptide를 사용하였다. 본 연구에서 사용한 Necrosis를 유발하는 물질은 세포사를 유발하는 단백질로 알려진 Noxa로부터 유래된 MTD(mitochondrial targeting domain) peptide로 10개의 아미노산으로 구성되어있다. MTD peptide는 세포 내부에 존재 할 경우 mitochondria의 PT pore를 열어 많은 양의 Ca+을 cytosol로 방출하여 세포의 ion

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balance가 깨지면서 necrosis를 유발한다. 하지만 세포 외부에 존재할 경우 아무런 영향을 미칠 수 없기 때문에 세포 내부로 들어 갈 수 있는 system을 필요로 한다. 이와 같은 특성은 항암제 개발에 있어 이점으로 작용할 수 있다. 기존 항암제의 경우 암세포만을 표적 할 수 없었기 때문에 정상 세포에 영향을 미쳐 피부발진, 머리 빠짐, 구토와 같은 부작용이 있었다. MTD를 특정 세포에만 전달하는 system을 이용한다면 이와 같은 부작용을 감소시킬 것으로 기대한다. 본론으로 돌아가 세포는 plasma membrane이라하고 하는 세포막을 통해 외부와 내부의 환경을 차단한다. 외부의 물질이 세포내부로 들어가기 위해서는 세포막을 통과하는 system이 필요한데, R(8)라는 펩타이드는 (+) charge를 이용해 membrane을 뚫고 세포 내부로 들어간다고 알려졌다. 본 실험에서는 R(8)와 MTD를 결합시킨 R(8):MTD를 세포 외부에 처리해 주었을 때 세포사가 유발된다는 것을 알게 되었다. 하지만 R(8):MTD는 모든 세포의 plasma membrane을 통과 할 수 있기 때문에 정상세포 또한 죽일 수 있다. 본 연구자는 특정 세포만을 죽일 수 있는 새로운 전달 시스템을 검색하였고, 암에서 발현되는 수용체와 결합하는 pepetide를 이용해 MTD를 세포 내로 전달할 수 있도록 하였다.

외부의 신호가 세포 내부로 전달 되기 위해서는 세포막에 존재하는 수용체를 거쳐야 한다. 외부에 존재하는 신호의 종류는 크게 증식, 죽음, 이동에 관한 신호가 존재한다. 암 세포처럼 빠른 증식을 보이는 세포는 증식에 관한 수용체가 많이 발현된다. EGFR family는 EGF와 결합하는 수용체로 증식이 빠른 세포에서 높게 발현된다. 그 중 ERBB2는 다양한 종류의 암에서 발현이 되어 표적 치료제의 표적이 된다. 이 ERBB2와 결합하는 peptide인 KWYS를 MTD와 결합시켜 ERBB2를

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발현하는 세포만 특이적으로 죽이는 KWYS:MTD를 개발하였다. 하지만 ERBB2는 증식이 빠른 정상세포에도 존재하기 때문에 암세포만을 특이적으로 표적 하기에는 어려움이 있었다. 본 연구에서는 ERBB2 이외에도 다양한 암의 marker를 검색하였고 NRP-1이라고 하는 단백질이 암에서 높게 발현 된다는 것을 발견하였다. NRP-1과 결합하는 펩타이드인 RPARPAR을 MTD와 연결하여 TU17:MTD를 개발하였고, 혈관으로 펩타이드를 주입하였을 때 암 특이적으로 이동하여 암의 증식을 억제한다는 결과를 얻었다. 하지만 NRP-1 또한 혈관 내피세포에서 발현되기 때문에 혈관을 망가트리는 부작용이 나타났다. 아직까지 MTD를 backbone으로 사용한 연구에서 부작용이 없는 항암제의 개발이 이루어 지지 않았지만, 다양한 암의 마커 검색을 통해 새로운 항암제의 개발이 이루어 질 것으로 기대된다. 또한 MTD는 암뿐만 아니라 식도, 혈관, 요도에서 일어나는 내막증식과 같이 과도한 이상증식을 일으키는 질병에도 적용 시킬 수 있기 때문에 아직까지 무궁무진한 연구를 수행 할 수 있는 분야로 생각된다.

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ABSTRACT

The development of the new anticancer agents using synthetic peptides capable of binding to cancer specific markers

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Cells are the smallest component unit in the human body, in which various types of cells form organs, which are connected to one another to complete an entity. Living organisms must maintain homeostasis in the body including the sizes of organs, levels of hormones, and balance of body fluids in order to continue life. In the process of keeping homeostasis, cells perform their respective functions, age and die out at proper times, and are created anew. An old cell should divide and create new ones, in which process the mother cell gives the same information as its genetic information (chromosomal DNA) to its daughter cells. When there is something wrong with the genetic information, a disease or cancer happens. Cells with abnormalities lose their original functions, create a new organization through proliferation, and

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spread around into other organs, which is the mechanism of cancer. Cells of abnormal proliferation eat into normal cells in the surroundings and continue to grow until the affected organs stop functioning right, which leads the homeostasis of the body off the track and eventually to death. Entering the homo-hundred age, the current humankind has increased in the number of cell division compared with their duration of survival and in the time of exposure to carcinogens, thus facing greater probabilities of DNA errors.

According to statistics, average life span of 80 years means 34% probability of cancer [1], which means that one of the three to four members in a family will experience cancer. Since cancer can happen at any parts of the body comprised of cells, there are a variety of symptoms, diagnosis and treatment methods according to the location of cancer. The best way of treating cancer is to remove cancer tissues with a surgical operation. When it is impossible to do an operation due to the certain functions and locations of cancers, however, other anticancer therapies will be applied. Anticancer therapies are mainly divided into physical radiotherapy and medication-based chemotherapy. Radiotherapy administers radiation of high energy to kill cancer tissues, but the long-term application of radiotherapy is not desirable since excessive exposure to radiation can serve as a mutagen to alter the genetic information of normal cells. Chemotherapy kills cancer cells with toxic medication. This study focused on chemotherapy. The life and death of cells is determined

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inside cells, but they are more affected by chemical signals delivered from the outside. Cells are killed by signals from the outside in three major mechanisms: first, apoptosis is a system in which cells commit suicide silently without affecting their surrounding tissues. It is a way of removing the tissues that have become useless in the developmental process, aged tissues, and damaged tissues; second, necrosis is a phenomenon of cells bursting into death in a very extreme environment both physically and chemically. Recent research shows that there are cases of cell death with the programming of necrosis (necroptosis); and third, autophagy is a phenomenon of cells digesting their organelles to survive and then dying with no possibility of survival any longer when they should be removed in an environment of depletion of energy or due to abnormality in their organs. The conventional anticancer drugs used a high degree of apoptotic agents to cause cell death without influencing the surrounding cells. Genetic variations causing cancer are, however, under the huge influence of loss of death-related genes as well as the acquisition of growth-related genes. Of them, loss can be easily found in apoptosis-related genes to manage the suicide system of cells. In addition, once out of the Immune surveillance system, cancer cells can easily have variant cells and thus develop resistance cancer cells to apoptotic agents. In recent research on anticancer drugs, there is a gradual shift to the development of drugs to cause other mechanisms of cell death,

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necrosis and autophagy, to kill resistant cancer cells with resistance to apoptotic agents. Necrosis -inducing agents have a couple of advantages including killing cells with tolerance to the conventional anticancer drugs and making it difficult to develop tolerance to them due to the very short route of cell death.

In this thesis, I used MTD peptide to cause necrosis in order to remove cancer with resistance to apoptotic agents. The MTD (mitochondrial targeting domain) peptide derived from Noxa known as the protein causing apoptotic cell death. MTD peptide consists of ten amino acids. Inside cells, the MTD peptide opens up the PT pores of mitochondria, discharges large volumes of Ca+ into the cytosol, and breaks the ion balance of cells, resulting in necrosis. Outside cells, however, the MTD peptide itself has no means to influence and thus needs a system to go into cells. This characteristic can serve as an advantage in the development of anticancer drugs. The conventional anticancer drugs were not able to target only cancer cells and affected normal cells, causing a skin rash, hair loss, and vomit. If there is a system to deliver the MTD only to certain cells, it will reduce such side effects. In cells, plasma membrane blocks the external and internal environments. External materials need a system to pass through the plasma membrane in order to go into cells. A peptide called R(8) is known to go through the membrane with (+) charge and enter the inside of a cell. In this experiment, R(8) was combined with MTD.

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The resulting R(8):MTD was treated outside a cell and caused necrosis. However, R(8):MTD can go through the plasma membranes of all cells, which means that it can also kill normal cells. Thus, I searched a new delivery system to kill only certain cells and ensured the delivery of MTD into cells with a peptide combining with a receptor manifested in cancer.

The external signals should go through a receptor in the cell membrane in order to reach the inside of a cell. External signals mainly concern proliferation, death, and migration. Fast proliferation like cancer cells, many receptors are manifested for growth. The EGFR family includes receptors to be combined with EGF and has a high level of manifestation in cells of fast proliferation. ERBB2 member of EGFR family is manifested in a range of cancer cells. I succeeded in developing KWYS:MTD to kill cells with the manifestation of ERBB2 specifically by combining MTD with KWYS, which is a peptide to be combined with ERBB2. In the study, I also searched an cancer markers in addition to ERBB2 and discovered that a protein called NRP-1 recorded a high level of manifestation in cancer. After developing TU17:MTD by combining MTD with RPARPAR, which is a peptide to be combined with NRP-1, I injected the peptide intravenously into mice bearing cancer tissue, and found that TU17:MTD suppressed the progression of cancer. NRP-1, however, caused the side effect of damaged blood vessels as it was manifested inside endothelial cells of blood vessels. No previous studies that used MTD as a

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backbone succeeded in developing an anticancer drug with no side effects; but, it is expected to develop a new anticancer drug through the search of various cancer markers. In addition, MTD can also be applied to diseases causing excessive abnormal proliferation such as intimal hyperplasia in the esophagus, blood vessel, and urethra as well as cancer, thus presenting an area for various researches.

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CHAPTER1

INTRODUCTION

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1.1. Cell and Gene

All cells in the body have the same genome. How can the cells in the body have diversity in the same genome? If the cell was a biological computer, DNA acts as a command to run a program, and genome is a storage of commands. By controlling the command (specific coding DNA) in a cell with the same genome, Cells can perform many functions such as proliferation and growth as well as repair of damaged cells, removal of aged cells, and cell migration. The various cells and organs create from single fertilized oocyte in the same way. However, there are many risks such as expressing potentially unnecessary genes, because cells contain too many genes more than they need. Normal cells undergo many generations of cell division, resulting in a high incidence of errors due to the repetitive replication of the complete gene, exposure to mutagen, carcinogen, and various damages. The occurrence of an error does not lead directly to cancer, but when mutations occur in a critical region of genes related to proliferation or death, they produce abnormally overgrown cells in the body. These cells lose their basic functions and turn into abnormal cells that only intend for proliferation, and these cells are called cancer.

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1.1.1. Cell fate and signaling

Metazoan animals are composed of tens of thousands of individual cells and differentiated into various types of cells to perform their distinct functions. Differentiated cells live for a certain period of time that they can function normally, and then die when they get abnormality or senescent. How do cells know when to differentiate and when to die? If cells determine its fate without cell-cell interactions, it will get seriously confused like hyperplasia (abnormal grown) in tissues and organs. For this reason, the fate of a cell is affected by the cell cycle clock inside the cell, but it is mainly determined by external factors.

1.1.2. Receptors and growth factors

The external and internal environment of the cell separated by plasma membrane (also known as phospholipid bilayer). The inside of the cell separated by plasma-membrane is called cytosol. Various sub-cellular organelles are also enveloped by membranes and separated from cytosol. The existence of the membrane is to maintain the internal environment. For example, lysosomes require an acidic environment (pH 4.6 to 5), as many lysosomal enzymes are acidic hydrolases. Another function of the membranes is to prevent the foreign substances invasion. Foreign substances are transferred into the cell through channels and gates because cell membranes are selectively permeable (semi-permeable barrier). These gates are called receptors, including transmitters that carry external signals.

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Growth factors, External regulatory proteins that transmit signals to intracellular organelles maintain cell function and population. There is various type of growth factors since the overall functions of cells such as cell growth, proliferation, death, and migration are affected by these factors. As the types of growth factors vary, the types of corresponding receptors also vary. Furthermore, depending on the types of cells and a combination of receptors, the matched growth factors are different. Just like that the epidermal growth factor binds to epidermal growth factor receptors present in epidermal cells and transmits signals into the cells (Table 1-1).

1.1.3. Signal transduction (cell signaling)

What is the change inside the cell when the growth factor bind to the receptor? Growth factor signaling is a trigger for changing programs in the cell (like a biological computer). Cells have biological circuits composed of proteins. When the growth factor bind to the receptor, Intracellular domain of the receptor immediately undergoes a physical or chemical change such as phosphorylation. Subsequently, downstream proteins are also re-constituted by upstream partners, and the signal amplify. The benefits of using such biological circuits are possible to transmit the amplified signal to organelles that are difficult to move in the cytosol.

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Growth Factor	Receptor	cell responding to GF	cancer type	Reference
PDGF (Platelet-derived growth factor)	PDGFR	endothelial, Vascular smooth muscle cells, fibroblast, other mesenchymal cells	osteosarcoma, gliomas	[2], [3]
FGF (Fibroblast growth factor)	FGFR1, FGFR2	endothelial, Vascular smooth muscle cells, fibroblast, other mesenchymal cells	breast, gastric, endometrial carcinomas	[4]
EGF (Epidermal growth factor)	EGFR	many types of epithelial cells, some mesenchymal cells	breast, head and neck, stomach, colorectal, esophageal, prostate, bladder, renal, pancreatic, ovarial carcinomas; glioblastoma	[5]
HGF/SF (Hepatocyte growth factor, scatter factor)	Met	various epithelial cells	Head and neck squamous carcinoma, thyroid carcinoma, lung adenoma	[6]
IGF (Insulin-like growth factor)	IGFR	wide variety of cell types	Breast cancer, colorectal cancer	[7]
IL-6 (Interleukin 6)	IL-6R	mast cells, smooth muscle cells and skeletal muscle cells	Myeloma ,Head and neck squamous cell carcinoma	[8]

Table 1-1. Growth factors and receptors that are involved in cancer development

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1.1.4. Oncogene

Some problems arise in major proteins of complex and elaborately designed biological circuits, the cells become confused, and then programs are modified to abnormal. The gene that can potentially cause cancer through disturbance of the biological circuit is called an oncogene. What are the major proteins that can produce cancer and how many are there? Starting from this question, researchers have discovered many types of oncogenes, and they realized that certain mutations are predominant in specific cancer.

1.1.5. Carcinogenesis by oncogene

How do normal cells that are systematically regulated turn into cancer cells? As mentioned above, the cells of the metazoan animals have a complete genome, which causes errors due to repeated division or exposure of the carcinogen. Cells with errors in the genome are returned to their original state by repair or remove when the repair is impossible. A few abnormal cells that have errors in oncogenes avoid surveillance through some events. Abnormal cells that escape from surveillance accumulate more mutations, and gradually transformed themselves into cancer cells. Cancer-causing events include insertion of viral oncogenes amplification or mutation of genes by exposure to carcinogen, and etc. First, viruses have a system that replicates and produce their genes after infection on the host cell, and some viral DNA can insert into chromosome DNA of host cells

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(called integration). As Integration of the viral transcription promoters or oncogenes can occur anywhere into host cell chromosome, host cells produce tremendous amount of onco-proteins or fusion-proteins. Such these modified proteins because reprogramming of the system of cells, result in the proliferation of abnormal cells without normal functions. Second, exposure to mutagen causes cancer. Mutagenic substances include specific chemicals, radiations and etc. Exposure to mutagen causes a point mutations; but, the number of genes and size, and even the physical structure in cancer cells are very similar to normal genes. How can these mutations cause cancer? Point mutations generally found in the active site of the protein in cancer patients. The active site is the most essential part of the enzyme in the region where the substrate binds and then catalyze the chemical reaction. A mutation occurs in an active site when the protein is over-activated or inactivated, resulting in abnormalities of the biological circuit. The oncogenes known to play in a critical role in specific cancers summarized in the table below (Table 1-2).

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Oncogenes	Genetic Alterations	Description	Reference
Cell Cycle Control: G1/S Checkpoint			
Abl	Translocation	Self-Suffciency in Growth Status	[9]
Cdk-2	Amplification, increased expression	Self-Suffciency in Growth Status	[10], [11]
Cdk-4	Point mutation	Self-Suffciency in Growth Status	[12]
Cyclin D	Amplification, translocation	Self-Suffciency in Growth Status	[13]
Cyclin E	Amplification	Self-Suffciency in Growth Status	[11]
HPV-E7	Viral Infection	Self-Suffciency in Growth Status	[14]
G2/M DNA Damage Checkpoint			
Aurora A	Amplification, increased expression	Self-Suffciency in Growth Status	[15]
HPV-E6	Viral Infection	Evading Apoptosis	[16]
Mdm2	Amplification	Evading Apoptosis	[17]
Ras signaling pathway			
B-Raf	Point mutation, amplification, increased expression	Self-Suffciency in Growth Status	[18]
Fos/Jun	Increased expression	Evading Apoptosis, Self-Suffciency in Growth Status	[19]
ILK	Increased expression	Evading Apoptosis, Tissue Invasion & Metastasis	[20]
Ras	Point mutation	Self-Suffciency in Growth Status	[21]

Table 1-2. List of oncogenes

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1.1.6. Tumor suppressor gene

Tumor suppressor genes in the biological system are also known that inhibit the growth of cells (anti-growth gene). Since many types of tumor suppressor genes play a role in monitoring cell cycle error and mutation of genes, the loss of tumor suppressor genes induces many mutations in the gene. Furthermore, the loss of tumor suppressor genes cannot be restored and then is passed on to offspring. When this gene is inactivated or lost, cells quickly proliferated by activating the oncogenes. In general, loss of the tumor suppressor gene is stronger than the activation of the oncogene for the formation of cancer.

1.1.7. Carcinogenesis by Tumor suppressor gene

The cell in metazoan animals has the two alleles that interact to produce the phenotype, and these alleles can be the same (homozygous) or different (heterozygous). When one dominant allele and recessive allele paired, the dominant phenotype appears. To express the recessive phenotype, it must be paired two recessive alleles. As the many tumor suppressor genes are a recessive gene, cancers can be arisen when both alleles are inactivated or lost. However, how can two alleles be lost despite the presence of gene repair systems in the cell? Loss of heterozygosity (LOH) is a genetic event observed in many cancer types. LOH occurs due to mitotic nondisjunction, recombination, gene conversion, and so on. For one example, recombination refers to the shuffling of chromosomal arms from

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two homologous chromosomes. Recombination between chromosomes occurs during meiosis, but also during active cell proliferation. It was termed mitotic recombination to distinguish meiotic recombination. By recombination, the chromosomal arm carrying the wild type allele replace with the chromosomal arm carrying the mutant type allele. When LOH arise from a mutant allele of the tumor suppressor gene in the chromosome, the wild type allele is removed, and eventually, the cell growth cannot be controlled. The certain tumor suppressor genes known to play a critical role in cancers are summarized in the table below (table 1-3).

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Tumor suppressor genes	Genetic Alterations	Description	Reference
Akt Signaling Pathway			
PI3K	Point mutation	Evading Apoptosis	[22]
LKB1	Point mutation	Sustained Angiogenesis	[23]
PTEN	Point mutation	Evading Apoptosis	[24]
TSC1/TSC2	Point mutation	Self-Suffciency in Growth Status	[25]
Cell Cycle Control: G1/S Checkpoint			
p15	Point mutation	Self-Suffciency in Growth Status	[26]
p16	Point mutation	Self-Suffciency in Growth Status	[26]
p57	Point mutation	Self-Suffciency in Growth Status	[27]
Rb	Point mutation	Self-Suffciency in Growth Status	[28]
Cell Cycle Control: G2/M DNA Damage Checkpoint			
BRCA1	Point mutation	Self-Suffciency in Growth Status, Insensitivity to Anti-Growth Signals	[29]
Chk1	Point mutation	Insensitivity to Anti-Growth Signals	[30]
Chk2	Point mutation	Insensitivity to Anti-Growth Signals	[31]
p53	Point mutation	Evading Apoptosis, Insensitivity to Anti-Growth Signals	[32]

Table 1-3. List of Tumor suppressor genes

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1.1.8. The protectors of the cell cycle

It is already mentioned that cells are affected by external factors in order to proliferate and divide. Depending on the ratio of mitotic growth factor and anti-mitotic growth factor around the cell, the cell decides whether to divide or not. When there is a lot of growth factor around the cell, the cell enters the cell division cycle. Cell division cycle is composed of mitosis (M), Gap1 (G1), synthesis (S), and Gap2 (G2). G1 phase is the first phase, and the cells determine their fate whether to continue cell cycle and enter S phase or stop cell cycle and enter the subscription phase. The deciding point called restriction point(R point), and this point is at the end part of the G1 phase. The next step is the DNA synthesis step (S-phase) and soon enters the G2 phase, the gap between the S-phase and the M-phase. The last step is the mitotic phase, the replicated chromosomes, and cytoplasm separate into two new daughter cells. As DNA is placed in a vulnerable environment to damage such as DNA synthesis and mitosis, there are surveillance checkpoint mechanisms at each step in the cell cycle. Typical checkpoint gene is the Rb gene (retinoblastoma gene) that act as a critical player to control cell cycle clock at R point. Phosphorylation of Rb is regulated by cyclins and CDKs, which regulate the cell cycle clock. The hypo-phosphorylated Rb is complex with E2Fs (transcription factors for cell proliferation); but, Rb function is lost with hyperphosphorylation by the complex of E cyclin -CDK2 in the next step. In other words, Rb can play the role of a protector in the

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dephosphorylation state, and its function is lost by phosphorylation. That is why the loss of Rb gene cannot act as a protector to guard the R point. Other checkpoint proteins are also protecting cells from deformation with similar mechanisms.

1.1.9. Cell cycle clock

Cells have a finite number of divisions. After the number of times the cells can divide, they enter senescence or crisis, and eventually will die. According to the previous chapter, the fate of cells is regulated by external factors, but it also has a system that regulates the cell cycle inside the cell. It is called a cell cycle clock, which regulates the number of cell divisions. The most famous cycle clock is telomere, which is the repeat sequence present at the end of the chromosomal DNA. Since Chromosome DNA exists in an unstable state on a very long linear form of DNA, the gene information is easy to be lost without protectors. To prevent the loss of information, Telomere acts as a protective film at the end of the chromosomal DNA. Telomeric DNA is formed several thousand of repeating 6 base sequence (ex. 5'-TTAGGG-3') at the end of the chromosomal DNA, and only single strand end of the chromosomal DNA is extended 100 to 200 bp. This 3 'overhang structure is configured T-loop that curls inside to protect linear DNA. As telomeric DNA structure is unique and complicated, about 50 to 100 bp of the end of telomeric DNA is lost during replication. According to this logic, the

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length of telomeric DNA is shortened every of cell division, and end part of the linear DNA cannot be protected after telomeric DNA depletion. As unprotected linear DNA forms an end-to-end fusion and produces a giant DNA that is different from normal, cell face the inevitable death or crisis by the protector such as p53. This means that even if cells acquire an oncogene or lose a Tumor suppressor gene, infinite proliferation cannot be possible.

1.1.10. Immortalization of cancer cell

How can cancer overcome the cell cycle clock mechanism of telomere and continue to divide? Cancer reaching 85 to 90% was found to recover from Telomere's length and get out of a crisis. There are not only cancer cells that proliferate indefinitely. Even in the case of embryonic stem cells, the length of telomere is maintained at a constant level to allow continuous growth. Such cells increase the activity of telomerase that is a type of reverse transcriptase and synthesizes telomere. As described above, since cells retain all genetic information, even normal cells can increase the activity of genes that make telomerase and change them into cancer cells. However, it is not yet known how cancer increases telomerase activity.

1.1.11. Progressive changes of cancer cell from normal cell

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When cancer tissue is found in the body, the surrounding cells are in a state of intermediate cancer and normal. Cells acquired for the first time oncogene do not behave like cancer, but they become increasingly aggressive and dangerous cells as they move away from original sites through repeating of cell division. Because more variations are accumulated in the cells as acquiring the oncogene is easier in the cells losing the monitor function. The cancer tissue that arises through this process intended for proliferation, the expression levels of the growth related genes are high and the levels of death related genes are low. The more advanced cancer shows a higher level of migration related genes that facilitate invasion of other tissues. The developmental stages of cancer are summarized as follows: (1) hyperplastic stage induced by excessive stimulation of normal cells, (2) dysplastic stage, which is different from normal cells, (3) benign cancer which is abnormally proliferating in the state without invasion of lamina or stroma, (4) metastatic cancer that can invasion into other tissues. Cancer seems to be progressing step by step from (1) to (4) (Figure 1-4) [33].

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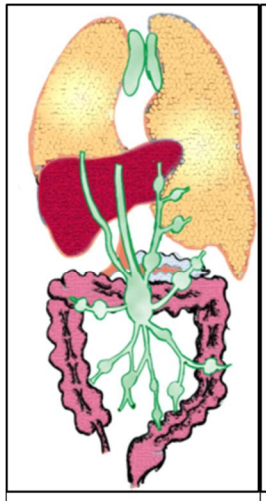	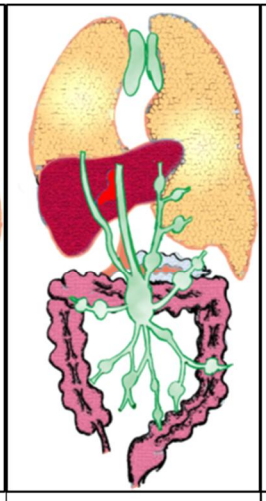	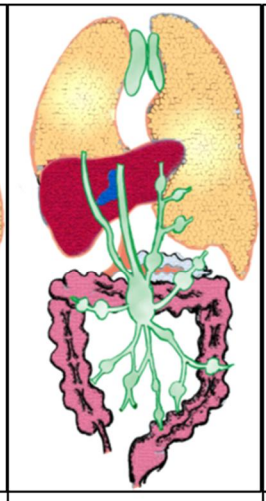	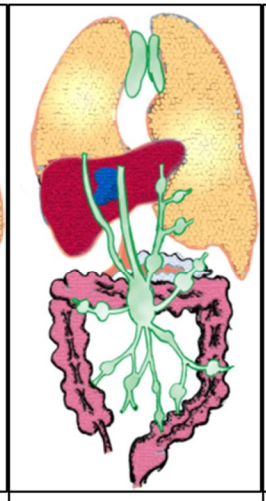	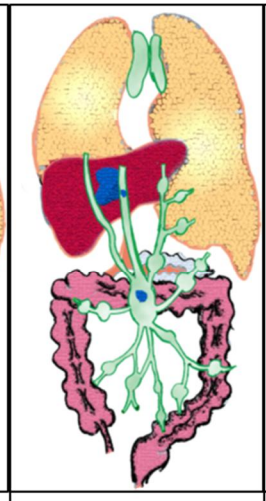	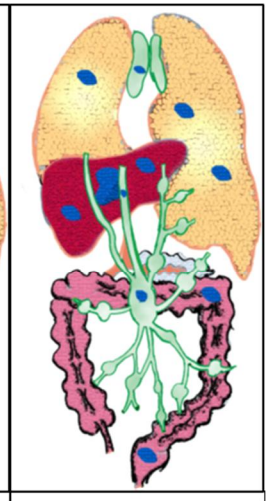
Normal	**Hyperplasia:** Increase in the number of cells, but cells morphology and relationship remains normal.	**Dysplasia:** Cells proliferate excessively and they are no longer normal characteristics. but the cells have not yet progressed to cancer .	**in situ Cancer:** Cells are transformed into very abnormal structure, and lost their function by some mutations. But it is still confined to the epithelial layer from which it arose.	**Invasive cancer:** Cancer cells invade neighboring tissues and shed cells into the blood or lymph.	**Metastatic cancer:** Cancer cells move or spread from primary caner tissue to another organs and proliferate.

Table 1-4. Scheme of cancer development process

Hyperplasia tissue (marked in red), dysplasia tissue and cancer tissue (marked in blue)

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1.1.12. Microenvironment of cancer

If cancer cells continue to proliferate in one place, there is not enough energy or growth factor to survive the cells. Also, cancer cells are difficult to proliferate by monitoring of immune cells. The cancer cells are interfere with the signals of surrounding normal stromal cells and immune cells. Thus cancer cells release various cytokines or micro-RNAs and assimilate surrounding normal cells into cancer. Immune cells found around cancer are mostly regulated T (T_{reg}) cells or myeloid-derived suppressor cells (MDSC) that promote cancer growth. Such immune suppressive cells deplete the nutrients required by the normal immune cells and induce ROS to prevent cancer-filtrating immune cells proliferation [34]. Also, while Immune suppressive cells are present around cancer, they act as a barrier to prevent infiltration immune cells of the cancer tissue. Stromal cells around cancer also have a beneficial effect on cancer growth. Cancer cells that penetrate the stromal absorb the growth signal secreted by the stromal cells and draw nutrients from the stromal cells when the energy is depleted. Also, the surrounding environment is appropriately changed to survive cancer through angiogenesis, hypoxic condition, and lactic acid generation.

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1.1.13. Cancer traits

To summarize the previous chapters, cancer cannot be formed by acquiring an oncogene. To form cancer in the body, various mutations of the genes must occur through loss of a tumor suppressor gene, and the surrounding environment also has to change to a cancerous condition. Not all cancer types have all the features described in the previous chapters. Although the logic of cancer development is similar, the characteristics of cancer differ depending on the location, environment, cell type, and so on. Due to the variety of genes that affected cancer development even in the same organ, the stage and classification of cancer are set according to the type of altered gene. Moreover, even within same cancer, different cancer cells often have different genetic mutations. In same cancer, one group of cells is capable of proliferation, the other group of cells is easy to migrate, and another group of cells is easy to survive in extreme environments. As the cells suitable for survival among the mixed various cancer cells proliferate, many cancers have characteristics of monoclonality. For this reason, we can diagnose cancer through a marker for specific cancer cells and treat appropriate chemotherapy.

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1.2. Diagnosis of cancer

In order to confirm the presence of cancer, it should be possible to distinguish between cancer and normal tissues. In the 19th century, it was possible to observe tissue on a cell basis with the invention of the microscope, and it was possible to observe cells that focus only on proliferation, which is the most characteristic of cancer cells. The reason why the cancer classification is classified morphologically is that microscopic observation is still the most performed tool to date. Since then, the advancement of medicine has led to the development of diagnostic methods such as PET-CT, marker-based test and so on. As the characteristics of various cancers have been discovered, diagnostic methods and therapies have also been developed. Currently, the diagnosis of cancer is not only based on morphological characteristics but also biological characteristics.

1.2.1. Histological diagnosis of cancer

Histological diagnosis of cancer is a very classic and widely used method. A biopsy is a test that can show the most important factor in the diagnosis of cancer such as the morphology of cancer cells and the invasion to the basement membrane. Compared to other testing methods, there is less misdiagnosis as it is the only method that is a direct way to see the cancer cells. However, the process of acquiring specimens from patients is invasive and accompanies with pain. In

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addition, there is a disadvantage that the gene characteristics of cancer cannot be considered. To overcome these shortcomings, recently, a technique of staining a cancer specific antigen using a gene specificity antibody has been applied to histological examination. As a result, it has become possible to diagnose not only the size, shape, and invasion of cancer but also characteristics of cancer.

1.2.2. Imaging diagnosis of cancer

Imaging diagnosis is the only way to know the size and location of cancer without invasion. Also, imaging diagnosis is essential when the location of cancer for histological examination is needed. Imaging diagnosis is not limited to observing the shape of organs, but detection techniques are being developed based on the characteristics of cancer. For example, PET-CT was developed to detect cancer with high glucose metabolism since cancer cells prefer to use glucose as an energy source. The development of such devices, makes easier to find rapidly proliferating tissues beyond simple size comparisons, and enables to detect early stage of cancer. Although it is the most commonly used as a noninvasive method for cancer screening, accurate diagnosis is difficult because it can sometimes detect the abnormal proliferation of the tissue. Since it is difficult to determine whether the type of cancer is benign or malignant by imaging, the final diagnosis should be made through histological examination so far.

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1.2.3. Molecular diagnosis of cancer

A molecular diagnosis is a way to know the presence and characteristics of cancer cells in the body. There is a little or no pain because the body fluid and blood are collected for olecular diagnostic test. Although the location and size of cancer cannot be determined, it is easy to distinguish the type of genetically modified cancer. In addition, this method also provide the genetic information of cancer can be used for treatment of individual patients. Molecular diagnosis includes tests such as changes in growth factors, secretion proteins in body fluids, and detection of cancer-specific antigens.

1.2.4. Cancer-specific marker

Cancer diagnostic markers are very useful indicators for diagnosis and classification of cancer. It applies to all cancer diagnostic tests, among them, molecular diagnostics is highly dependent on cancer specific markers. Increased oncogenes, reduced tumor suppressor genes, and micro-environments described in Chapter 1 can all be cancer markers. The ideal marker for diagnosis should be non-invasive and easy to measure. It should be able to predict the development, the prognosis of the disease at the molecular level such as DNA, RNA, and protein in the blood, urine, and tissue from a patient. When cancer can be diagnosed using these markers, a personalized prescription can be made according to the characteristics of

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cancer cells. It is also possible to develop new drugs that directly target cancer or regulate cancer-related molecular levels in the body using probes that can bind to these markers. However, in early cancer, there are few substances secreted by body fluids and too little to diagnose. Therefore, the most common method for staining a sample obtained from a patient with a specific antibody has been developed. It is still difficult to diagnose cancer using a marker in a noninvasive stage. In order to detect and diagnose various types of cancer, it is urgent to find new markers. Cancer specific markers according to cancer types are summarized in the table below (Table 1-5).

1.3. Chemotherapy

The conventional treatments of cancer can be divided into surgery, radiotherapy, and chemotherapy. So far as possible, the cancer is removed by surgery and remains a small number of cancer cells can be removed by radiation or chemotherapy to achieve the highest cure efficiency. However, where the surgery is not possible, or the metastasis progresses to the whole body, the patient will have to rely on chemotherapy. The goals of chemotherapy are complete remission, the extension of life, and relief of symptoms.

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Markers	Cancer types	Analyzed	Reference
Alpha-fetoprotein (AFP)	Liver cancer and germ cell tumors	Blood	[35]
Beta-2-microglobulin (B2M)	Multiple myeloma, chronic lymphocytic leukemia, and some lymphomas	Blood, urine, or cerebrospinal fluid	[36]
Beta-human chorionic gonadotropin (Beta-hCG)	Choriocarcinoma and germ cell tumors	Urine or blood	[37]
BCR-ABL fusion gene (Philadelphia chromosome)	Chronic myeloid leukemia, acute lymphoblastic leukemia, and acute myelogenous leukemia	Blood and/or bone marrow	[38]
BRAF V600 mutations	Cutaneous melanoma and colorectal cancer	Tumor	[39]
C-kit/CD117	Gastrointestinal stromal tumor and mucosal melanoma	Tumor	[40]
Nuclear matrix protein 22	Bladder cancer	Urine	[41]

Table 1-5. List of cancer diagnosis markers used in clinical practice

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Conventional methods of cancer therapy inhibit cell proliferation, and most anti-cancer agents lead to apoptosis of rapidly proliferating cells. Because cancer cells proliferate more rapidly than normal cells, they act more on cancer cells than on normal cells. Also, apoptosis is a mechanism of eliminating the cells that are no longer needed during the development of normal cells, and cell death progress without affecting the surrounding cells. However, there are many side effects on conventional anticancer agents because of the presence of the fast growing cells in normal. The anti- cancer agents do not respond to cancer resistance to apoptosis.

1.3.1. Cell death mechanism of anticancer agents

There are many ways in the cell death mechanism to remove the cancer cells in the body. The types of cell death are roughly divided into apoptosis, necrosis, and autophagy. The body's immune response, therapeutic effects, side effects, etc. differ depending on how cell death occurs. Anticancer drugs have to get a few side effects and should not deteriorate the condition of the body. Currently, the major components of anticancer drugs are an apoptotic reagents, but it has many disadvantages so that the treatment is not adequate, and cancers get resistant easily. Recently, many agents that induce necrosis and autophagy have tried to be developed anticancer agents. In this study, we focused on necrosis inducing agents.

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1.3.2. Apoptosis

In the past, cell death referred to the rupture of cells by external stimuli. After that, apoptosis, where the cells themselves commit suicide, is known and the cell death begins to be distinguished. For the first time, apoptosis has been referred to cell death required for normal development, which is precisely induced at specific times and removes unwanted tissue. In addition, apoptosis is induced to minimize the effects on surrounding tissues when the cells are unable to function due to aging, cell infection with a virus, and the occurrence of the cancer cell. As the research progresses, it has become known that apoptosis can be induced by not only endogenous cell death signals but also by specific stimuli or drugs. The characteristics of apoptosis are that as the cells shrink, DNA fragmentation and several small blebs are formed. The dead cells that have formed apoptotic body are removed from adjacent cells or immune cells (Figure 1-1). Thus, apoptosis is referred to as silent suicide without affecting surrounding cells.

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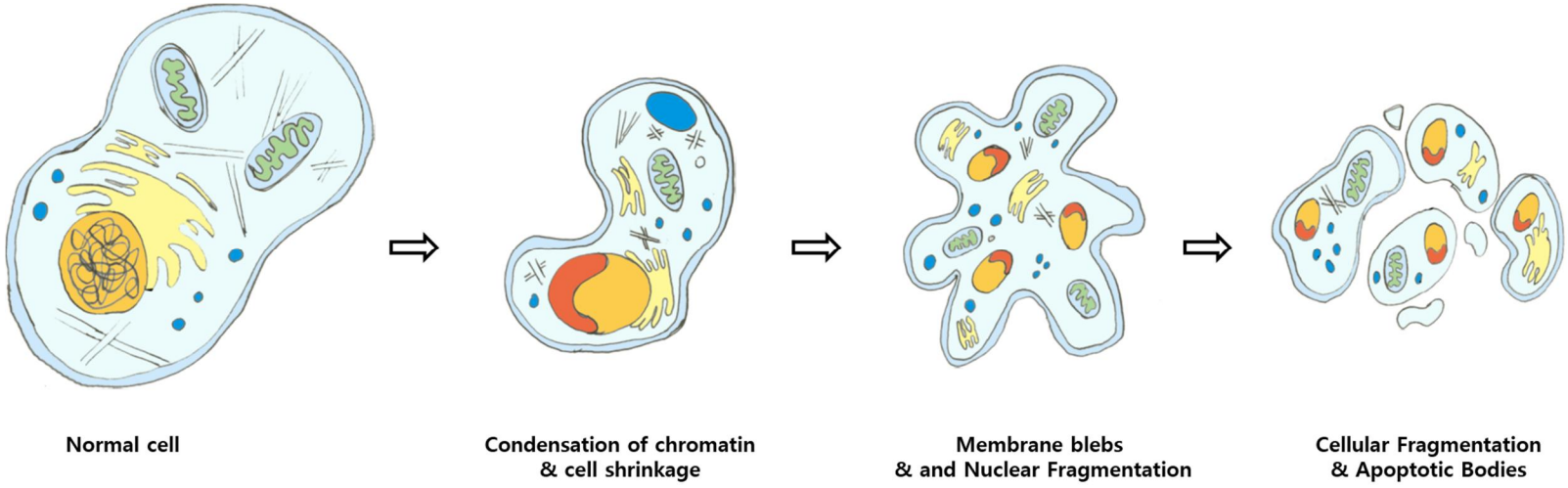

Figure 1-1. Scheme of apoptosis in Eukaryotic cell

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1.3.3. Necrosis

Necrosis had been known as an accidental death due to extreme stimuli such as temperature changes or physical attacks. Necrosis results in the rupture of cell membrane and release of cytosol products into the extracellular space (Figure 1-2). However, recently discovered necroptosis has been shown to be programmed form of necrosis. Necroptosis also includes a defense mechanism for removing infected cells to protect themselves from external stimuli such as viral infection and other pathogens. Additionally, studies on necroptosis have shown a significant interaction between apoptosis and necrosis pathway. Cell death signals can bypass to necrosis when apoptosis signal inhibited by a viral infection or blocking proteins. This finding has shown that apoptosis is not always a desirable form of cell suicide. Necrotic cell death leads to rupture of cell membrane and release of endogenous DAMPs (damage-associated molecular pattern) such as ATP, DNA, and HMGBI. DMAPs can recruit leukocytes, cytokines, and chemokines. They provoke local inflammatory responses by activating the innate immune system. However, the downstream death signaling events remain poorly characterized.

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Figure 1-2. Scheme of necrosis in Eukaryotic cell

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1.3.4. Autophagy

Autophagy (literally translated as self-eating) is the mechanism of cells to digest their organelles for cell survival. Autophagy plays a critical role in the clearance or recycling of damaged organelles and proteins aggregates (Figure 1-3). Additionally, autophagy has been implicated in antigen presentation to immune cells. However, Excessive activity of autophagy can induce autophagic cell death beyond protective action. Autophagy is also defined as programmed cell death because it also controlls biological processes such as development, maintain homeostasis and protection of pathogen. Autophagy stimulates programmed cell death without inducing inflammation like apoptosis.

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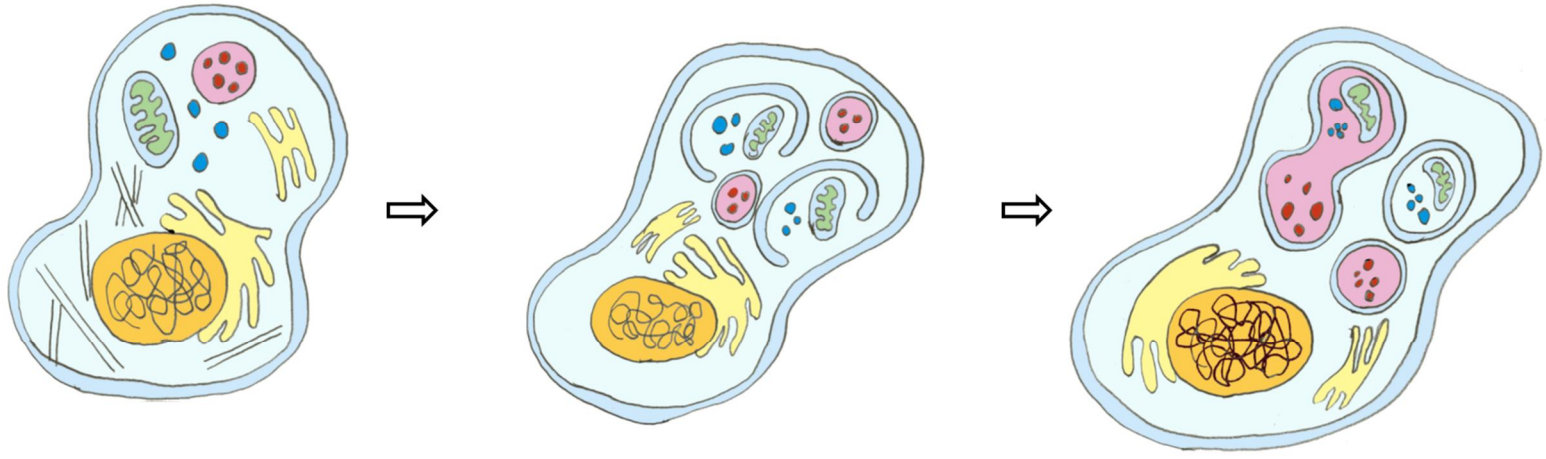

Figure 1-3. Scheme of autophagy in Eukaryotic cell

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1.3.5. The therapeutic effects on cancer depending on the type of cell death

Which cell death mechanisms are more beneficial in removing cancer cells? Apoptosis has the advantage that it does not affect the surrounding cells, as mentioned above (Chapter 1.3.2). Apoptotic inducing agents are mostly developed as anticancer drugs for this benefit. However, the apoptosis signal transduction pathway is very multi-complicated and involves various proteins. Complex signaling pathways act to promote resistance to anticancer agents. In addition, apoptosis signals are often suppressed in many types of cancers to focus on proliferation. On the other hand, since necrosis has a very short signaling pathway, necrosis-inducing drugs will have a much lower tolerance than apoptotic agents. In addition, DAMPs released during necrosis can invigorate immune system activation. Inactivation of the immune system is observed in the many cancers to avoid immune surveillance since normal immune cells detect and remove abnormal cells. However, strong necrosis can cause cytokine syndrome and sepsis. Finally, autophagy prevents normal cells from being transformed into cancer through the removal of organelles with abnormalities. It plays a role in presenting antigens, which causes immune surveillance and prevents cancer development. However, it has been reported that cancer can proliferate by obtaining energy through autophagy when cancer cannot obtain energy source from surrounding enviroments. Autophagy has not been studied so far because it may become poisonous or medicinal depending on the situation. Thus, as all three pathways have their pros and cons, any route

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of drugs are meaningful for anticancer development. This study focused on substances that induce necrosis to overcome the shortcomings of the apoptotic agents.

1.3.6. Sluggish action and side effects of anti-cancer drugs

Another problem with conventional anti-cancer drugs is that they can kill not only cancer cells but also normal cells. The majority of anticancer drugs block the replication of genes, suppression of proliferation and division, and hamper energy metabolism, but it cannot have cancer selective- killing activity. As they have the same effect on normal cells, resulting in very serious side effects (Table 1-6). Therefore, the patient's age or health condition is inevitable to proceed with chemotherapy. Since the use of high-concentration drugs is limited, it is not expected to significant therapeutic effect. New anticancer drugs are emerging to improve conventional anti-cancer therapies, including targeted drugs that combine cancer markers and therapeutic agents.

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Symptoms	5FU	Vincristine	Vinblastine	Cisplatin	Paclitaxel	Docetaxel	Etoposide	Doxorubicin	Bleomycin
Bone marrow dysfunction	○		○	○	○	○	○	○	
Nausea and vomiting	○			○				○	
Stomatitis and diarrhea	○				○	○	○	○	
Constipation		○	○						
Pigmentation	○						○		○
Peripheral neuritis		○	○	○	○	○			
Mental disorder									
Liver failure				○					
Kidney failure									
Heart failure					○	○		○	
hair loss		○	○	○	○	○	○	○	○
Reference	[42]	[43]	[44]	[45]	[46]	[47]	[48]	[49]	[50]

Table 1-6. Side effects of conventional anticancer drugs

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1.4. Targeted anti-cancer agent

Surgical, radiation therapy, and chemotherapy were used for conventional cancer treatment. However, except surgery, other therapies do not target cancer cells alone so that they affect normal cells and cause various side effects such as hair loss, skin rash, and vomiting. To overcome these limitations, therapeutic agents targeting cancer specific markers has been developed so that cancer drugs can attack only cancer cells without affecting normal cells. Therapeutic cancer markers are not the same as cancer diagnosis markers. The diagnostic markers include any genes specifically expressed in cancer, and new substance that is made by cancer. However, to target living cancer cells, only substances present on the surface of cells can be used as therapeutic markers. Cancer markers are broadly divided into two categories. Genes that present only cancer cells are classified as tumor specific antigens (TSA), and genes that present on some tumor cells and also some normal cells are classified as tumor associated antigens (TAA). However, there is almost no gene for which only cancer cell expresses. The table below summarizes the TAA expressed on the surface of cells (Table 1-7). As shown in the table, to survive the cancer cells in competition with the normal cells, the receptor related to cell proliferation and angiogenesis is highly expressed in the cancer cell surface.

When the targeted anti-cancer agent is administered to the patient, it is possible to selective treatment in consideration of the characteristics of

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individual cancer cells, thereby reducing the side effect and increasing the efficiency of removing cancer cells. The types of targeted anti-cancer agents that are currently used in clinic include therapeutic antibodies, cancer targeting peptides, and so on.

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Receptor	TSA/TAA	cancer type	reference
EGFR	TAA	glioma, skin, stomach, Urothelial cancer	[51]
ErbB2	TAA	breast, head and neck, stomach, colorectal, esophageal, prostate, bladder, renal, pancreatic, ovarial carcinomas; glioblastoma	[52]
NRP-1	TAA	Ovarian, Colorectal, Stomach, Thyroid, Testis,Lymphoma, Melanoma	[53]
IGF-1R	TAA	Colon cancer	[54]
cMET/HGFR	TAA	Lung cancer	[55]
Gastrin-Releasing Peptide Receptor (GRPR)	TAA	breast cancer	[56]
cystic fibrosis transmembrane conductance regulator (CFTR)	TAA	Ovarian cancer	[57]
Anaplastic lymphoma kinase (ALK)	TAA	Esophageal squamous cell carcinoma,giloblastoma, Breast, Renal, thyroid cancer	[58]
Membrane-bound hepatocellular receptor tyrosine kinase class A2 (EphA2)	TAA	Ovarian cancer, melanoma	[59]
Prostate-Specific Membrane Antigen (PSMA)	TAA	prostate cancer	[60]
Epithelial cell adhesion molecule (ep-CAM)	TAA	colorectal cancer	[61]
CD44-v7	TAA	bladder, cervical cancer	[62]
CD133	TAA	colorecta cancer	[63]

Table 1-7. Tumor associated antigen (TAA) present on the cell surface

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1.4.1. Therapeutic antibodies

Antibody is an immune substance in the body capable of recognizing a specific target, called an antigen. Antibodies produced from B cells play a role in recognizing and removing foreign substances from the outside of our body. Antibodies can recognize a variety of foreign substances because of the large number of combinations that can be made during antibody formation. Antibodies are composed of a combination of the heavy chain and light chain. Variable, diversity, and joining lesions exist in each chain, and the number of combinations that can be produced by the antibody is about 10^{12} to 10^{14}. In addition to recognizing the antigen, the function of the antibody include the removal of antigen by antibody dependent cell-mediated cytotoxicity (ADCC), antibody dependent cellular phagocytosis (ADCP), and complement dependent cytotoxicity (CDC). So cancer cells with antigens targeted by antibodies can be specifically removed. A representative example of cancer -specific targeted therapeutic antibody is Herceptin. Rapidly proliferating breast or lung cancer cells overexpress Her-2 protein, which acts as a receptor on the cell surface that binds to EGF and transmits cell proliferation signals. Although the Her2 receptor does not exist only in cancer cells, because cancer cells show excessive proliferation compared to normal cells, Herceptin acts as a highly effective anticancer agent. However, since the Herceptin was injected from the outside of the body, antibodies against Herceptin were generated and showed resistance to antibody treatment. Also, the production of antibodies has to

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go through a very complicated process, and the produced antibodies are difficult to deform and take a long time to be developed as anticancer agents. These are pointed out as a drawback of all antibody therapies. In order to compensate for this drawback, a targeted therapeutic agent was developed using a peptide that has lower antigenicity than antibody therapeutic agents and can recognize cancer cell specific markers.

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Antibody	Target	Cancer type	Mechanism of action	Reference
Bevacizumab (Avastin)	VEGF	colorectal cancer, non-squamous non-small cell lung cancer, glioblastoma, and renal cell carcinoma	Inhibition of VEGF	[64]
Ipilimumab (Yervoy)	CTLA-4	metastatic melanoma	Inhibiton of CTLA-4	[65]
Rituximab (Rituxan)	CD20	Non-hodgkin's lymphoma	ADCC, direct induction of apoptosis	[66]
Durvalumab (Imfinzi)	PD-L1	non-small cell lung cancer, and urothelial carcinoma	Inhibition of PD-L1	[67]
Avelumab (bavencio)	PD-L1	Merkel cell carcinoma, urothelial carcinoma	Inhibition of PD-L1, ADCC	[68]
Pembrolizumab (keytruda)	PD-1	advanced melanoma, non,small cell lung cancer and hodgkin's lymphoma	Inhibition of PD-1	[69]
Nivolumab (Opdivo)	PD-1	Melanoma, urothelial carcinoma, and non-small cell lung cancer, and Hodgkin's lymphoma	Inhibition of PD-1	[70]
Trastuzumab (Herceptin)	Her2(ErbB2)	breast cancer and gastroesophageal junction carcinoma	Inhibition of Her2, ADCC	[71]

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Antibody	Target	Cancer type	Mechanism of action	Reference
Pertuzumab (perjeta)	Her2(ErbB2)	breast cancer	Inhibition of Her2, ADCC	[72]
Alemtuzumab (campath Lemtrada)	CD52	B-cell chronic lymphocytic leukemia	ADCC, CDC	[73]
Gemtuzumab	CD33	Acute myeloid leukemia	Calicheamicin-mediated DNA damage	[74]
Cetuximab (Erbitux)	EGFR(ErbB1)	Colorectal cancer, breast cancer , lung cancer	Inhibition of EGFR, ADCC, CDC	[75]
Rilotumumab	HGF/SF	gastric/Gastresophageal Junction adonocarcinoma	Inhibition of HGF	[76]

ADCC, antibody dependent cell-mediated cytotoxicity; CDC, complement dependent cytotoxicity

Table 1-8. FDA approved therapeutic monoclonal antibodies for chemotherapy

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1.4.2. Targeted peptide anti-cancer agent

Many receptor signal substances are proteins, and downstream regulators also consist of proteins. If we can regulate the activity of proteins from outside, we can control the life and death of cells. However, experiments using proteins have difficulties in various aspects. It is difficult to purify the protein in an active form because it has to adjust the conditions such as protein solubility, quaternary structure, pH, and so on. Even in the purification process, unnecessary sequences like his-tag and GST-tag can be added. It is very difficult to apply it to the treatment because the molecular weight is massive, the antibody is easily formed, and the half-life is very short in the body after the successful purification. Peptide therapeutics have been developed to recognize specific markers similar to antibody therapeutics. As they have short amino acid sequences, peptide therapeutics have less immune response than antibody therapeutics; thus, peptides have a long lasting therapeutic effect. The peptides that can be synthesized in the desired sequence can have advantages such as library design, transformation to D form, and attachment of probes. Through these advantages, the most effective sequences can be selected, and peptide tracking and half-life can be known. The half-life of peptides can also be adjusted through modification of the structure. Many peptide-based studies have identified peptide sequences that can apply to a variety of diseases, and specific peptide sequences are summarized in the table below (Table 1-9).

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Sequence	Name	Target	Receptor	application	Reference
FLGWLFKVASK	A4W-GGN5N11	cancer	Melanocortin4 receptor	Anti-cancer	[77]
FLGALFKWASK	V8W-GGN5N11	cancer	Melanocortin4 receptor	Anti-Cancer	[77]
CRGDKGPDC	iRGD	Tumor fibroblasts, tumor cell	Av intergrins, NRP-1/2	Anti-tumor	[78]
CGNKRTRGC	LyP-1	Tumor endotherlials, macrophages, Tumor cells	P32(gC1qR), NRP-1/2	Anti-tumor	[79], [80]
AKRGARSTA	Lineal TT1	Tumor endotherlials, macrophages, Tumor cells	P32(gC1qR), NRP-1/2	Anti-tumor	[81]
CRNGRGPDC	iNGR	Tumor endotherlials	Amnopeptidase N, NRP1/2	Anti-tumor	[82]
CGNKRTR	tLyP-1	Tumor endotherlials,NRP positive cells	NRP-1/2	Anti-tumor	[80]
CDCRGDCFC	RGD-4C	Angiogenic blood	αvβ3/ αvβ5	Anti-tumor	[83]
CNGRCVSGCAGRC	NGR	Angiogenic blood	Aminopeptidase N(CD13)	Anti-tumor	[84], [85]
YHWYGYTPQNVI	GE11	SMMC-7721 human hepatoma cells	EGFR	Anti-tumor	[86]
LTVSPWY		Her2-postive breast cancer SKBR3 cells	Her2	Anti-cancer	[87]
SWELYYPLRANL		MCF-7 human breast carcinoma,MDA-MB-435	E and N cadherin	Anti-cancer	[88]

Table 1-9. List of targeting peptides used for anti-cancer experiment.

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1.5. Development of New anti-cancer agents using synthetic peptides

Most of the approved anti-cancer chemical or antibody drugs are apoptosis-inducing agents targeting fast-growing tumor, normal cells, or specific signaling proteins including receptor tyrosine kinases. Thus, these drugs have many side effects such as skin irritation, nausea, anemia, and hair loss. In addition, cancer is easily tolerated when using conventional chemotherapeutic agents.

In this study, to overcome these shortcomings conventional chemotherapeutic agents, I designed a new anticancer peptide fused to MTD that targets cancer cells and can penetrate the cell membrane, and kill cancer cells.

First, I used MTD peptides that found in my study (Chapter2) as necrosis inducing substance. Many cell death inducing peptides have already been identified. However, most necrosis inducing-peptides cannot target specific cells because they destroy cell membranes. Peculiarly, MTD does not play such non-specific killing until it enters the cells, since MTD peptide needs to induce the release of Calcium mitochondria in the cytosol in order to kill cancer cells. For this reason, I hypothesized that fusing cell-targeting sequences with MTD could cause necrosis only in specific targeted cells.

Second, I searched for some genes with high expression in cancer to find cancer specific binding sequences. I choose Neuropilin-1 (NRP-1) and ErbB2 as candidates highly expressed in cancer. The first candidate, ErbB2 is a receptor that

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transmits a strong cell proliferation signal through interaction with EGF and is overexpressed in many cancers. The second candidate, NRP-1 is an angiogenesis-related protein and plays an important role in cancer formation and metastasis. Several sequences that bind to two candidate genes are already identified in other group's studies. I used some modified sequences to find very effective MTD fused ErbB2 binding peptides or NRP-1 binding peptides a higher effect on fusion with in this study.

1.5.1. Backgrounds of MTD

Mitochondria are important organelles in the cell, controlling of AIP (aryl hydrocarbon receptor-interacting protein) generation, calcium homeostasis, osmotic regulation, and cell death through mPTP (mitochondrial permeability transition pore). mPTP opening can lead to mitochondrial swelling, membrane depolarization, and cell death. MTD has 10 amino acids derived from c-terminal of NOXA, induced calcium release from mitochondria to the cytosol that leads to necrotic cell death [89]. MTD can induce necrosis by targeting mitochondria only within the cell. The great advantage of MTD is highly toxic inside the cell but has no toxicity outside the cell. Therefore, it is toxic only when it is transferred to the inside of the cell by combine with cancer targeting peptide. In addition, since the cell death mechanism of MTD is necrosis, it is useful for malignant cancer, which is resistant to the apoptotic reagents.

1.5.2. ErbB2 overexpression in cancer

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Cancer cells are more efficient at absorbing nutrient and growth factors than normal cells, as the growth factor receptors are over-expressed or activated than normal cells. Regulation of cell proliferation is controlled through competition between growth factors and inhibitors. EGFR family is activated by auto-phosphorylation when it binding to growth factor such as TGF-alpha, heparin binding EGF like growth factor (HBEGF), amphiregulin(AREG), betacellulin(BTC), epiregulin(EREG). EGFR family is classified as receptor tyrosine kinase because it is activated by phosphorylation of tyrosine residue. EGFR family consists of four members (EGFR1, ErbB2, ErbB3, and ErbB4). Each unit can combine with each member to form hetero-dimer. Since the type of ErbB hetero-dimer determines the binding affinity, various combinations of the receptor are required. Since ErbB2 (Her2) is an orphan receptor whose ligand has not been identified (maybe not able to bind to ligands by itself) [90], it has to form heterodimers with other receptors to get activity. However, ErbB2, which binds to other types of receptors at a high rate, causes amplification of strong signals. Due to ErbB2 expression plays a critical role in cell proliferation, it is overexpressed in many types of cancer (Table1-10).

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cancer type	Receptor	over expression percentage (%)	Reference
Mammary	EGFR	14-91	[91] [92]
	HER2	21	[93]
Bladder	EGFR	31-48	[94] [95]
	HER2	36	[96]
Colon	EGFR	25-77	[94] [97]
	HER2	50	[98]
Glioma	EGFR	40-50	[99] [94]
Non-small-cell lung	EGFR	40-80	[94],[100],[101]
Pancreatic	EGFR	30-50	[94], [102]
Ovarian	EGFR	35-70	[94], [103]
	HER2	32	[104]
Gastric	HER2	26	[105]
Lung	HER2	28	[106]
salivary	HER2	32	[107]
head and neck	EGFR	80-100	[94],[108]

Table 1-10.Overexpression rate of the EGFR and HER2 in cancers [5].

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1.5.3. NRP-1 overexpression in cancer

NRP-1 is involved in angiogenesis as a VEGF receptor expressed in vascular endothelial cells, and. NRP-1 have a multitude of functions dependent on the binding of specific ligands, and they affect cell survival, migration, and attraction [109]. NRP-1 can bind to VEGF to induce angiogenesis, by itself, or it can make hetero-dimers with plexins to transmit other signals into cells [110]. NRP-1 is known to be highly bound to VEGF165 and to modulate the activity of other VEGFRs. Also, it plays a role in angiogenesis as inducing smooth muscle cell migration through binding to PDGF-BB. However, the functions and mechanisms of NRP-1 are largely unknown in tumorigenesis and metastasis. Many papers have identified sequences targeting NRP-1, and RPARPAR (CendR) is also one of them. In this paper, we performed experiments using fusion peptide that combines with MTD and CendR [111].

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CHAPTER 2

Minimal killing unit of the mitochondrial targeting domain of Noxa

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2.1. ABSTRACT

Noxa is a key player in p53-induced cell death via mitochondrial dysfunction, and the mitochondrial-targeting domain (MTD) of Noxa is responsible for the translocation of Noxa to mitochondria and for the induction of necrotic cell death. The purpose of this study was to define the minimal killing unit of MTD in vitro and in vivo. It was found that the peptides R8:MTD(10), R8: MTD(9), and R8:MTD(8) can kill various human tumor cells (HCT116, HeLa, MCF-7, BJAB), but that R8:MTD(7) abolishes the killing activity of MTD mainly because of the loss of mitochondrial targeting activity. I find it interesting that R8:MTD(8) was found to kill tumor cells but showed a limited killing activity on normal peritoneal macrophages. Furthermore, R8:MTD (10), R8:MTD(9), and R8:MTD(8) limitedly suppressed tumor growth when injected i.v. into BalB/C mice bearing CT26 cell-derived tumors. These results indicate that MTD(8) is the minimal killing unit of MTD.

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2.2. INTRODUCTION

Noxa was originally identified as a p53 target gene and to contain two functional domains, the BH3 and MTD domains [112, 113]. It has been demonstrated that the BH3 domain is responsible for inducing apoptotic cell death by selectively inhibiting Mcl-1 and A1/Bfl-1 in mitochondria [114-116]. Previously, we showed that the deletion of the MTD domain abolished HeLa cell death due to the abnormal localization of Noxa [113], and that MTD peptide fused with cell-penetrating peptide kills various tumor cells with necrotic morphological features [6]. R8:MTD peptide consisting of eight arginine residues for cell penetration, and 10 a.a. MTD of Noxa causes an abrupt cytoplasmic calcium release spike from mitochondria, which results in necrotic cell death [89]. Furthermore, R8:MTD peptide has strong necrosis-inducing activity as it has been reported to kill most tumor cells tested in vitro within 10-30min [89]. In the present study, we investigated whether the deleted form of MTD fused to R8 can induce necrotic cell death in vitro and in vivo. We found that R8:MTD(9) and R8: MTD(8), but not R8:MTD(7), can cause necrotic cell death in various tumor cells. Furthermore, R8:MTD(10), R8:MTD(9), and R8:MTD(8) were found to show limited tumor growth suppressing activity in vivo.

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2.3. Materials and Methods

2.3.1. Reagents

Mitochondrial-targeting domain or deleted MTD peptides were synthesized and purified by HPLC to obtain peptides of purity~98% (Anygen, Korea). Peptides were suspended in saline and stored at 20 C. Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute medium (RPMI) 1640, and fetal bovine serum (FBS) were purchased from GenDEPOT (Barker, USA). XTT kits (cell death assay kits) were purchased from Promega (Madison, USA). Unless otherwise stated, all other reagents were obtained from Sigma (St. Louis, USA).

2.3.2, Cell Culture

HeLa, HCT116, and MCF-7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea), and BJAB cells were generously donated by Genentech Inc. (South San Francisco, USA). HeLa cells were cultured in DMEM, CT26, MCF-7, and BJAB cells in RPMI 1640, and HCT116 cells in MacCoy's 5A (all media contained 10% FBS, 100 units/ml penicillin and 100mg/ml streptomycin) at 37 C in a humidified 5% C02atm.

2.3.3. Animals

Specific pathogen-free BalB/C mice (male, 6weeks old) were purchased from Samtaco Co. (Daejon, Korea). Animals were housed under normal laboratory conditions (21-

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24 C, 40-60% RH) under a 12-h light/dark cycle with free access to standard rodent food and water.

2.3.4. Isolation of Peritoneal Macrophages

Peritoneal macrophages were isolated from mice injected intraperitoneally with 2ml of thioglycollate medium (4% (w/v)) three days prior to peritoneal lavage, which was conducted using 10ml of RPMI 1640. Harvested cells were washed with RPMI 1640 three times and then cultured in RPMI 1640 supplemented with 10% FBS, 2mML-glutamine, 100 U/ml penicillin, and 100mg/ml streptomycin. Cells were then plated on culture dishes and incubated for 2h at 37 C in a 5% CO2 humidified incubator. After removing non-adherent cells, mono-layered macrophages were treated with MTD or deleted MTD.

2.3.5. XTT Assay

Cells were cultured in 96 well plates (2 105/well or 1 104/well) overnight and treated with MTD or deleted MTD. Cell viabilities were assessed using XTT-based colorimetric assay kits purchased from Promega (Madison, USA), according to the manufacturer's instructions.

2.3.6. Aspartate Aminotransferase and Alanine Aminotransferase Assay

For assaying the activities of ALT and AST, sera were prepared from mice treated with peptides by centrifuging blood at 2500 g for 15min at 4 C. Enzyme activities were determined according to the manufacturer's instructions.

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2.3.7. Enhanced Green Fluorescent Protein(EGFP)-fused MTD(10-7)

Previously reported vector EGFP-Noxa (41-50) [113] (indicated as EGFP-fused MTD(10) in this study) was used for PCR template to construct EGFP-fused deleted MTDs. The vector pEGFP-Noxa (41-50) was digested with Bgl II and then amplified with PCR primers (CTGAAGCTTGAGTCCGGACTTGTACAGCTC and GTCAAGCTTCT-TCTGAATCTGATATCCAAACTC for EGFP-fused MTD(9); CTGAAGCTTGAGTCCGGACTTGTACAGCTC and GTCAAGCT-TCTGAATCTGATATCCAAACTC for EGFP-fused MTD(8); CTGAAGCTTGAGTCCG-GACTTGTACAGCTC and GTCAAGCTTAATCTGATATCCAAACTCTTC for EGFP-fused MTD(7)). The amplified PCR products were digested with Dpn1 and BglII and then self-ligated for transformation. The plasmid vectors expressing EGFP-fused deleted MTDs were confirmed by DNA sequencing. The vectors of EGFP alone or EGFP-fused MTDs were transfected into HeLa cells for overnight. Mitochondria were visualized with DsRed2-Mito expression vector (Clontech, Mountain View, CA, USA). Images were captured using a laser scanning confocal microscope (Leica Microsystems TCS NT).

2.3.8. Mouse Tumor Models

Animal experiments were performed according to our institutional guidelines. Subcutaneous tumors were generated in BalB/ C mice by injecting 1.5105 CT26 cells in 100ml saline subcutaneously. Tumor cells were grown for 2weeks until tumor volumes reach to around 100mm3. MTD, deleted MTD, or saline were then injected i.v. through a tail vein. Tumor dimensions in the MTD group (n=5), the deleted

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MTD-treated group (n=5), and the saline-treated group (n=5) were measured using a caliper, and tumor volumes were calculated using the longest diameter x width 2 x 0.5 on the indicated days.

2.3.9. Histological Analysis

Tumor tissues were fixed for 12h in 10% formaldehyde solution and embedded in paraffin. Sections were then stained with hematoxylin and eosin and mounted.

2.3.10. Statistical Analysis

Results are reported as meansSDs. ANOVA was used to evaluate differences between more than two groups and when a significant difference was observed, Dunnett's t-test was used to compare the means of two groups. Statistical significance was accepted for p values of <0.05.

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2.4. Results and Discussion

In our previous studies, we found four leucine residues (second, third, fifth, and ninth sites) in MTD are important for mitochondrial localization and that two amino acid residues (fifth and ninth) in MTD are critical for cell-killing activity [89]. To further define the minimal unit of MTD required for cell-killing activity, we designed several deleted forms of R8:MTD(10) peptide, as shown in Table 1. R8:MTD(10) and R8:MTD(9) peptides contain all four leucine residues. The first leucine residue (second site) was deleted in R8:MTD(8), and R8:MTD(7) contained only two leucine residues (fifth and ninth sites) of MTD. To examine the cell-killing activities of R8:MTD(10), R8:MTD(9), R8:MTD(8), and R8:MTD(7), HeLa (cervix adenocarcinoma), HCT116 (colorectal carcinoma), MCF-7 (breast adenocarcinoma), or BJAB (B cell lymphoma) cells were treated with these peptides in vitro. R8:MTD(10) and R8:MTD(9) showed similar concentration dependently killing activities in all tumor cells tested, whereas R8: MTD(8) showed less killing activity than R8:MTD(10) or R8:MTD(9) (Figure 2-1). However, R8:MTD(7) had no cell-killing activity in any cell-line tested. These results indicate that the leucine residue at the second site of MTD is not required for the killing activity of MTD, but that the leucine residues at third, fifth, and ninth sites are required. To investigate whether R8:MTD(10) and deleted R8:MTD peptides could kill normal cells, R8:MTD(10) or deleted R8:MTD peptides were treated at various concentrations to normal mouse

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Name	sequences	Amino acid
R8:MTD(10)	RRRRRRRRKLLNLISKLF	18
R8:MTD(9)	RRRRRRRR LLNLISKLF	17
R8:MTD(8)	RRRRRRRR LNLISKLF	16
R8:MTD(7)	RRRRRRRR NLISKLF	15

Table 2-1. Sequences of R8:MTD(10-7) peptides

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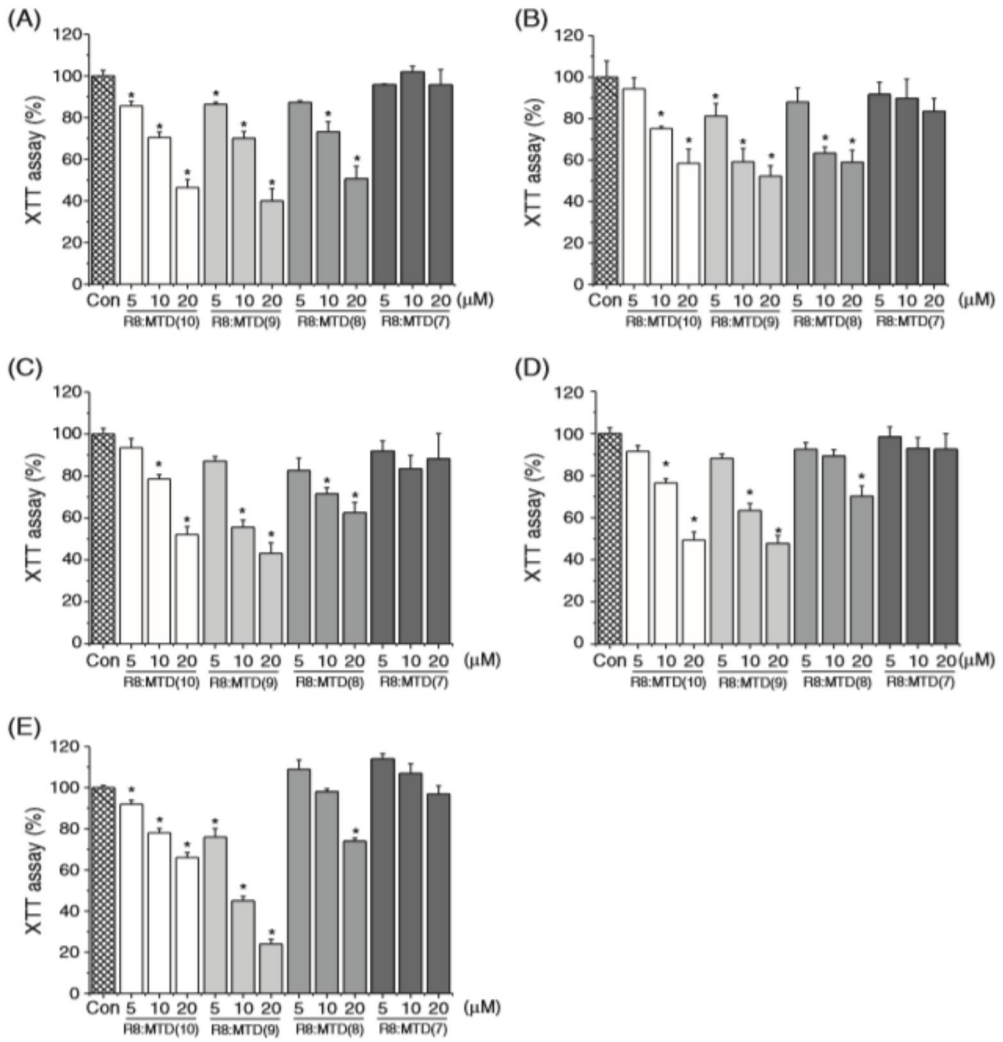

Figure 2-1. The effects of R8:MTD(10) and R8: MTD(9-7) on cell viabilities. (A) HCT116, (B) HeLa, (C) MCF-7, (D) BJAB, and (E) CT26 (1x10^4/well) cells were treated with R8:MTD(10), R8:MTD(9), R8:MTD(8), or R8:MTD(7). After 15min of treatment, cell viabilities were measured by XTT assay. XTT assay results of non-treated controls were considered to be 100%. *, $p<0.01$ versus non-treated controls.

peritoneal macrophages, and cell viabilities were monitored by sodium 2,3,-bis(2-

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methoxy-4-nitro-5-sulfophenyl)5-[(phenylamino)-carbonyl]-2H-tetrazoli-um (XTT) assay. As was expected, R8:MTD(10) and R8:MTD(9) peptides showed cellkilling activity in normal mouse peritoneal macrophages, because R8 cannot discriminate between normal cells and tumor cells. However, R8:MTD(9) was less potent at killing normal peritoneal macrophages. R8:MTD(7) was, as expected, not able to kill normal mouse peritoneal macrophages. An interesting finding was that R8:MTD(8) could not kill normal mouse peritoneal macrophages but did kill various tumor cells (Figure 2-2). The molecular mechanism responsible for the ability of MTD(8) to differentiate normal and tumor cells is obscure, although we suggest the possibility that it is due to different threshold levels in these cells. The expression of EGFP-fused MTD4Lmt (leucine replaced with alanine at second, third, fifth, and ninth of MTD in HeLa cells showed no mitochondrial localization and no cell death[6]. Also, R8:MTD4Lmt(10) mutant peptide failed to kill cells (data not shown), suggesting that loss of killing activity of R8:MTD(7) might be due to a failure to target to mitochondria. To determine whether the loss of killing activity of R8:MTD(7) is due to the failure to localize to mitochondria, plasmid vectors expressing EGFP-fused MTD(10, 9, 8, or 7) and DsRed2-Mito were introduced into HeLa cells. HeLa cells expressing EGFP alone showed dispersed pattern of EGFP expression. Co-localization of EGFP fused MTD(10) with DsRed2-Mito-stained mitochondria in

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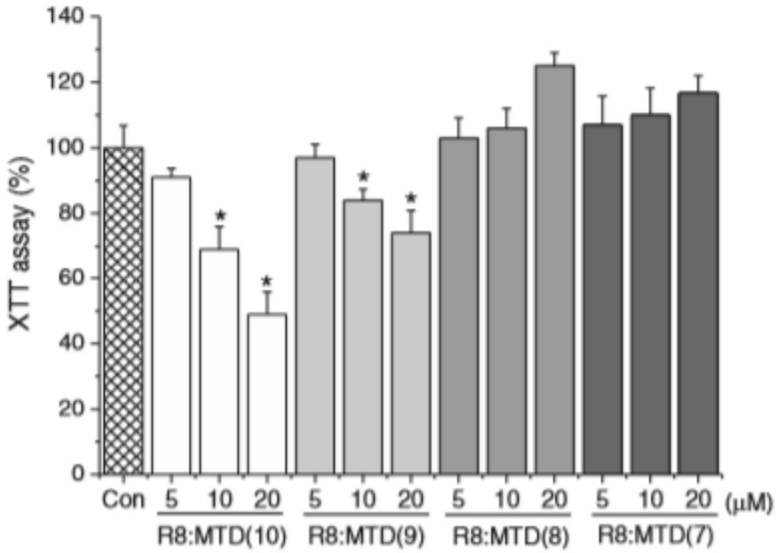

Figure 2-2. The effects of R8:MTD(10), R8:MTD(9), R8:MTD(8), or R8:MTD(7) on cell viability. Peritoneal macrophages (2x10^5/well) weretreatedwithR8:MTD (10),R8:MTD(9),R8:MTD(8),orR8:MTD(7).After15minoftreatment,cellviabilities were measured by XTT assay. XTT assay results of non-treated controls were considered to be 100%. *, $p<0.01$ versus non-treated controls.

HeLa cells is consistent with the previous results [113][Figure 2-3(A)]. EGFP-

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fused MTD(9) showed similar localization to that of EGFPfused MTD(10), indicating that MTD(9) maintains mitochondrial targeting ability. Moreover, EGFP-fused MTD(8) still maintained mitochondrial punctuate localization. However, EGFP-fused MTD (7) showed completely dispersed localization, meaning that MTD(7) completely lost the mitochondrial targeting ability [Figure 3(A)]. To further confirm that killing activities of EGFPfused MTDs are directly correlated to the mitochondrial localization of EGFP-fused MTDs, HeLa cells were transfected with plasmid vectors expressing EGFP-fused MTDs. The cell viabilities in HeLa cells transfected with these vectors were measured by XTT assays. The cell viabilities were decreased in HeLa cells transfected with the vectors expressing EGFP-fused MTD(10), MTD(9), or MTD(8) in a dose-dependent manner; however,EGFP-fused MTD(7) did not decrease the cell viability [Figure 3 (B)]. Together, these results demonstrate that MTD's killing activity depends on the mitochondrial localization of MTD. To examine tumor suppressing activities of R8:MTD(10), R8:MTD (9), and R8:MTD(8) in vivo, syngenic mice bearing tumors developed after the s.c. injection of CT26 cells were i.v. injected with R8:MTD(10) (2.5mmol/mouse), R8:MTD(9) peptide (5mmol/mouse), or R8:MTD(8) peptide (5mmol/mouse) using the protocol shown in Figure 2-4(A). Mice treated with R8:MTD(10), R8:MTD(9), or R8: MTD(8)MTD showed only limited or no suppression of tumor growth [Figure 2-4(B)], although a histochemical

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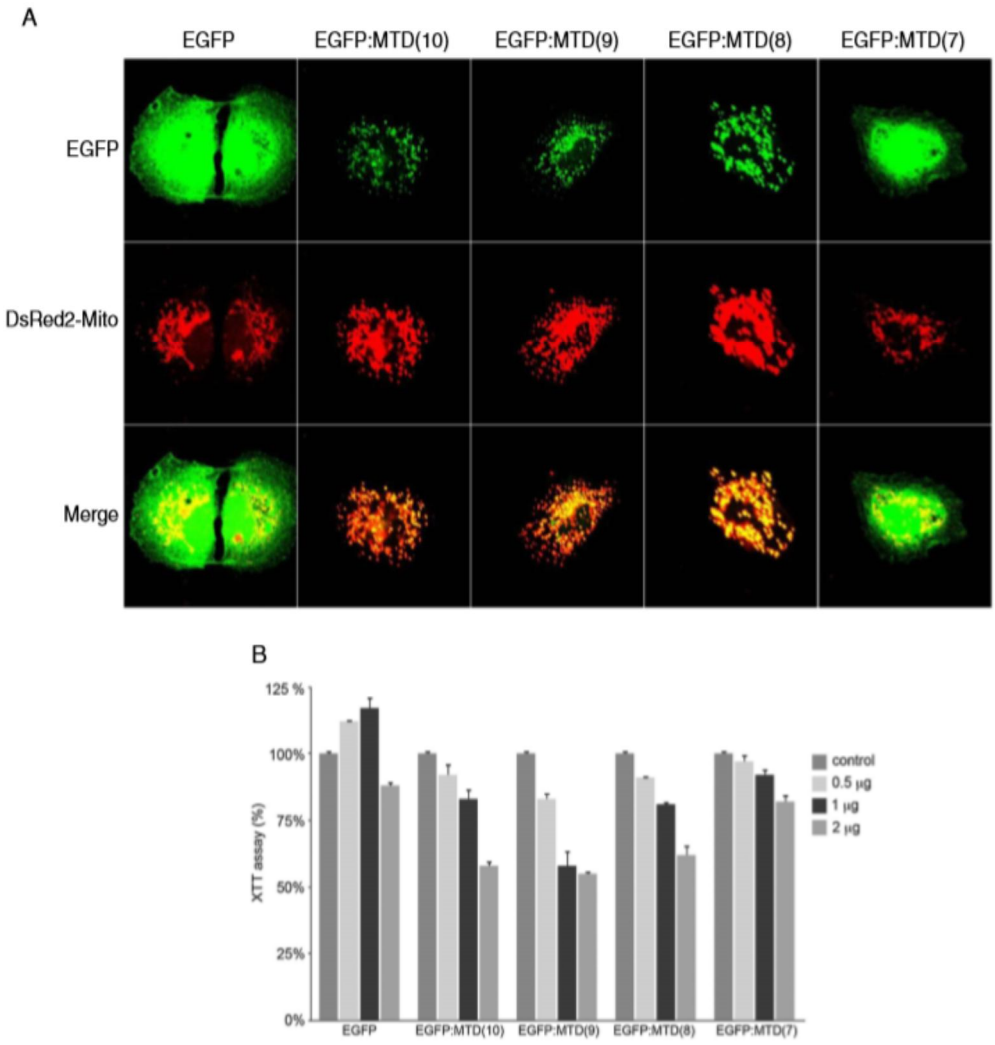

Figure 2-3. Cellular distribution of EGFP-fused MTDs. Expression vectors of EGFP alone or EGFP-fused MTDs (indicated as EGFP:MTDs) were cotransfected with expression vector of DsRed2-Mito into HeLa cells for overnight. (A) Images were captured using a laser scanning confocal microscope (Leica Microsystems TCS NT), and (B) cell viabilities were measured with XTT assays.

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examination revealed necrotic tumor cell death in mice treated with R8:MTD(10), R8:MTD(9), or R8:MTD(8) but not in mice treated with PBS [Figure 2-4 (C)]. We suggest that R8:MTD(10),R8:MTD(9),and R8:MTD(8) did not suppress tumor growth because of insufficiencies of these peptides in tumor tissues. The eight arginines of R8:MTDs peptides are incapable of differentiating tumor and normal cells, and thus, R8: MTDs peptides are insufficiently delivered to tumor tissues.

Although serological analysis of ALT and AST showed no apparent liver damage in mice injected with R8:MTD(10), R8:MTD(9), or R8: MTD(8) at the dosages used in this study [Figure 2-4(D)], R8:MTD (10) and R8:MTD(9) were found to be toxic when greater amounts were administered (data not shown). Together, these results suggest that MTD peptide could be a valuable basis for the development of a new type of anti-cancer agent, and that its specific delivery to tumor tissues is a key developmental target. In summary, the present study shows that all tested cells, including tumor cells and normal macrophages, were killed by R8:MTD(10) or R8:MTD(9), and that R8:MTD(7) could not kill any ofthecellstested.However,R8:MTD(8)killed all tumor cells tested in vitro but showed a limited killing activity on normal peritoneal macrophages. Therefore, we believe that MTD(8) might represent the minimal unit required for the killing activity of MTDs. In this study, R8 was used as the cell penetrating transporter peptide, in this case, MTD. However, R8 does not provide secure delivery of R8:MTD to tumor tissues [117-120]; and thus, R8:MTD is likely to

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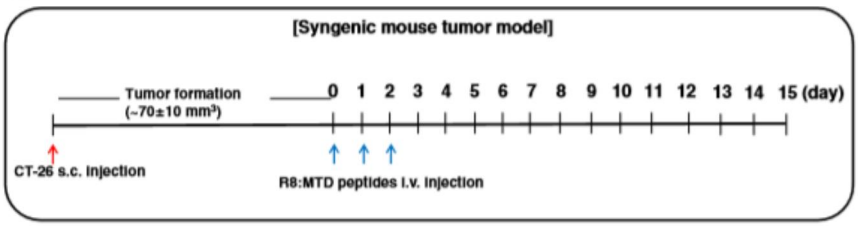

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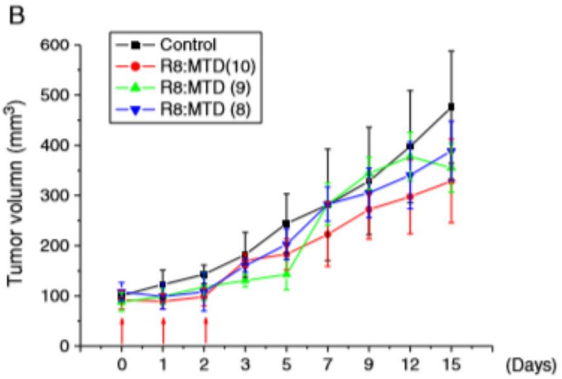

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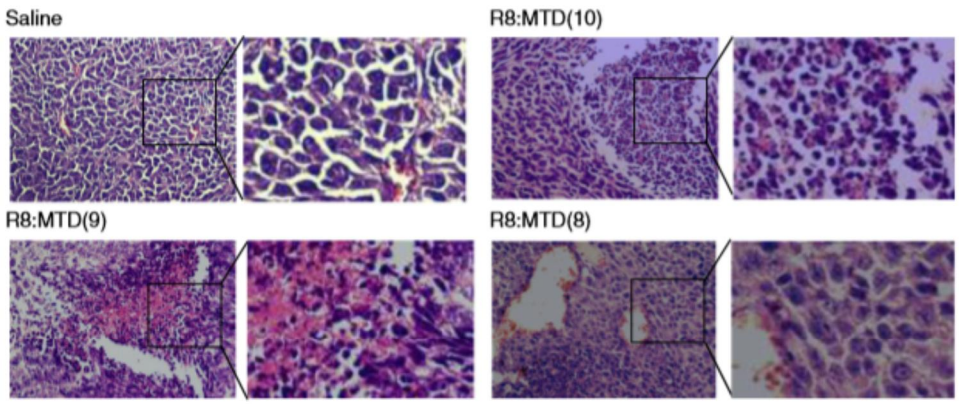

D

Treatment	Serum aminotransferase (karman units/ml) AST	ALT
NC	3.4 ± 0.8	41.1 ± 5.2
R8:MTD(10)	3.3 ± 0.8	36.8 ± 4.1
R8:MTD(9)	4.5 ± 2.3	40.5 ± 4.2
R8:MTD(8)	5.8 ± 4.2	38.2 ± 5.2

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Figure 2-4. Treatment of R8:MTD peptides in the syngenic mouse tumor model. (A) Tumors were generated over 2weeks after injecting CT26 cells s.c. into the backs of BalB/C mice. When tumor volumes reached around 7010mm3, R8:MTD(10) (2.5mmole/mouse), R8:MTD(9) (5mmole/mouse), R8:MTD(8) (5mmole/mouse), or saline was injected i.v. through a tail vein three times as indicated. (B) Tumor dimensions in the peptide-treated groups (each group, n=5) and saline-treated group (n=5) were measured using a caliper, and tumor volumes were calculated using the longest diameter x width 2 x 0.5 on the indicated days. (C) Tumors were obtained from the mice treated with saline or R8:MTDs after sacrifice and stained with hematoxylin and eosin. Images (original magnification x400) of tumor regions were obtained (left); the boxed regions show enlarged images. (D) Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured in the sera of mice treated with the indicated peptides.

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be toxic or lethal if high amounts are administered. Thus, a secure method of delivery to tumor cells should be devised to facilitate the use of MTD peptides as anti-cancer agents.

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CHAPTER 3

Designing a cancer therapeutic peptide by combining the mitochondrial targeting domain of Noxa and ErbB2-targeting moieties

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3.1. Abstract

Many anticancer drugs target epidermal growth factor receptors to inhibit receptor tyrosine kinases and tumor growth. Here, we show that an ErbB2targeting pronecrotic peptide (KWSY:MTD) selectively kills tumor cells expressing ErbB2 in vitro. An antibody against ErbB2 inhibits KWSY:MTDinduced cell death. KWSY:MTD causes membrane permeability which allows propidium iodide entry into the cytosol and the release of HMGB1 into the media, indicative of necrosis. Mitochondrial swelling occurs in response to KWSY:MTD. Moreover, in vivo analysis using a mouse model shows that KWSY:MTD partially suppressed growth in tumor tissue bearing ErbB2expressing cells, but did not have obvious toxicity in mouse liver or kidney tissue. Taken together, KWSY:MTD has potential as an ErbB2-targeting anticancer drug.

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3.2. Introduction

Proteins in the epidermal growth factor receptor (EGFR) family play key roles in cell proliferation, differentiation, and tumorigenesis. Among all EGFR family proteins, ErbB2 is known to be overexpressed in a wide spectrum of cancers, including breast cancer, gastric cancer, non- small cell lung carcinoma, and ovarian cancer [121, 122]. Amplification of ErbB2 triggers strong tumorigenic signals, such as activation of the PI3K/AKT pathways, through the dimerization of ErbB1 and ErbB3 [121]. ErbB2 signaling also protects cells that are exposed to apoptosis-inducing molecules such as 5 0-FU. In addition, overexpression of ErbB2 in tumor cells promotes transformation into a malignant tumor, resulting in poor clinical outcomes in cancer patients [122]. Tyrosine kinase inhibitors such as afatinib and lapatinib have been developed and used for treatment of patients with cancer cells that overexpress ErbB2 [123]. The monoclonal anti-ErbB2 antibody trastuzumab is another anticancer drug for breast cancer overexpressing ErbB2 [121]. These inhibitors have demonstrated significantly improved outcomes for treatment of cancer patients, indicating that ErbB2 is a key target for therapy [124-126]. However, these anticancer drugs cause mutations in ErbB2 in tumor cells that interfere with the binding of anticancer drugs to ErbB2, and these tumor cells become drug-resistant tumor cells [121, 123, 127]. Thus, it is necessary to develop a novel anticancer drug that is not affected by ErbB2 mutation or resistance [122, 128]. Many tumor-homing peptides provide a

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tool to selectively deliver killing materials to tumor cells [129]. KCCYSL has been identified as an ErbB2-targeting peptide by bacteriophage display screening [130]. It has also been adapted for use as a diagnostic agent for breast carcinomas and ovarian carcinomas and to deliver anticancer drugs to ErbB2-expressing cells by conjugating KCCYSL to imaging agents and anticancer agents, respectively [131, 132]. The mitochondrial targeting domain (MTD), consisting of 10 amino acids derived from the C terminus of the pronecrotic peptide Noxa, directly targets the mitochondria in cells and leads to necrosis when conjugated to cell-penetrating peptides; however, it does not induce necrosis outside of cells due to the lack of cell permeability of the MTD [89, 133]. In our previous study, we showed that TU17:MTD, in which the MTD is fused to the NRP-1-targeting motif (RPARPAR), effectively inhibited tumor growth without side effects [134]. In this study, we developed a novel peptide that targets ErbB2-overexpressing cells and induces necrosis by generating a hybrid peptide of the MTD and KCCYSL. We found that this molecule, termed KWSY:MTD, induces cell death by targeting ErbB2expressing tumor cells in vitro and in vivo.

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3.3. Materials and methods

3.3.1. Peptide synthesis

All synthesized peptides were purchased from GenScript (Piscataway, NJ, USA) or A&PEP (Chungbuk, Korea). All peptides were purified by rapid reverse phase-HPLC to homogeneity (i.e., > 90%) and evaluated by mass spectrometry. The peptides weredissolved in water or50% DMSO. Peptide sequences used in this study are presented in Fig. 1C.

3.3.2. Antibodies and recombinant proteins

Anti-ErbB2 (Cat. #2165S) and anti-MAPK (Cat. #4376S) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-HMGB1 antibody (Cat. #. ab18256) were purchased from Abcam (Cambridge, UK). Recombinant EGF protein was purchased from Sigma-Aldrich Korea (Cat. #E9644, Seoul, Korea). Recombinant 6xHis-profilin protein was purified in our laboratory using Ni-nitrilotriacetic acid agarose column (Cat. #30230, Qiagen, Hilden, Germany).

3.3.3. Cell viability assay

FaDu, MCF7, A431, Jurkat, 293, A549, U373MG, 4T1, CT26, and HePa-1c1c7 cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea). For FaDu, MCF7, A431, Jurkat, 293, A549, U373MG, 4T1, CT26, and HePa1c1c7 cell lines, 2 9 104 cells were cultured in 96-well plates overnight. Cells were treated with

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peptides for 2 h. Cell viability was assessed by the MTS Assay Kit (CellTiter 96 aqueous solution Cat. #G3580, Promega, Madison, WI, USA) according to the manufacturer's instructions. All data are represented as the mean SD (n = 3).

3.3.4. Propidium iodide staining

Cells (2 9 105 cells per well) were treated in the presence or absence of the peptides for 2 h, then incubated with 10 μg/mL1 propidium iodide (PI) (Sigma-Aldrich Korea, Seoul, Korea) for 15 min at room temperature in the dark. PI-positive cells were then counted by the Muse Cell Analyzer (EMD Millipore Corporation, Hayward, CA, USA).

3.3.5. HMGB1 release

Supernatants collected from 4T1 cells treated with peptides were precipitated with 4 volumes of 20 ° C 10% TCA by centrifugation, followed by a wash of the pellets with 20 mM DTT in acetone at 20 ° C. HMGB1 was detected by western blot analysis with the anti-HMGB1 antibody (Abcam Cat. #. ab18256, Cambridge, UK).

3.3.6. Isolation of mitochondria

Mitochondria were isolated from 6-week-old BalB/C mouse livers as described previously [16]. Briefly, mouse livers were minced in washing buffer (250 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 5 mM HEPES [pH7.4], 0.1 mM PMSF, and 4 IM Rotenone) and homogenized with the Teflon Potter Elvehjem tissue grinder (Sigma-

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Aldrich Korea, Seoul, Korea). The homogenate was centrifuged at 1000 9 g for 10 min at 4 ° C. The supernatant was centrifuged at 10 000 9 g for 10 min at 4 ° C. The pellet was washed twice with regeneration buffer (250 mM sucrose, 10 mM HEPES [pH 7.4], 5 mM sodium succinate, 25 IM EGTA, 2 mM potassium phosphate, 0.1 mM PMSF, and 4 μM Rotenone). The final pellet was resuspended in regeneration buffer for further experiments.

3.3.7. Mouse tumor models

Animal experiments were performed in accordance with the guidelines of Chosun University Institutional Animal Care and Use Committee. The Animal Ethics Committee approved the protocols for mouse tumor experiments (Approval numbers: CIACUC2013-S0005, CIACUC2015-S0019, CIACUC2017S0031, and CIACUC2017-S0032). For the syngeneic tumor model, BalB/C mice (male, 6-week old) purchased from OrientBio (Sungnam, Korea) were inoculated with 2 9 105 cells of 4T1 or CT26 into the subcutaneous layer. Two hundred microliters KWSY:MTD (0.2 mM) or saline were administrated via the tail vein. For the xenograft tumor model, 2 9 107 Fadu cells were inoculated into the subcutaneous layer of NOD.CgPrkdcSCIDIl2rgtm1Wjl/SzJ mice (6-week old) purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Tumor, liver, and kidney tissues were collected on the last day of observation and were processed for hematoxylin and eosin staining.

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3.3.8. Statistical analysis

Statistical data are expressed as mean SD. Statistical analysis was carried out with ANOVA to evaluate the difference between two groups. Statistical significance was accepted for P values < 0.05.

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3.4. Results

3.4.1. Design of ErbB2-targeting necrosis-inducing peptides

To develop an ErbB2-targeting necrosis-inducing peptide, the MTD was fused to an ErbB2-targeting motif (KCCYSL) to selectively deliver the necrosis-inducing peptide to ErbB2-expressing cells; this fused peptide was named KCCYSL:MTD (Figure 3-1A). When the A431 cell line, an ErbB2-expressing line (Figure 3-2B), was treated with KCCYSL:MTD, the viability of A431 was decreased in a dose-dependent manner in the range from 10 to 40 IM. However, KCCYSL alone and the MTD alone showed no changes in the viability in A431 cells (Figure 3-1B). Although KCCYSL:MTD exhibited killing activity in A431 cells, the cytotoxicity of KCCYSL:MTD was insufficient. Moreover, KCCYSL: MTD has low solubility in water and high viscosity, making absorption in biological systems difficult. Thus, we modified the ErbB2-targeting motif to enhance cell killing activity and to improve the physical properties of solubility and viscosity. To screen for the best hybrid peptide with regard to killing activity and targeting, two ErbB2 overexpressing cancer cell lines, FaDu and MCF7 (Figure 3-2B), were treated with peptides (Figure 3-1C, D). KCCYSL:MTD exhibited no killing effect in FaDu and MCF7 cells, indicating that the combination of KCCYSL and the MTD may inhibit the targeting ability of KCCYSL to the ErbB2 protein. Two modified peptides, KWSY and KSWY, combined with the MTD showed the best killing activities in both FaDu and MCF7 cells, although KWCYS, KCWY, and KSSYSL with the MTD all showed some killing activities in MCF7 cells (Figure 3-1C,D).

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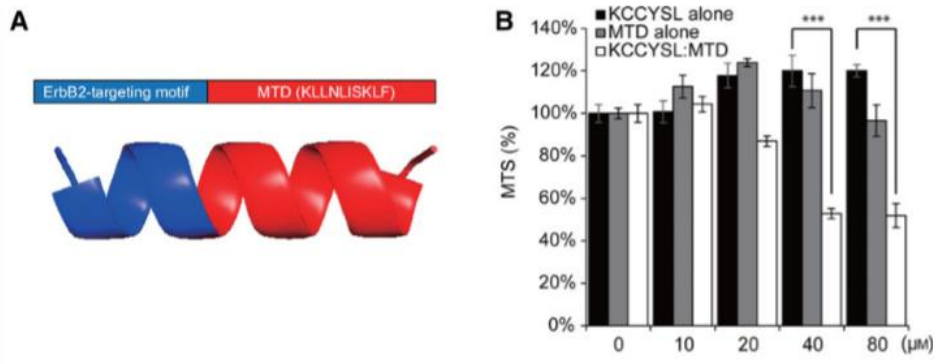

C

Amino acid sequence (target motif of ErbB2:MTD)	IC50 (μM)	
	FaDu	MCF7
KCCYSL: KLLNLISKLF	>100	>100
KWCYS: KLLNLISKLF	72.19 ± 3.26	ND
KCWY: KLLNLISKLF	>100	37.79 ± 7.54
KWSY: KLLNLISKLF	16.72 ± 4.95	ND
KSWY: KLLNLISKLF	15.15 ± 6.26	ND
KSSYSL: KLLNLISKLF	56.55 ± 9.22	41.09 ± 4.79
KSSYS: LLNLISKLF	>100	80.66 ± 6.77

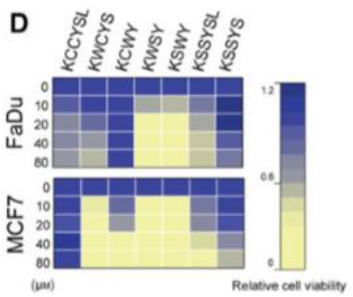

Figure 3-1. Combination of the ErbB2-targeting peptide and the MTD to kill ErbB2expressing cancer cells. (A) Diagram of the fusion peptides. (B) Cell viability was measured by the MTS assay in A431 cells treated with KCCYSL alone, the MTD alone, and KCCYSL:MTD for 2 h. (C) Sequences of the MTD combined with KCCYSL or modified ErbB2-targeting motifs. IC50 was calculated by the MTS assay presented in D. ND; not determined. (D) FaDu and MCF7 cells were treated with the indicated peptides, and relative cell viability is presented as color density. ***P < 0.001.

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3.4.2. KWSY:MTD kills ErbB2-expressing cancer cells

To identify the optimal peptide from the set of modified hybrid peptides, tumor cells that display high or low expression of ErbB2 were treated with peptides (Figure 3-2A). KWCYS:MTD and KWSY:MTD (range of 20-40 μM) were sufficient to induce cell death in tumor cells with high expression of ErbB2 such as the A431, 293, and A549 cell lines, whereas these peptides did not induce cell death in cells with low expression of ErbB2 such as the U373MG, CT26, and HePa-1c1c7 cell lines. The killing activity of the peptides in cells derived from mouse breast cancer was also tested to investigate whether the peptide can selectively kill ErbB2-expressing mouse cell lines. KWSY:MTD effectively killed the ErbB2-high-expressing mouse breast cancer cell 4T1, but KCCYSL:MTD did not kill when used at 20- 40 IM (Figure 3-2A). To test whether peptides selectively kill cancer cells and not normal cells, isolated normal splenocytes from mice were treated with the peptides. As shown in Figure 3-2A, KWSY:MTD did not kill splenocytes even at higher concentrations (> 80 μM), whereas KCCYSL:MTD and KWCYS:MTD exhibited some killing activity when used at 40-80 μM. The IC50 (the concentration that induces 50% inhibition) of the KWSY:MTD peptide for normal splenocytes was 20fold higher than that for Jurkat cells (Figure 3-2B). Together, these results suggest that KWSY:MTD has stronger killing activity in ErbB2-high-expressing tumor cells rather than in normal cells.

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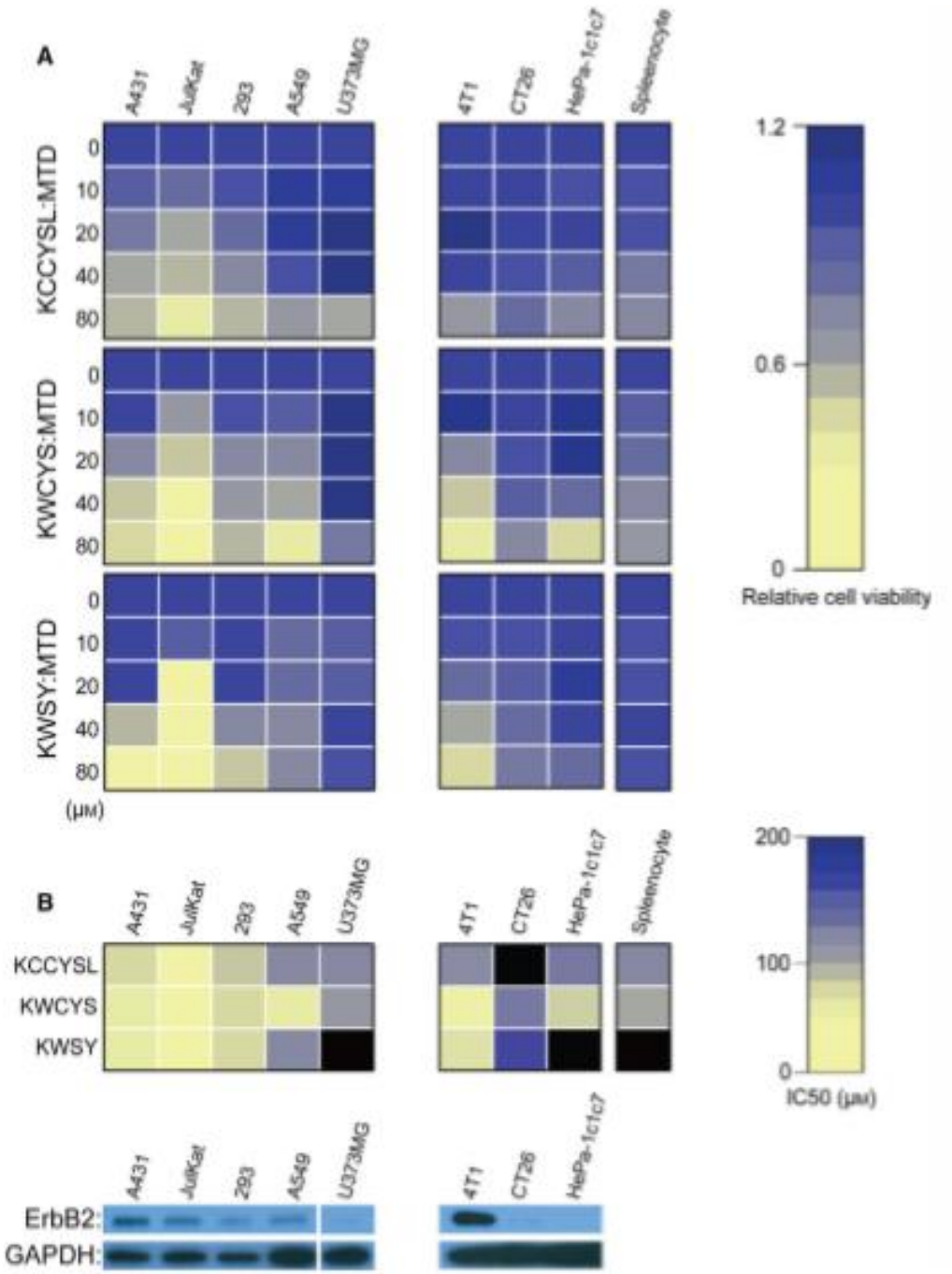

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Figure 3-2. Killing activities of KCCYSL:MTD, KWCYS:MTD, and KWSY:MTD in ErbB2high-expressing or ErbB2-low-expressing cells. (A) Indicated cell lines were treated with KCCYSL:MTD, KWCYS:MTD, or KWSY:MTD, and color density presents relative cell viability. (B) The IC50 for peptides in indicated cells is presented as color density; black indicates no killing activity (Top panel). Lysates from cell lines indicated on top panel were subjected to western blot analysis using anti-ErbB2 and anti-GAPDH antibodies (Bottom panel)

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3.4.3. Blocking EGFR inhibits KWSY:MTD-induced killing

To test the specificity of KWSY:MTD for targeting ErbB2, an anti-ErbB2 antibody was used to compete for binding to ErbB2 with KWSY:MTD, which is expected to decrease the killing activity of KWSY: MTD. Indeed, when FaDu cells pretreated with an anti-ErbB2 antibody were treated with KWSY:MTD, the level of cell death was greatly inhibited. However, the control antibody (anti-MAPK antibody) also mildly blocked the cell death induced by KWSY:MTD (Figure 3-3A,B). Moreover, KWSY:MTD-induced cell death was inhibited in EGF-pretreated cells compared to that in control protein (recombinant 6xHis profilin)treated cells (Figure 3-3A). These results suggest that KWSY:MTD-induced cell death is dependent on the level of ErbB2 expression in tumor cells and that KWSY:MTD is binding to ErbB2.

3.4.4. KWSY:MTD induces necrosis in tumor cells

Our previous studies have demonstrated that TU17: MTD and R8:MTD cause necrosis in cancer cells; thus, I expected that KWSY:MTD would also induce necrosis due to the necrosis-inducing motif, MTD [133, 134]. High mobility group 1 (HMGB1) is a protein present in the nucleus as a chromatin-binding protein and is released outside the cell when necrotic cell death such as non-programmed cell death is induced. The release of HMGB1 outside the cell is a biochemical indicator of necrotic cell death [134, 135]. Necrotic bodies were evidently formed in less than 10 min after 20 IM of KWSY:MTD treatment (Figure 3-3B), and many PI-positive 4T1 cells were observed in response to KWSY:MTD; however, a low level of PI-

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positive 4T1 cells was observed in response to KCCYSL alone, the MTD alone, and KCCYSL: MTD (Figure 3-4A,B). HMGB1 was released into media in response to KWSY:MTD, supporting the idea that KWSY:MTD is a necrosis-inducing peptide (Figure 3-4C). However, the reason why KWSY:MTD released more HMGB1 protein outside the cell than KWCYS:MTD is not clear, although KWCYS:MTD caused more PI-stained cells than KWSY:MTD. The opening of the mitochondrial permeability transition pore (mPTP) is known to be an important event in apoptosis or necrosis. When the mPTP is opened, the mitochondrial matrix swells, and the mitochondrial membrane integrity is damaged. Death factors in the mitochondrial matrix or intermembrane space are released into the cytosol, leading to rupture of the cell membrane [89, 136-139]. As shown in Figure 3-4D, the swelling of the mitochondrial matrix was monitored through changes in optical density at 540 nm in isolated mouse mitochondria. Consistent with previous reports [113, 140], calcium ions significantly induced mPTP opening, which was blocked by cyclosporine A (CsA). KWSY: MTD and KWCYS:MTD decreased the optical density of isolated mitochondria at 540 nm despite the low degree of mPTP opening compared to that induced by calcium ions; however, KCCYSL alone and the MTD alone did not change the optical density at 540 nm. These results indicate that KWSY:MTD and KWCYS: MTD open mPTP, causing necrosis in tumor cells.

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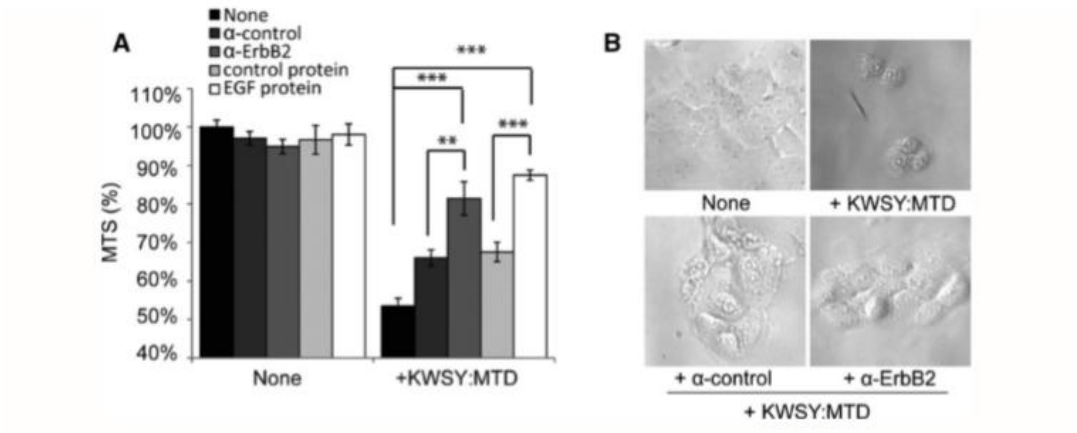

Figure 3-3. Blocking of KWSY:MTD-induced killing by anti-ErbB2 antibody and EGF. FaDu cells pretreated with an anti-ErbB2 antibody (1μg/mL1), EGF (100 ng/mL1), control protein (recombinant 6xHis-profilin, 100 ng/mL1), or control antibody (anti-MAPK, 1μg/mL1) for 1 h were treated with KWSY:MTD (20μM) for an additional 1 h. Cell viability was measured by the MTS assay (A), and cell images were taken using inverted optical microscopy (B). **P < 0.05, ***P < 0.001.

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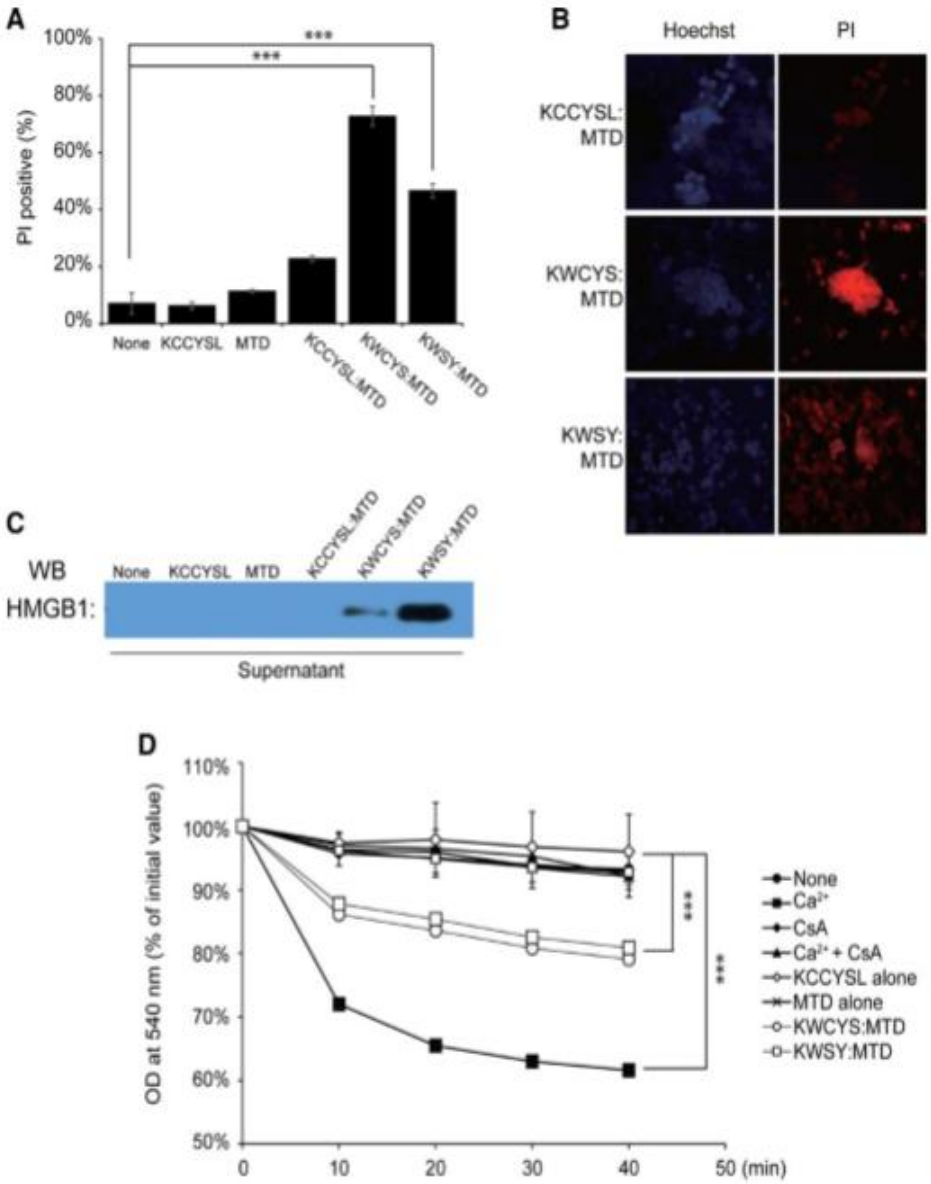

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Figure 3-4. KWSY:MTD induces necrosis. 4T1 cells were treated with the indicated peptides (20 μM) for 2 h. PI-positive cells were counted (A), and PI fluorescence images were obtained with Olympus fluorescence microcopy (B). HMGB1 in conditioned media from cells treated with peptides was detected by western blotting using an anti-HMGB1 antibody (1 : 1000) after precipitation with TCA and acetone (C). (D) Isolated mitochondria from mouse livers were treated with the indicated peptides (20 μM), calcium ions (200 μM), or CsA (20 μM), and optical densities at 540 nm were monitored every 10 min. ***P < 0.001.

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3.4.5. In vivo antitumor activity of KWSY:MTD in ErbB2 overexpressing cancer

To assess the ErbB2-specific anticancer effects of KWSY:MTD on tumor growth in a syngeneic tumor model, CT26 cells (an ErbB2-low-expressing cell line) and 4T1 cells (an ErbB2-high-expressing cell line) were used to generate tumor tissues in BalB/C mouse. I expected that the killing activity of KWSY:MTD in tumor tissues bearing CT26 cells would be minimal, whereas KWSY:MTD would exhibit robust killing activity in tumor tissues bearing 4T1 cells. Indeed, inhibition of tumor growth by KWSY:MTD in tumor tissue bearing CT26 cells was not seen when KWSY: MTD was intravenously administrated into mice via the tail vein (Figure 3-5A), whereas KWSY:MTD exhibited growth suppression in tumor tissue bearing 4T1 cells (Figure 3-5B). The FaDu cell line is a human pharynx squamous cell carcinoma, which has higher expression levels of ErbB2 (Fig. 2B), and FaDu cells were sensitive to KWSY:MTD at 10-20 lM (Figure 3-1C). To examine the anticancer effects of KWSY:MTD in a xenograft model using FaDu cells, tumor tissues of FaDu cells were formed on the backs of NOD-scidIL2Rgnull mice that lack mature T cells, B cells, and NK cells [141]. The killing activity of KWSY:MTD in tumor tissues bearing FaDu cells was significant (Figure 3-5C), and was evident in H&E staining (Figure 3-5D). In addition, H&E staining of kidney and liver tissues of KWSY:MTD treated mice or non-treated mice exhibited no damage. Together, these results indicate that KWSY:MTD targets and kills the tumor cells expressing ErbB2 but not noncancerous cells in the liver and kidney.

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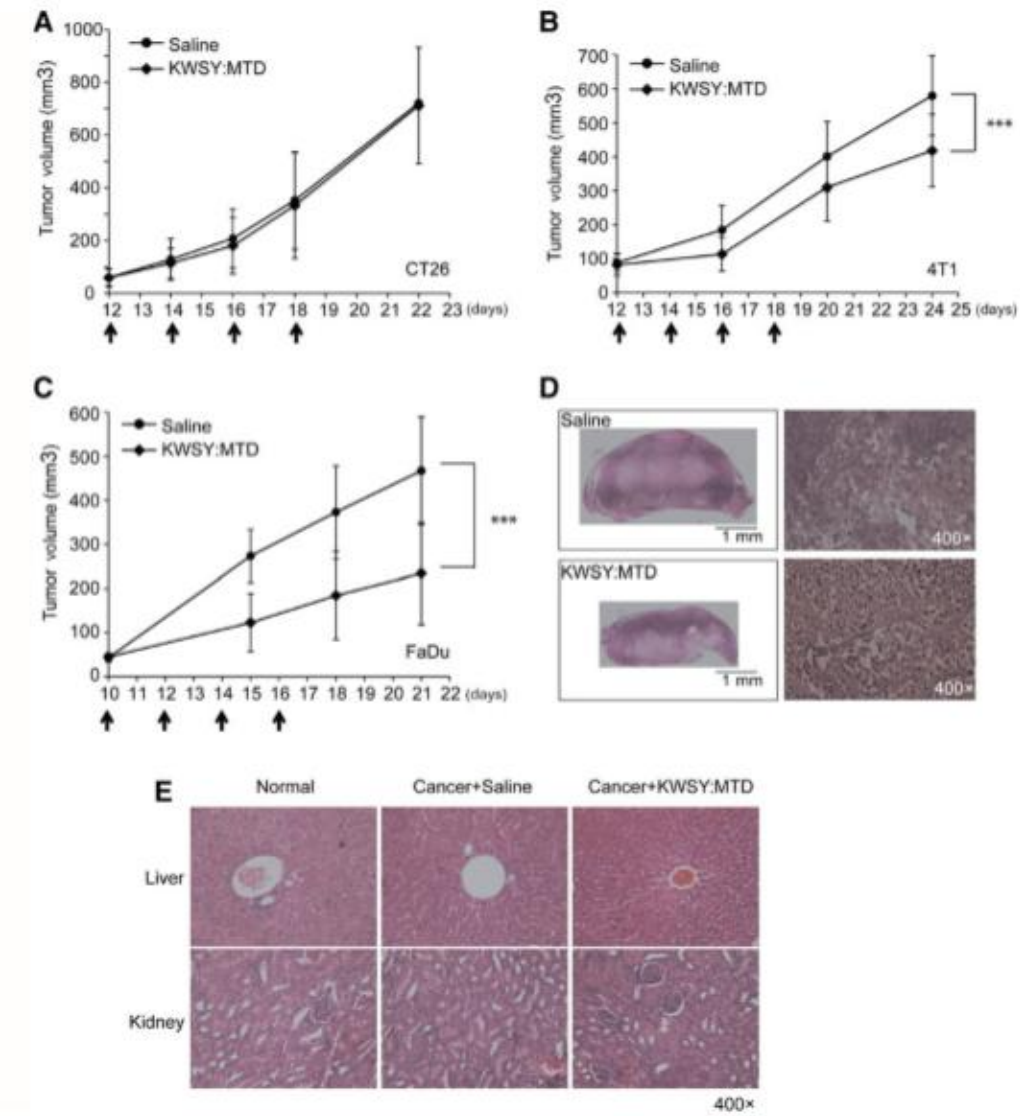

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Figure 3-5. KWSY:MTD suppresses FaDu-bearing tumor growth in mice. KWSY: MTD (200 of 0.2 mM per mouse) or PBS was intravenously injected every 2 days four times (indicated as arrows) into the tail vein of CT26-bearing mice (n = 10) (A) or 4T1-bearing mice (n = 10) (B). KWSY:MTD (200 μL of 0.4 mM) or PBS was intravenously injected into the tail vein of FaDu-bearing SCID mice (n = 6). (D) Isolated tumors from FaDu-bearing SCID mice were stained with H&E, and images (magnified 40x or 400x) were taken and merged using Adobe Photoshop CS5. (E) Livers and kidneys isolated from KWSY:MTD or PBS injected BalB/C mice were stained with H&E, and images magnified 400x were obtained using a light microscope. ***P < 0.001.

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3.5. Discussion

In this study, I developed a novel ErbB2-targeting peptide combined with the MTD, KWSY:MTD. I found that KWSY:MTD kills the ErbB2-high-expressing tumor cells by necrosis, but shows limited killing activity in noncancerous cells or ErbB2-low-expressing tumor cells in vivo and in vitro. The KWSY sequence was, in this study, modified from KCCYSL, a well-known ErbB2-targeting motif. KCCYSL was identified as an ErbB2-binding peptide through phage-display screening [142], and was used for cancer imaging agents and delivery tools to target ErbB2-expressing cells [130-132, 143]. In this study, direct coupling of KCCYSL with the MTD was not satisfactory because high amounts of KCCYSL:MTD were needed to kill the ErbB2-high-expressing FaDu cells. This could be due to the steric hindrance of KCCYSL binding to ErbB2 by the MTD or the lack of a linker sequence such as GSG [131, 132]. However, KWSY:MTD is likely to selectively target the ErbB2-high-expressing cells, such as FaDu and 4T1 cell lines, based on the observation that the killing activity of KWSY:MTD on FaDu was blocked by an anti-ErbB2 antibody or EGF (Figure 3-3). However, it is not yet clear whether KWSY is sufficient for targeting ErbB2 or if KWSY and some part of the MTD is necessary. Deletion forms of KWSY:MTD conjugated with fluorescent dye could be used to pin-point the ErbB2-binding portion of KWSY:MTD in the future. KWSY:MTD showed the highest killing activity in Jurkat cells but moderate killing activity in 4T1 cells, although ErbB2 expression levels in A431 cells and 4T1 cells were higher than that in Jurkat

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cells. The reason why the killing activity of KWSY:MTD does not correlate closely with the expression levels of ErbB2 in Jurkat cells and 4T1 cells is not clear. It is speculated that it could be different penetration efficacy of KWSY:MTD between Jurkat cells and 4T1 cells, possibly due to the cell surface interfering molecules (e.g., proteins or lipids) of A431 cells and 4T1 cells. The interfering molecules in A431 cells and 4T1 cells could cause the differential binding affinity of KWSY:MTD to ErbB2 or directly slow down the membrane penetration of KWSY:MTD in A431 cells and 4T1 cells by unknown mechanisms.

An anti-ErbB2 antibody can prevent the KWSY: MTD-induced cell death, which indicates that the function of the MTD is dependent on ErbB2 expression level. In addition, EGF inhibit the KWSY:MTD induced cell death. This finding is unexpected, as EGF binds to ErbB1 (EGFR) but not to ErbB2 [144]. It provokes to speculate that the binding of EGF to ErbB1 induces the hetero-dimerization with ErbB2 and spatially blocks the KWSY:MTD binding region on ErbB2 by ErbB1. Alternatively, EGF-induced hetero-dimerization of ErbB1 and ErbB2 may induce structural changes in ErbB2, and prevent the binding of KWSY:MTD to ErbB2. It would be interesting to define which part of ErbB2 is the KWSY:MTD binding region in future. As it is not known which part of KWSY:MTD contains the recognition motif for ErbB2, there remains the question of whether KWSY alone can target ErbB2 or not. Additionally, the MTD alone did not induce mitochondrial swelling and may not maintain its helix structure well with only 10 amino acids. Based on these

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facts, adding a KWSY to the MTD seems to not only stabilize the structure of the MTD but also enable a function that recognizes ErbB2. The resulting KWSY:MTD is unable to enter the cell unless it encounters ErbB2 and cannot cause cell death from outside the cell. These findings imply that KWSY:MTD might reduce off-target effects because it cannot directly lyse cells, unlike hybrid peptides that combine lytic peptides and cancer target sequences. The approach applied herein to develop KWSY: MTD can be adapted to other tumor-targeting motifs to design a hybrid peptide with higher tumor selectivity and fewer off-target effects and lower toxicity. We previously showed that direct coupling of an NRP-1targeting motif and the MTD (TU17:MTD) successfully suppresses tumor growth; we also demonstrated that the MTD has much better killing activity in TU17:MTD compared to (KLAKLAK)2 [134]. Thus, we believe that the MTD provides a platform to design a new anticancer therapeutic agent with fewer side effects. Conventional anticancer drugs slowly induce apoptosis, resulting in transformation and tolerance in cells; this leads to the expectation of low killing efficiency in cancer cells [145, 146]. We observed synergistic or additive effects of combinations using R8:MTD and other anticancer agents in cancer cells (data not shown). As the mechanism of cell death induced by KWSY:MTD is similar to that by R8:MTD, I expect that anticancer effects will be increased when KWSY:MTD and other anticancer agents are used together. Barriers caused by the microenvironment of cancer and peptidases in the blood are major obstacles to the delivery of peptide-based anticancer drugs [147-149]. We also

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observed that the cytotoxicity is decreased when KWSY:MTD is injected into vessels of tumorigenic mice. The peptides may be rapidly inactivated by peptidases and serum elements in the blood. To overcome this problem, we propose modifying KWSY:MTD to a diastereomeric form. Alternatively, we expect local delivery with a catheter will increase anticancer effects. In conclusion, although KWSY:MTD is still limited by instability in the blood, it displays promise as an anticancer agent. If its limitations can be overcome, it will have a very strong anticancer effect by inducing the necrosis pathway.

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CHAPTER 4

Necrosis-inducing peptide has the beneficial effect on killing tumor cells through neuropilin (NRP-1) targeting

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4.1. ABSTRACT

The therapeutic efficacy of most anti-cancer drugs depends on their apoptosis inducing abilities. Previously, we showed that a peptide containing the mitochondrial targeting domain (MTD) found in Noxa, a BH-3 only protein of Bcl-2 family, induces necrosis. Here, a fusion peptide of neuropilin-1 (NRP-1) targeting peptide and MTD peptide, designated tumor homing motif 17:MTD (TU17:MTD), was found to induce necrosis in cancer cells in vitro and to cause the regression of tumors when intravenously injected into mice bearing subcutaneous CT26 colorectal carcinoma tumors. The necrosis within tumor tissues was evident upon administering TU17:MTD. TU17:MTD penetrated into tumor cells by targeting to Neuropilin-1, which could be blocked by anti-NRP-1 antibody. The efficacy of TU17:MTD on tumor regression was higher than that of TU17:D(KLAKLAK)2, a fusion peptide of NRP-1 targeting peptide and a pro-apoptotic peptide. The necrotic cell death within tumor tissues was evident at day 1 after administering TU17:MTD systemically. Transplanted subcutaneous substantially reduced in size within two weeks and 5 days, respectively, with no apparent side effects. Together, these results propose that the pro-necrotic peptide MTD may present an alternative approach for development of targeted anti-cancer agents.

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4.2. Introduction

Over the last three decades, studies on apoptosis have revealed that the signaling pathways of apoptosis are very complicated involving various mediators such as caspases, Bcl-2 family proteins, and IAP proteins [150, 151]. Most anti-cancer drugs, including chemotherapeutic agents, such as, cisplatin, etoposide, and paclitaxel, and targeted anti-cancer drugs, such as, imatinib induce apoptotic cell death. However, many tumor cells develop escape mechanisms that involve the deletion or modulation of key mediators of apoptosis, and eventually develop a resistance to anti-cancer drugs. This emergence of tumor resistance restricts the therapeutic efficacies of pro-apoptotic anti-cancer drugs, and is one of the major causes of relapse after anti-cancer drug treatment [152]. The other unfavorable aspect of pro-apoptotic anti-cancer drugs is that they suppress or deplete subgroups of cells in the immune system, especially T-cells [152]. This is mainly due to their direct toxic effects on immune cells [153, 154]. Pro-apoptotic anti-cancer drugs can trigger minimal immunogenic reactions because apoptosis is a type of cell death that minimizes the release of intracellular danger signals into the surrounding environment, thereby, ameliorating inflammatory immune reactions [150, 151]. Although a large number of studies have revealed the detailed signaling pathways involved in the apoptotic mechanism, at the biochemical and genetic levels, including the role of caspases, Bcl-2 family proteins, and IAP proteins [150, 151], strategies to overcome the limitations of

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pro-apoptotic anti-cancer drugs are still not established yet. In contrast to apoptosis, necrosis, which is characterized by the swelling of subcellular organelles and a loss of cytoplasmic membrane integrity, release of intracellular danger signals into surrounding environments and the activation of immune responses, was disregarded as a passive and harmful form of cell death for several decades. Recent evidence, however, indicates that necrosis is a regulated form of cell death. Some apoptosis associated genes like receptor-associated protein (RIP)1 and RIP3 have been shown to be involved in the regulated necrosis or necroptosis [155-157]. RIP3 phosphorylates the mixed lineage kinase-like protein (MLKL), resulting in membrane localization of MLKL by releasing four-helix bundle domain of MLKL, which then causes membrane disruption and finally necroptosis [158-160]. However, the key mediators of necrosis remain largely unknown. I reported a 10-residue long, pro-necrotic peptide derived from the mitochondrial targeting domain (MTD) located in the C-terminal region of Noxa, a BH-3 only protein of Bcl-2 family [89]. The MTD region of Noxa is responsible not only for mitochondrial targeting of Noxa [113] but also for mitochondrial fragmentation induced by Noxa [161]. Two leucine residues (L45 and L49) in the region of MTD play a key role in mitochondrial fragmentation induced by Noxa [161]. Moreover, I showed that the MTD peptide conjugated with a cell penetration peptide like eight arginine (R8:MTD) potently induces necrosis in many tumor cells via an abrupt spike in cytoplasmic calcium release from mitochondria. The two leucine

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residues of the MTD that played a key role in mitochondrial fragmentation are also key residues in induction of necrosis by R8:MTD [13]. R8:MTD peptide induced necrosis in various tumor cells in vitro within 10 ~ 30 minutes in a caspase-independent manner. Although the molecular mechanisms of R8:MTD-induced necrosis are largely unknown, it may directly damage mitochondria, rather than activating a cell death signaling cascade [89]. Here, I describe a novel pro-necrotic peptide anti-cancer agent based on the combination of MTD with tumor-homing motifs, and suggest that pro-necrotic agents such as MTD may be an alternative way to overcome the limitations of pro-apoptotic anti-cancer drugs.

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4.3. Material and methods

4.3.1. Cell culture

All cell lines used in this study were purchased from Korean Cell Line Bank (Seoul, Korea), and were maintained by a standard culture protocol according to Korean Cell Line Bank's culture conditions.

4.3.2. Peptide synthesis and reagents

The peptides used in this study were synthesized from AnyGen (Gwangju, South Korea). Anti-NRP-1 antibody was purchased from Santacruz Biotechnology (Dallas, Texas, USA). Other chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

4.3.3. Isolation of peritoneal macrophages and splenocytes

Peritoneal macrophages were isolated from mice injected intraperitoneally with 2 ml (4% (w/v) thioglycollate medium) three days prior to peritoneal lavage, which was conducted using 10 ml of RPMI 1640. Harvested cells were washed with RPMI 1640 three times and then cultured in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ ml streptomycin. Cells were plated on culture dishes and incubated for 2 hours at 37° C in a 5% CO2 humidified incubator. After removing non-adherent cells, monolayered macrophages were treated with TU17:MTD.

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For splenocyte isolation, spleens were removed from BALB/c mice, placed in a Petri dish containing phosphate-buffered saline (PBS, pH 7.4), and pushed through a 200-mesh stainless steel sheet mesh. Resulting cells were suspended in PBS and centrifuged at 400 × g for 10 min at 20° C. Red blood cells were removed by incubation with hypotonic (0.87% (w/v) NH4Cl in PBS) lysis buffer for 3 minutes. Cells were then washed twice with PBS and were re-suspended in RPMI 1640 medium.

4.3.4. XTT assay

Cells were cultured in 96 well plates (2 × 10^5) overnight and treated with TU:MTDs. Cell viabilities were assessed using a XTT-based colorimetric assay kit, according to the manufacturer's instructions.

4.3.5. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assays

To assay the activities of ALT and AST, mouse serum was prepared by centrifuging blood at 2500 × g for 15 minutes at 4° C. Enzyme activities were determined, according to the manufacturer's instructions.

4.3.6. Mouse tumor models

All animal studies followed the guidelines of the Chosun University Institutional Animal Care and Use Committee. The Animal Ethics Committee at the Chosun University approved the protocols for animal experiments (Approval number: CIACUC2012-A0008, 2013-S0005 and CIACUC2015-S0019). Specific pathogen-free BALB/c mice (males, 6 weeks-old) were purchased from Samtaco (Daejon, Korea). Animals

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were housed under normal laboratory conditions (21-24° C and 40-60% relative humidity) under a 12-hour light/dark cycle with free access to standard rodent food and water. Subcutaneous tumors were in BALB/c mice by injecting 1.5 × 105 CT26 cells in 100 μl saline into the subcutaneous layer of the mouse. Tumor cells were grown for 7 to 8 days. TU:MTDs (approximately10 mg/kg) or saline were injected i.v. into a tail vein. Tumor volumes were calculated as length × width2 × 0.5 as previously described [13].

4.3.7. Time-lapse confocal microscopy

For time-lapse imaging of the penetration of TU17:MTD-FAM peptides, HeLa cells or CT26 cells plated on the coverslips were placed in the chamlide magnetic chamber (Live Cell Instrument, Seoul, Korea). Cells were excited at 488 nm wavelength in the Laser Scanning Confocal Microscope (Olympus Corporation, Tokyo, Japan). Images of cells were obtained at every 1- or 2 minutes intervals for 30 minutes after addition of the indicated peptide to the medium.

4.3.8. Targeting of TU17:MTD to tumor tissues

BALB/c mice harboring subcutaneous tumor tissue were injected with PBS, TU17:MTD-FAM, or TU17:MTDFAM plus TU17:ΔMTD. One hour upon peptide injection, the mice were sacrificed to harvest tumor tissues and blood samples. The fluorescence intensities of tumor tissues were measured by Xenogen IVIS 200 imaging system, and were quantified by using a living imaging software (Caliper

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Life Sciences, Hopkinton, MA, United States). The fluorescence intensities of blood samples were measured using Sunrise microplate reader (Tecan, Mannedorf, Switzerland).

4.3.9. Statistical analysis

Results are reported as means ± SDs. ANOVA test was used to evaluate differences between more than two groups. When a significant difference was observed, Dunnet's 't' test was used to compare the means of two groups. Statistical significance was accepted for p values < 0.05.

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4.4. Result

4.4.1. TU17:MTD, a peptide containing MTD, kills tumor cells

To design a MTD peptide anti-cancer drug, the MTD peptide was fused to various known tumor-homing motifs through its N-terminal or C-terminal region [120], and a linker was introduced between these two motifs to impart flexibility and minimize steric hindrance (Figure 1A). The MTD peptides fused with tumor-homing motifs (hereafter designated TU:MTDs) were synthesized as linear or cyclic entities using L-amino acids. Some TU:MTDs were found to suppress tumor growth, but not to reduce tumor sizes. TU17:MTD was found to have a stronger suppressive effect on tumor growth than did the other TU:MTDs (Figure 4-1B). The tumor-homing motif of TU17:MTD has a "RPARPAR" sequence containing the C-end rule (CendR) element that has known to bind to neuropilin-1 (NRP-1) [78, 162], although the "RPARPAR" sequence is located at the N-terminus of the MTD rather than at the C-terminus. Thus, I further tested the effects of TU17:MTD on tumor growth in vitro and in vivo. When developing anti-cancer drugs, minimization of the adverse effects on normal cells is one of the major concerns. Therefore, I determined whether TU17:MTD could discriminate tumor and normal cells in vitro. When TU17:MTD was added to HeLa (cervix adenocarcinoma), HCT116 (colorectal carcinoma), MCF-7 (breast adenocarcinoma), A549 (lung carcinoma), PC3 (prostate adenocarcinoma), or BJAB (B cell lymphoma) cells, it reduced cell viability in a concentration-dependent manner, as measured by XTT assay (Figure 4-1C). In contrast, the same

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concentrations of TU17:MTD did not induce cell death in normal peritoneal macrophages or splenocytes (Figure 4-1D). TU17:MTD and TU172:MTD, a second form of TU17:MTD in which the linker sequence GG is replaced by GFLG, presented a reduced killing activity compared to that of R8:MTD. I didn't observe any significant differences between TU17:MTD and TU17-2:MTD in terms of in vitro killing activity, suggesting that replacement of GG by GFLG has no advantages. Previously, I have shown that replacement of four leucine residues in MTD (KLLNLISKLF) to alanine, indicated as MTD4A (KAANAISKAF), abolishes the killing activity and mitochondrial targeting activity of MTD [89]. As expected, TU17:MTD4A abrogated its killing activity (Figure 4-1E), indicating that the killing activity of TU17:MTD is caused by MTD but not by tumor homing motif (RPARPAR) or linker sequence (GG).

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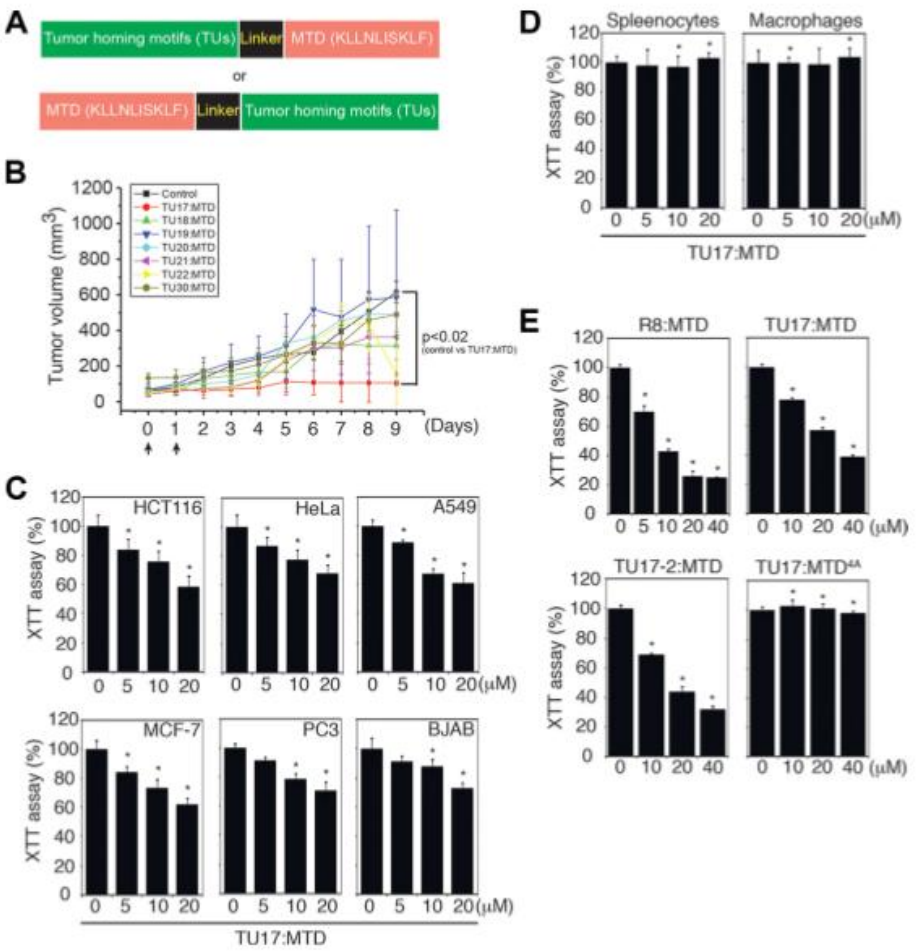

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Figure 4-1. Identification of TU17:MTD as a tumor targeting peptide. (A) Schematic diagram of the fusion peptides consisting of the tumor-homing motif, linker, and mitochondrial targeting domain (MTD). (B) Tumors were generated over 2 weeks after s.c. injection of CT26 cells into the backs of BALB/c mice. When tumor volumes reached palpable size, the indicated TU:MTDs (100 μL of 1 mM TU:MTDs) or PBS were administrated i.v. twice or three times. Tumor dimensions in the TU:MTDs-treated groups (n = 3) and PBS-treated group (n = 3) were measured using a caliper and tumor volumes were calculated at the indicated days using longest diameter × width2 × 0.5. Arrows indicate the points of peptide injection. P values < 0.02 (control group vs TU17:MTD at day 5, 6, 7, 8, and 9) (C) HCT116, HeLa, A549, MCF-7, PC3, and BJAB cells were treated with TU17:MTD (0 ~ 20 μM) for 30 minutes, and cell viability was monitored using XTT assays. *P < 0.05 (D) Primary splenocytes and macrophages were treated with TU17:MTD (0 ~ 20 μM) for 30 minutes, and cell viability was monitored by XTT assay. *P < 0.05 (E) CT26 cells were treated with R8:MTD, TU17:MTD, TU17-2:MTD or TU17:MTD4A (0 ~ 40 μM) for 4 hours, and cell viability was monitored by XTT assay. Results in C to E are expressed as means ± SD (triplicates), and are representative of at least two inde pendent experiments. P values are for experimental groups versus controls and were calculated using Dunnett's t-test. *P < 0.05.

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4.4.2. TU17:MTD, but not TU17:D(KLAKLAK)2, induces necrosis in tumor cells

To examine whether TU17-2:MTD induces necrosis or apoptosis in tumor cells, I observed the morphological and biochemical features of apoptosis and necrosis in CT26 cells treated with TU17-2:MTD or TU17:D(KLAKLAK)2. I used D(KLAKLAK)2 peptide, an well-known apoptosis-inducing peptide, to design TU17:D(KLAKLAK)2 [162-165]. To show that TU17:D(KLAKLAK)2 causes apoptosis in CT26 cells, activation of caspase-3 and caspase-8, which is a biochemical indicator of apoptosis, was examined by western blot. The results showed that TU17:D(KLAKLAK)2 but not TU17-2:MTD could activate caspase-3 and caspase-8 (Figure 4-2A). On the other hand, CT26 cells treated with TU17-2:MTD, but not with TU17:D(KLAKLAK)2, released HMGB1, a biochemical indicator of necrosis, into culture medium in vitro (Figure 4-2B). Morphological changes of the nucleus and cell membrane permeabilization in response to TU17:D(KLAKLAK)2 or TU17-2:MTD were further observed to distinguish the modes of cell death. Permeabilization of cell membrane, a morphological indicator of necrosis, analyzed by PI-staining was observed mostly in CT26 cells treated with TU17-2:MTD but not in cells treated with TU17:D(KLAKLAK)2 (Figure 4-2C, and Figure 4-3A). Condensed nuclei, a morphological indicator of apoptosis, analyzed by Hoechst staining were observed mostly in CT26 cells treated with TU17:D(KLAKLAK)2 but not in cells treated with TU172:MTD (Figure 4-2C, and Figure 4-3B). These results indicated that TU17-2:MTD causes necrosis, whereas TU17:D(KLAKLAK)2 causes apoptosis.

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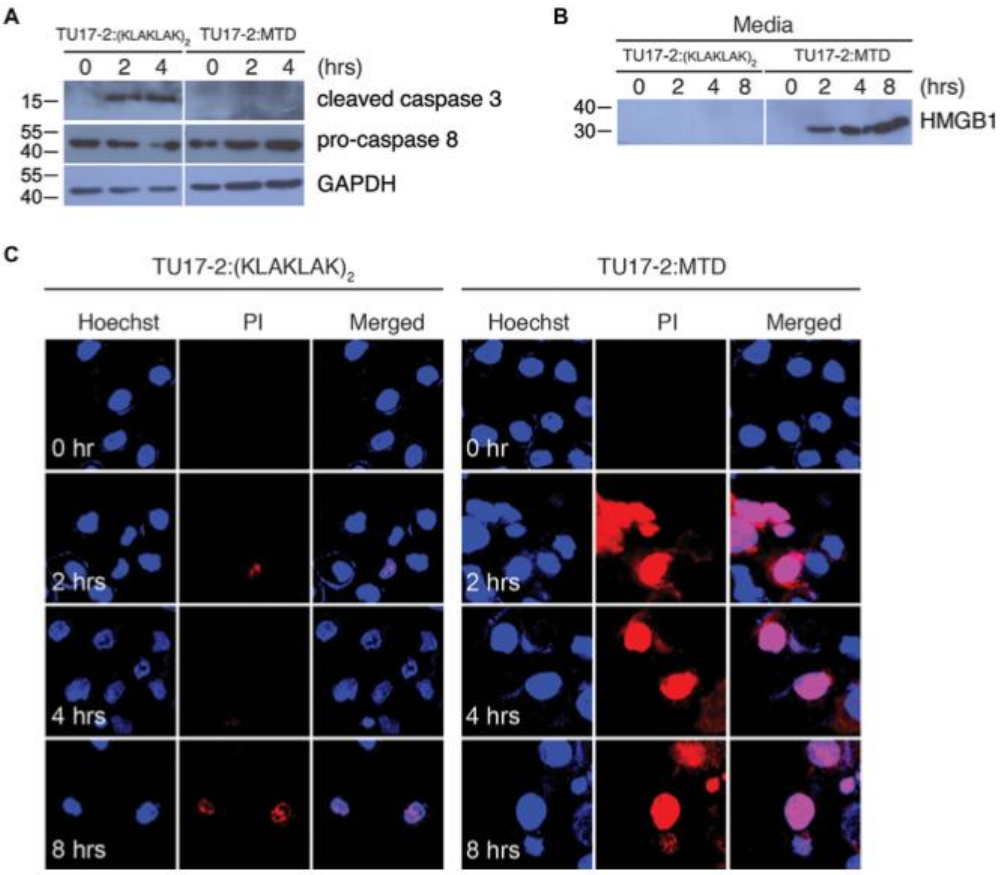

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Figure 4-2. TU17-2:MTD induces necrosis, whereas TU17-2:D(KLAKLAK)2 induces apoptosis. (A) Lysates of CT26 cells treated with TU17-2:D(KLAKLAK)2 (80 μM) or TU17-2:MTD (20 μM) were subjected to western blot with anti-caspase-3 (1:1000), antipro-caspase-8 (1:500) or anti-GAPDH (1:2000) antibodies. (B) Culture media from cells treated with TU17-2:D(KLAKLAK)2 (80 μM) or TU17-2:MTD (20 μM) were precipitated with TCA. Samples were subjected to western blot analysis by using an anti-HMGB1 (1:1000) antibody. (C) CT26 cells were treated with TU17-2:D(KLAKLAK)2 (80 μM) or TU17-2:MTD (20 μM), and were stained with Hoechst/PI.

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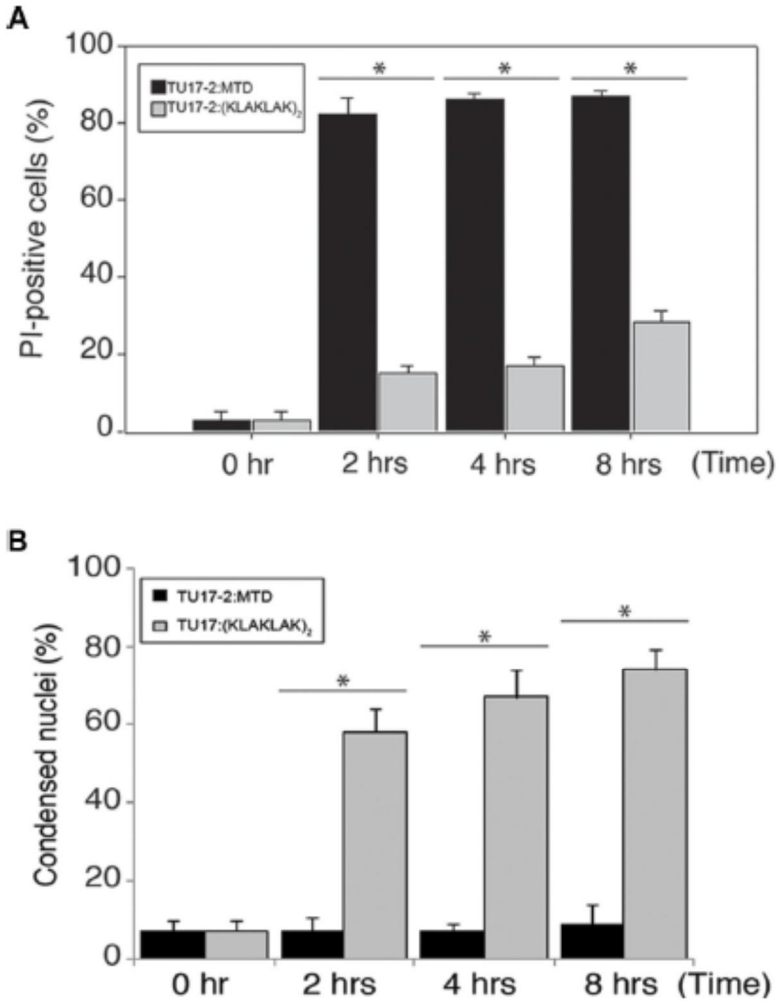

Figure 4-3. TU17-2:MTD and TU17-2:(KLAKLAK)2 cause necrosis and apoptosis, respectively, in CT26 cells. CT26 cells were treated with TU17-2:(KLAKLAK)2 or TU17-2:MTD, and were stained with Hoechst/PI. Images were obtained using an Olympus confocal microscope. PI-positive cells (A) or cells with condensed nuclei (B) were counted (n = over 200 cells). *$P < 0.05$.

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4.4.3. TU17:MTD regresses tumor volume in mice

To test the efficacy of TU17:MTD in tumor killing in vivo, I first generated subcutaneous CT26 cell tumors in BALB/c mice and started treatment when the tumors reached a palpable size. TU17:MTD or TU17-2:MTD (100 μl of 1 mM peptides/mouse/day) was intravenously administrated into the mice via tail vein. Tumor growth was greatly suppressed within 9 days, whereas control animals, which were given saline, exhibited continuous tumor growth (Figure 4-4A). In contrast, the administration of TU17:ΔMTD or TU17-2:ΔMTD (tumor-homing motif without MTD) or TU17:MTD4A resulted in progressive tumor growth (Figure 4-4A), showing that TU17:MTD and TU17-2:MTD showed comparable anti-tumor activity. In addition, I tested the extent of tumor volume reduction in response to TU17-2:MTD or TU17-2:D(KLAKLAK)2. TU17-2:MTD induced a significant reduction of the tumor volume, whereas TU17-2:D(KLAKLAK)2 showed no reduction of tumor volume, similar to the results obtained with treatment of TU17:MTD4A or PBS (Figure 4-4B). These results suggest that the necrosis-inducing activity of MTD, instead of apoptosis-inducing agents, may present a beneficial potential in anti-cancer drug development. To further investigate the changes in tumor tissues after the administration of TU17:MTD, tumor tissues were stained with H&E at 1, 2, and 15 days after TU17:MTD treatment was initiated. While tumor tissues in control mice were densely filled with tumor cells, massive necrotic cell death was observed in the tumor tissues of TU17:MTD-treated mice (Figure 4-4A). A significant reduction in tumor volume was evident at day 1 and 2 after injection. At day 15, tumors had greatly reduced in TU17:MTD-treated mice, but remained very large in control mice (Figure 4-5A).

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To evaluate the tumor-killing effects of TU17:MTD and TU17-2:MTD on tumor in vivo over extended time periods, I treated the animals with the peptides for up to 8 days after tumor was generated as shown in Figure 4-4. After completing the treatment, I observed for 30 days to assess whether the tumors would relapse or not. As expected, the tumors were regressed while the peptides were injected (total animal number of each group = 7). Upon completing the treatments, some mice (approximately 28%) presented a complete regression of the tumors, whereas others (approximately 71%) showed relapsed tumor growth (Figure 4-5B).

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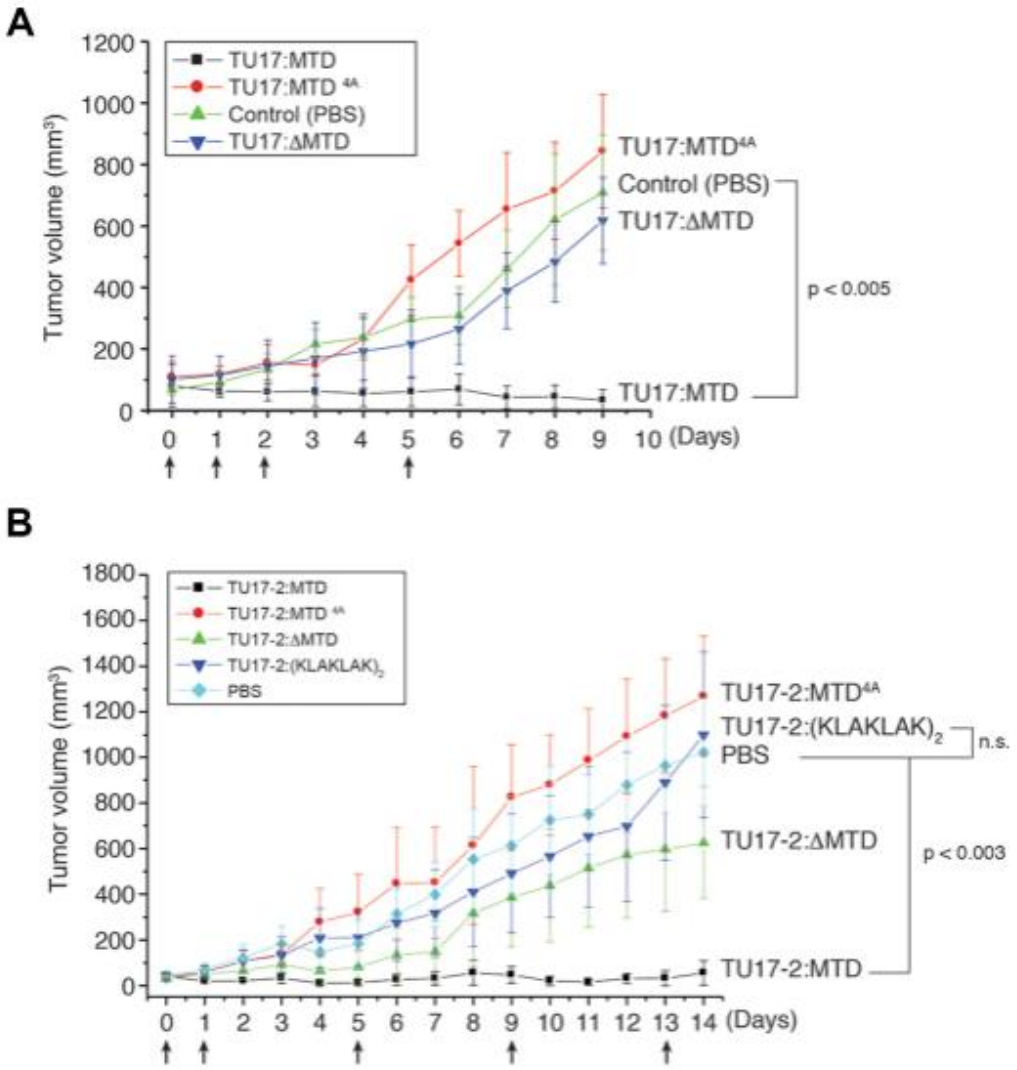

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Figure 4-4. Effects of TU17:MTD, TU17-2:MTD, and TU17-2:D(KLAKLAK)2 on tumor tissues in vivo. (A) Tumors were generated over 7~8 days after s.c. injection of CT26 cells into the backs of BALB/c mice. TU17:MTD, TU17:MTD4A, TU17:ΔMTD (200 μg/mouse/day, total volume: 100 μl) or PBS (100 μl) were administrated i.v. once daily as indicated (arrows). Tumor dimensions in mice from the TU17:MTD-treated group (n = 10), PBS-treated group (n = 10), TU17:MTD4A-treated group (n = 10), and TU17:ΔMTDtreated group (n = 10) were measured using a caliper and tumor volumes were calculated. Initial tumor size at day 0 was 70 ± 20 mm3. Arrows indicate the points of peptide injection. $P < 0.005$ (PBS vs TU17:MTD) (B) Mice bearing CT26 tumors were i.v. administrated with TU17-2:MTD (n = 6), TU17-2:D(KLAKLAK)2 (n = 6), TU17-2:MTD4A (n = 6), TU17-2:ΔMTD (n = 6), or PBS (n = 6) once daily as indicated (arrows). Initial tumor size at day 0 was 40 ± 10 mm3. Arrows indicate the points of peptide injection. $P < 0.003$ (PBS vs TU172:MTD), n.s. (non-significant).

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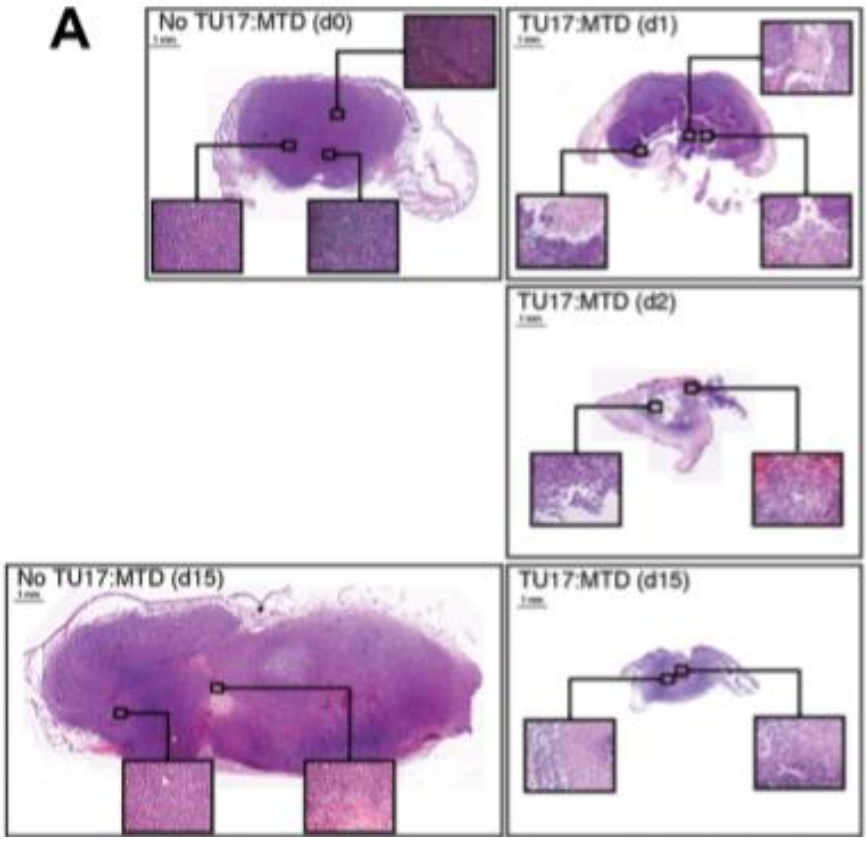

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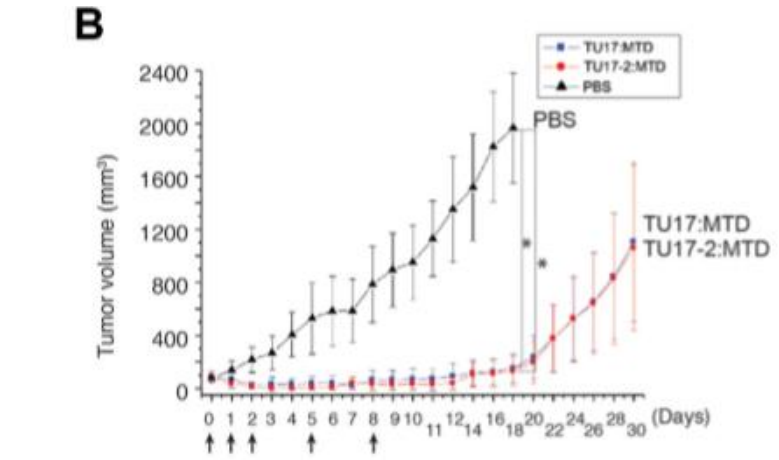

	Regressed	Non-responded	Replased
PBS (n=7)	0	7 (total)	-
TU17:MTD (n=7)	28.6% (n=2)	-	71.4% (n=5)
TU17-2:MTD (n=7)	28.6% (n=2)	-	71.4% (n=5)

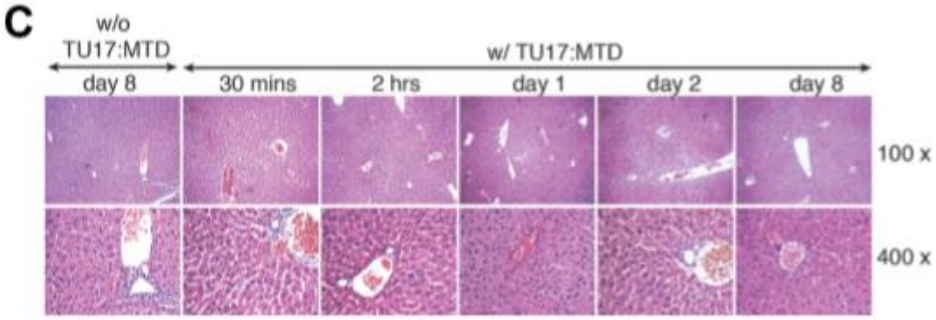

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Figure 4-5. TU17:MTD induces necrosis in tumor tissues. (A) Tumor tissues were obtained at day 1 (d1) post-TU17:MTD injection (a single injection), and on day 2 (d2) and day 15 (d15) from TU17:MTD-treated group. Tumor tissues were obtained at day 0 (d0) or day 15 (d15) from the PBS-treated group. Images of H&E stained sections (Magnification, 40 ×) covering the whole tumor tissues were taken under a light microscope, and photo-merged using Adobe Photoshop CS5. Inset magnification 200 ×. (B) Tumors were generated as described above using CT26 cells. TU17:MTD, TU17-2:MTD (200 μg/mouse/day, total volume: 100 μl) or PBS (100 μl) were administrated i.v. into the mice once daily as indicated (arrows). Tumor volumes in mice from the TU17:MTD-treated group (n = 7), TU17-2:MTD group (n = 7), or PBS-treated group (n = 7) were measured for 30 days. Initial tumor size at day 0 was 70 ± 20 mm3. *P values < 0.005 (PBS vs TU17:MTD or TU17-2:MTD at each) (C) Liver tissues, obtained from the TU17:MTD-treated group at the indicated time points or from the PBS-treated group at day 0 as a control, were stained with H&E, and images (100 ×, 400 ×) were taken under a light microscope.

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4.4.4. Anti-NRP-1 antibody blocks the entrance of TU17:MTD into CT26 cells and cell death induced by TU17:MTD

To identify the detailed mechanism by which TU17:MTD can specifically target the tumor cells, I first tested whether TU17:MTD conjugated with FAM at its C-terminal end (TU17:MTD-FAM) or TU17mut:MTDFAM, in which RPARPAR of TU17 is replaced by APAAPAA in TU17:MTD-FAM, can penetrate into tumor cells in vitro. The penetration of TU17:MTD-FAM into CT26 cells or HeLa cells was initially observed within 6 ~ 12 minutes of the peptide treatment. Thirty minutes after the treatment, TU17:MTD-FAM was observed within most of the CT26 cells and HeLa cells (Figure 4-6A and Figure 4-7A, respectively); however, TU17mut:MTD-FAM could not penetrate into CT26 cells and HeLa cells (Figure 4-6 C-D and Figure 4-7 A-C, respectively). The targeting specificity of TU17:MTD to tumor cells is likely to be determined by the "RPARPAR" sequence that is previously known to bind to NRP-1, a co-receptor for vascular endothelial growth factor (VEGF), expressed in tumor vessels and in most carcinomas [166-168]. Thus, I tested whether an antiNRP-1 antibody could inhibit the tumor targeting and killing activity of TU17:MTD or not. Cell death that was induced by TU17:MTD or TU17-2:MTD alone in CT26 cells was inhibited by the anti-NRP-1 antibody; however, R8:MTD-induced cell death could occurred irrespective of the presence of the anti-NRP-1 antibody. Moreover, TU17:ΔMTD could not induce the cell death irrespective of the presence of the anti-NRP-1 antibody (Figure 4-6B). Furthermore, the anti-NRP-1 antibody inhibited

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the penetration of TU17:MTD-FAM into CT26 cells and HeLa cells (Figure 4-6C and Figure 4-7B, respectively) in vitro, and the number of FAM-positive cells was substantially decreased by the anti-NRP-1 antibody in CT26 cells (Figure 4-6D) and HeLa cells (Figure 4-7C). These results indicate that TU17:MTD penetrates into tumor cells through NRP-1 and its killing activity depends on the penetration of cell membrane.

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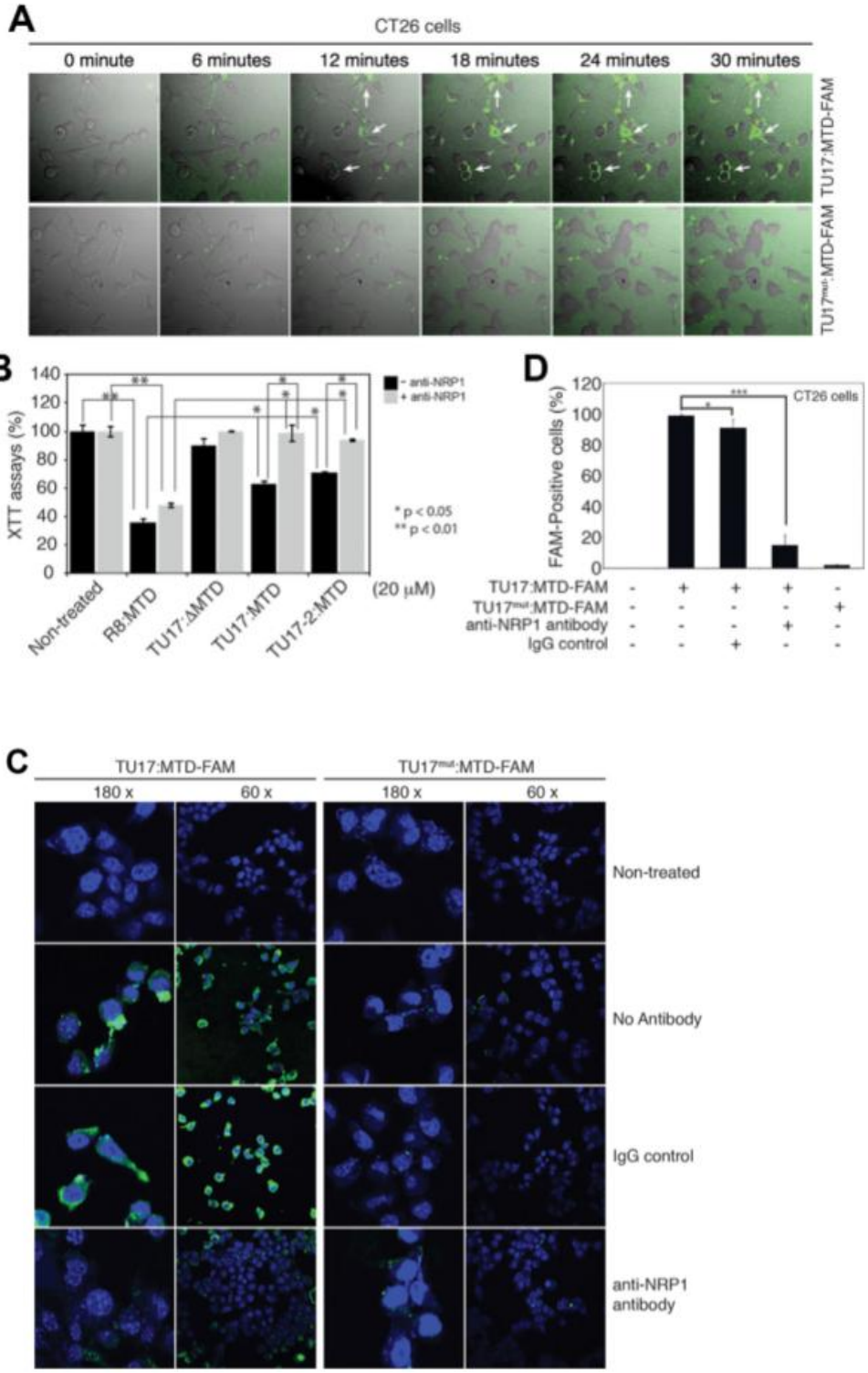

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Figure 4-6. Anti-NRP-1 antibody blocks TU17:MTD-induced necrosis. (A) CT26 cells (2 × 104 cells/cm2) were treated with TU17:MTD-FAM (500 nM) peptide or TU17mut:MTD (500 nM) peptide. Live cell images (magnification 60 ×) were obtained at 6-minute intervals for 30 minutes using an Olympus confocal microscope. (B) CT26 cells were treated with the indicated MTD peptides in the presence or absence of anti-NRP-1 antibody (1:100 dilution, pretreatment) for 1 hour. The viability was measured by using the XTT assay. *P < 0.05, **P < 0.01. (C) CT26 cells were treated with TU17:MTD-FAM or TU17mut:MTD-FAM in the presence or absence of the anti-NRP-1 antibody (1:100 dilution, pretreatment). At two hours after treatment, the samples were counterstained with the ProLong Gold containing DAPI, and were observed using Olympus confocal microscope. (D) CT26 cells (2 × 104 cells/cm2) were treated with TU17:MTD-FAM (500 nM) peptide or TU17mut:MTD (500 nM) peptide in the presence or absence of anti-NRP-1 antibody or IgG control as described in Figure 5C. Images were captured at 30 minutes after peptide treatment by using an Olympus confocal microscope. The percentage of FAM-positive cells (%) were determined (n = over 200 cells). *P < 0.05, ***P < 0.001.

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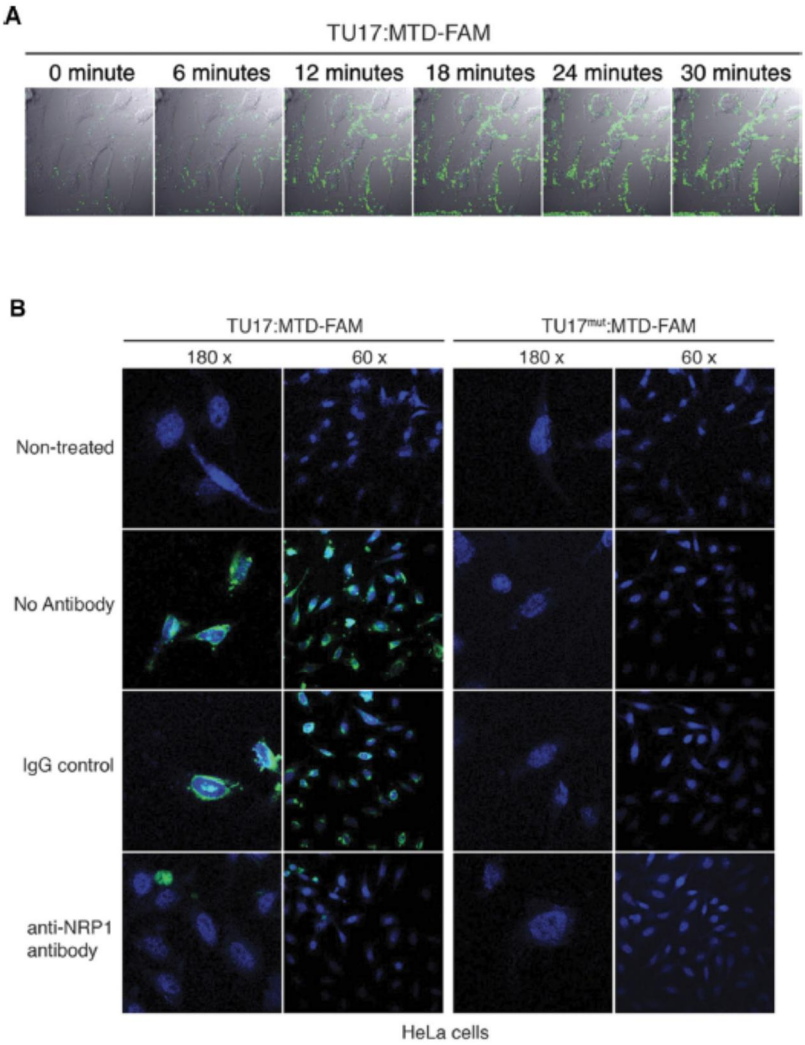

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Figure 4-7: Anti-NRP-1 antibody blocks the entry of TU17:MTD-FAM into tumor cells. (A) HeLa cells (2 × 104 cells/cm2) were treated with TU17:MTD-FAM (500 nM) peptide. Live cell images (magnification 60 ×) were obtained at 6-minute intervals for 30 minutes using an Olympus confocal microscope. (B) HeLa cells were treated with peptides as mentioned in Figure 5C. The images were captured at 30 minutes after treatment with Olympus confocal microscope. (C) The experiments were performed as described in Figure 5C. The percentage of FAM-positive cells (%) were determined (n = over 200 cells). *P < 0.05, ***P < 0.001

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4.4.5. Anti-NRP-1 antibody blocks TU17:MTD induced tumor regression

To further examine the targeting of TU17:MTD to tumor cells in vivo, TU17:MTD-FAM were injected into mice bearing tumor. Within 1 ~ 2 hours of treatment, the penetration of TU17:MTD-FAM was observed mainly in tumor cells but sparsely in the liver or kidney cells in vivo, whereas TU17mut:MTD-FAM was sparsely observed in tumor, liver or kidney cells (Figure 4-8A). Furthermore, the tumor targeting of TU17:MTD-FAM was blocked by TU17:ΔMTD (RPARPAR alone without MTD) in vivo (Figure 4-9A). TU17:ΔMTD will compete with TU17:MTD-FAM for NRP-1 targeting, resulting in reduction of TU17:MTD-FAM targeting to NRP-1 by TU17:ΔMTD. Indeed, total radiant efficiency measured by optical imaging in tumor tissues treated with TU17:MTD-FAM alone was greatly reduced by co-treatment with TU17:ΔMTD, although the fluorescence intensity in the blood of mice treated with TU17:MTDFAM alone was compatible with that in the blood of mice treated with TU17:MTD-FAM and TU17:ΔMTD (Figure 4-9B). These results indicate that the targeting specificity and penetration ability of TU17:MTD into tumor cells in vivo depend on the sequence of TU17 part but not MTD part. The killing activity of TU17:MTD or TU17-2:MTD on tumor tissues was substantially inhibited when the antiNRP-1 antibody was pre-injected into tumor-bearing mice in vivo (Figure 4-8B), and the extent to which TU17:MTD or TU17-2:MTD injection reduced tumor volume at day 1 was significantly decreased (Figure 4-8C). Together, these results indicate that TU17:MTD and TU17-2:MTD target, penetrate, and kill the tumor cells by targeting to NRP-1 on tumor cells.

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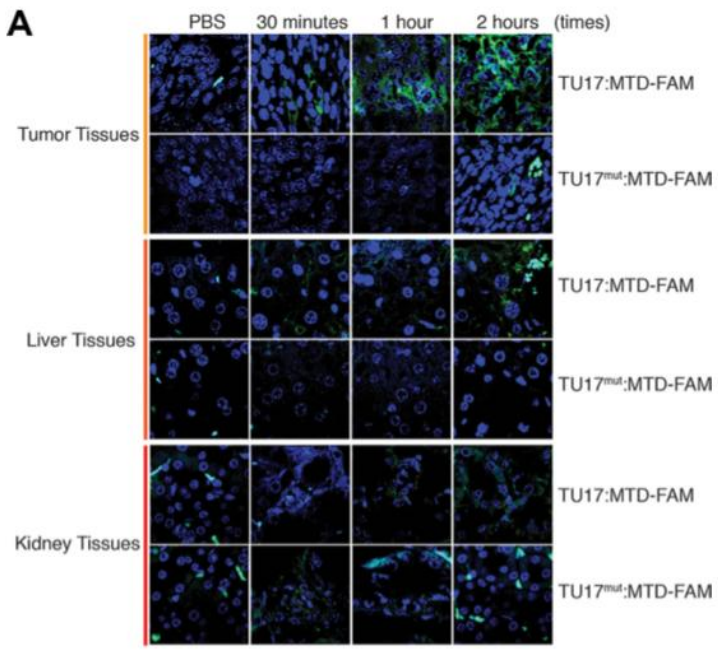

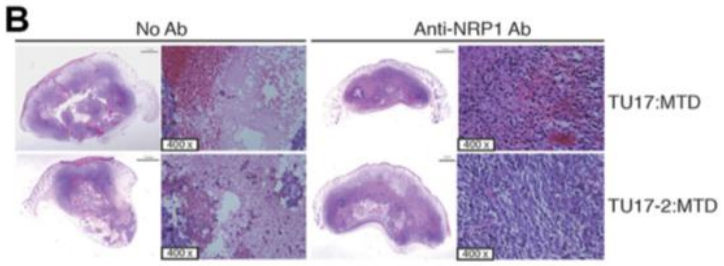

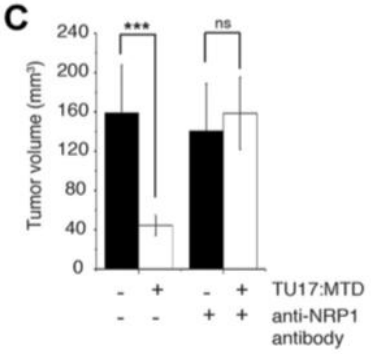

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Figure 4-8. Anti-NRP-1 antibody blocks necrosis in tumor induced by TU17:MTD. (A) TU17:MTD-FAM, TU17mut:MTD-FAM (100 μl of 1 mM), or PBS was i.v. injected into the mice bearing tumors generated with CT26 cells. Mice were sacrificed at 30 minutes, 1 hour, and 2 hours after injection. Tissues from the tumor, liver, and kidney were processed for paraffin remove with Histoclear solution. The sections were counterstained with ProLong Gold containing DAPI. Images (magnification 400 ×) were obtained using Olympus confocal microscope. (B) The anti-NRP-1 antibody (1:100, n = 5) or PBS (n = 5) was i.v. injected into the mice bearing the tumor at 2 hours before TU17:MTD administration. Mice were sacrificed at day 1 after treatments. The samples were stained with H&E. (C) The mice bearing CT26 cell tumor were i.v. injected with an anti-NRP-1 antibody (1:100, n = 5) or PBS (n = 5) at 2 hours before TU17:MTD administration. Mice were sacrificed at 1 day after treatments. Tumor volumes were measured. ***P < 0.001, ns (non-significant).

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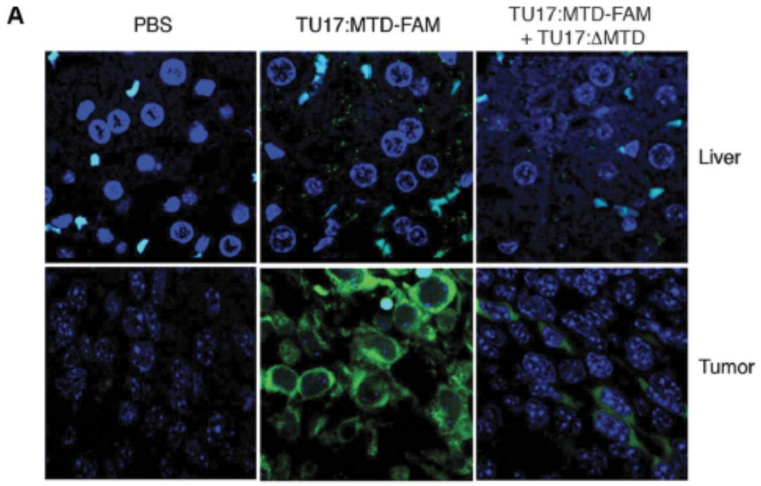

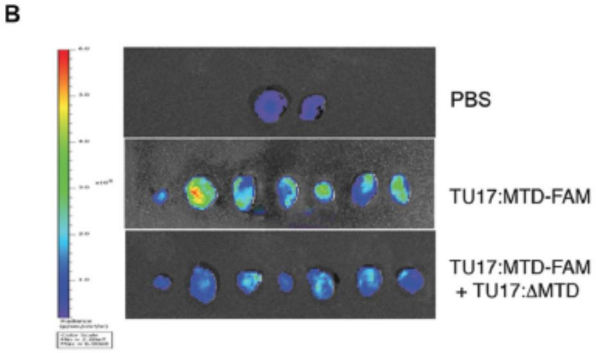

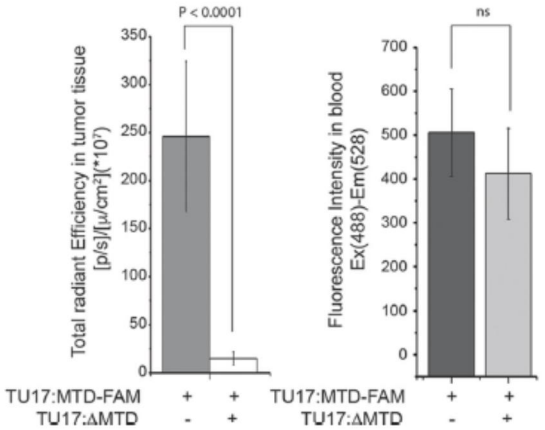

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Figure 4-9: Targeting of TU17:MTD to tumor tissue. (A) PBS alone (n = 2), TU17:MTD-FAM (100 μl of 1 mM, n = 7), or TU17:MTD-FAM plus TU17:ΔMTD (100 μl of 1 mM TU17:MTD-FAM plus 100 μl of 1 mM TU17: ΔMTD, n = 7) were i.v. injected into mice bearing CT26 tumors. At one hour after injection, the mice were sacrificed, and tumor tissues (magnification 400 ×) were processed to detect fluorescence using a confocal microscope after counterstaining with DAPI.(B) BALB/c mice bearing tumor were i.v. injected with PBS alone (n = 2), TU17:MTD-FAM (100 μl of 1 mM, n = 7), or TU17:MTD-FAM plus TU17:ΔMTD (100 μl of 1 mM TU17:MTD-FAM plus 100 μl of 1 mM TU17: ΔMTD, n = 7). At one hour after injection, the mice were sacrificed to obtain tumor tissues. The fluorescence intensities of tumor tissues were measured by Xenogen IVIS 200 imaging system, and were quantified by using a living imaging software (Caliper Life Sciences, Hopkinton, MA, United States). Blood from mice injected TU17:MTD-FAM (n = 7), or TU17:MTD-FAM plus TU17:ΔMTD (n = 7) as described above was harvested, and the fluorescence intensities were measured using Sunrise microplate reader (Tecan, Mannedorf, Switzerland). NS (non-signficant).

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4.5. DISCUSSION

Strategically, the action of peptide-based anti-cancer drugs depends on two functional motifs, a tumor-homing motif and a tumor-cell killing motif. If the tumor-cell killing motif is functional inside of the cells, the tumor-homing motif should satisfy both tumor specific targeting and penetration of cargo through the cell membranes of tumor cells. Although several studies have identified tumor-homing motifs that satisfy these conditions, tumor-homing motifs conjugated with a pro-apoptotic peptide, for example, D(KLAKLAK)2 [163] as shown in Figure 3, or with a chemotherapeutic agent [169-172] have shown limited anti-tumor efficacy, possibly due to the poor killing activities of pro-apoptotic motifs, short circulating half-life, and/or tumor cell resistance. The serum stability of peptide-based drugs without modifications are, in general, less than 1 hour [173-176]. For instance, the half-life of glucagon-like protein-1 (GLP-1) is less than 5 minutes because dipeptidyl peptidase-4 (DPP-4) rapidly inactivates GLP-1 peptide in the circulation [173, 177]. Thus, the increased half-life of peptide-based drug in the circulation is necessary to achieve the sufficient biological efficacy of the peptide in vivo. In addition, most pro-apoptotic molecules need at least several hours or even several days to induce apoptosis. Therefore, long-lasting peptide-based pro-apoptotic anti-cancer drugs are required to provide sufficient anti-cancer efficacy. Indeed, recent several reports showed that a long-lasting peptide conjugated to paclitaxel has the greatly improved efficacy on tumor growth

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[175, 178]. Thus, many efforts strived to design and search for the long-lasting peptide by modifying amino acid residues with unnatural residues or by conjugating AG10, fatty acids or albumin [179-182]. In view of this perspective, TU17:MTD appears to be advantageous in developing anti-cancer drug because TU17:MTD is able to kill tumor cells in tumor tissues within 30 minutes after treatment in vivo, which indicates that its serum stability of TU17:MTD may not be a limiting factor. Whether pro-necrotic anti-cancer peptide can induce cell death in tumor cells that are refractory to chemotherapeutic agents and/or targeted cancer drugs is an interesting question to pursue in future. I speculate that the pro-necrotic anti-cancer peptide could kill the refractory tumor cells, based on the finding that R8:MTD killed the tumor cells that are resistant to apoptosis inducing agents (data not shown). Thus, the pro-necrotic anti-cancer peptide would be an option to overcome the refractory tumor cells after treatment of apoptosis inducing anti-cancer drug. It is not clear at this point how TU17:MTD targets to NRP-1. RPAPRAR should be fused to C-term of cargo molecules based on C-end Rule described by Rouslahti's group [168]. However, the facts that the penetration and killing activity of TU17:MTD on tumor cells can be blocked by anti-NRP-1 antibody (Figures 4-6 and 4-8) indicate that TU17:MTD targets or possibly binds to NRP-1. If TU17:MTD binds to NRP-1, it could be suspected that a free arginine residue at N-term of RPARPAR behaves like a free C-term of arginine residue of C-end Rule. This possibility could be further investigated in future. Moreover, TU18:MTD

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(KLLNLISKLFGGRPARPAR) has the C-end Rule configuration in that MTD is conjugated to the N-term of RPARPAR, providing a free C-term of the arginine residue; however, TU18:MTD did show no or a little suppressive activity on tumor growth (Figure 4-1B). Another concern on TU18:MTD is that conjugation of additional amino acids at C-term of MTD reduces the killing activity of MTD. It might be the reason why TU18:MTD showed the weak suppressive activity on tumor growth compared to that of TU17:MTD. In summary, the results of the present study suggest that pro-necrotic peptides have considerable therapeutic potential as anti-cancer drugs. I hope that the MTD based pro-necrotic peptide represents a new platform for the development of cancer-targeting peptide drugs by combining tumor-homing motifs specific for cancer with the pro-necrotic MTD.

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Chapter5

CONCLUSION

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In this study, I designed MTD fused peptides to develop cancer specific anticancer agents. I confirmed that MTD leads to necrosis through mitochondria swelling when it is present in cytosol. I demonstrated that the MTD fusion peptides can induce necrosis only specific targeted cells. Also, these results indicate that combination of various target sequences with MTD can be used for investigation of new drugs such as elimination of intima hyperplasia of esophagus, blood vessels, and urethra as well as anti-cancer drugs. The results in this study indicate that MTD is effective for malignant cancer that is resistant to conventional anticancer drugs. Although the experiments of MTD fusion peptides have been successful in killing specific target cells, there are many hurdles such as high toxicity to be developed an anticancer drug High toxicity of MTD-fusion peptides seems to be no cancer-specific marker proteins yet. If cancer specific markers are found, MTD can be developed as a next-generation anticancer drug that overcomes the drawbacks of conventional anti-cancer therapeutics. In addition, it can be developed as a new immuno-therapeutic drug that targets immune suppression cells rather than cancer cells. It can complement the disadvantages of immunotherapy using antibodies such as Ipilimumab (Yervoy) and Pembrolizumab (keytruda), because peptides have the advantage of easy to transform for increasing activity and half-life.

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