Determination of Optimum Concentration of NGR Peptide With Anticancer Effect On Breast Cancer Microtissue

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***Abstract*—Breast cancer is a fatal disease, and it is one of the most common cancer types among women in the world. The traditional monolayer methods are used to treat diseases. However, the method is limited in terms of the cell to cell communication and responses of cells to drugs. One of the main goals of cancer treatments is to prevent tumor metastasis and prevent diffusion in various areas of the body, thereby it is needed to increase the effectiveness of the treatments and reduce side effects. Peptides can be used in cancer treatment. Most peptide studies are performed in monolayer culture. These cultures can not accurately represent the complex intercellular and intracellular environment in clinical studies. Peptide studies must be performed in scaffold-free conditions to mimic the natural responses of cells. The study has been performed as scaffold-free microtissues with different NGR peptide concentrations. Results have been evaluated in terms of diameters, and viability of microtissues. It is concluded that 2 mM NGR is the most effective concentration in MCF-7 microtissue treatment.**

Keywords—NGR Peptide, Breast Cancer Treatment, Scaffold Free Microtissues

I. *INTRODUCTION*

Cancer is a deadly disease, and it occurs because of the uncontrolled cell division and proliferation. Breast cancer is the most prevalent malignancy among women, which accounts for 7-10% of tumors occurring throughout the body and seriously threatening the patient's physical and psychological health [1]. Because of its large impact on the population, it represents a serious health problem that requires more research to describe the prognosis and treatment [2].

Cancer cells have signal systems that allow them to multiply uncontrollably. They continue to divide and multiply even when they touch other cells. They can influence their environment and create new vascular systems to get the necessary nutrients and oxygen.

2 Dimensional (D) cell cultures are often used in cancer researches. However, 2D cultures have many disadvantages. These disadvantages can be listed as the alteration of cellular morphology, and restriction of interactions between the cancer microenvironment and cells. 3D cultures developed to overcome these problems and provide biological responses similar to *in vivo* conditions.

Breast cancer is a common malignant tumor, and it is mostly treated with combined treatment methods such as surgery, chemotherapy, radiotherapy, hormone therapy , and molecular targeting therapy[3–5]. Although the drugs are used to treat breast cancer vary, cancer cells become resistant. Moreover, many drugs have serious side effects [6]. As a result, researching a drug with a good therapeutic effect, fewer side effects, and an effective inhibitory effect on breast cancer formation and development has become one of the most important issues[7].

Small size peptides are frequently used in the biomedical field due to their easy synthesis and modification. It has many advantages due to its ability to penetrate the tumor and its high biocompatibility with healthy tissues. Therefore, it has started to be used as promising treatment agents in cancer treatment. As it can connect to different recipients and is a part of various biochemical pathways, it acts as a diagnostic tool in cancer progression.

Peptides can be used in different ways to treat cancer [8–11]. They can be used as drugs such as angiogenesis inhibitors, tumor-targeting agents which carry cytotoxic drugs in targeted chemotherapy, and radiation therapy, hormones and vaccines.

There are number of proteins that are not expressed or barely expressed in normal blood vessels, but are upregulated in the angiogenic vessel endothelial cells, and tumor cells. One of the most important ones is the Aminopeptidase N (APN) /

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CD13 [12]. The CD13 isoform that is expressed in tumor blood vessels can act as a receptor for the Asn-Gly-Arg peptide (NGR). NGR peptide is an (APN)/CD13 binding peptide, and it is one of the first generation homing peptides. It consists of the amino acid sequence of Asparagin-Glycine-Arginine. Due to their neovasculature homing properties, NGR peptides were used by numerous investigators as ligands to deliver different compounds to tumors. Especially, peptides containing GNGRG, CNGRC, CVLNGR- MEC, acetylated-CNGRC and NGRAHA were used for delivering chemotherapeutic drugs, liposomes, cytokines, anti-angiogenic compounds, and DNA complexes to CD13-positive tumor neovasculature [13].

Findings of the previous cancer studies explain the selectivity and tumor homing property of the NGR peptide and have considerable effects in the development of NGR / CD13 system based vascular targeted treatments.

Previous studies have used NGR to increase the efficiency of other molecules. However, no study on the effectiveness of the NGR peptide alone on breast cancer microtissue has been identified. For this reason, determining the most effective concentration of the single uncomplicated NGR peptide on breast cancer microtissue was the aim of this study.

MATERIAL AND METHOD

A. Peptide Synthesis

The CNGRC amino acid sequence was synthesized by solid-phase peptide synthesis using RinkAmideNovaGel resin. During the synthesis step, the amino acids were added respectively to theCysteine-Asparagine-Glycine-Arginine-Cysteine. Kaiser test was used to determine whether the amino acids were attached or not. After the last amino acid was attached, cleavage was done to separate the resin from the synthesized peptide, and the peptide was dried in freeze dryer.

B. Cell Culture

MCF-7 cell line with passage numbers between 20-23 was used. In 2D cell culture, MCF-7 cells were incubated with Dulbecco's Modified Eagle Medium (DMEM, D2429, Sigma, St. Louis, Missouri, USA),which includes %10 Fetal Bovine Serum (FBS), %1 L-Glu and %1 Penicilin-Streptomycin. Agarose gel was created using commercial 3D petri dish to create cancer microtissues. After agarose gel incubation, $1x10^5$ MCF-7 cells were seeded. Approximately 500 µl medium was added to each cell as serum-free. 0 mM, 1 mM, 2 mM, and 4 mM of NGR peptide have been incubatedat 36.5°C and 5% CO_2.

C. Microtissue Size and Cell Viability Analysis

Microscope images were taken using by using Olympus brand fluorescent microscope. Image-J software (NIH) was used for diameter size analysis of cancer microtissues. On day 7, a Double Staining Kit (Dojindo, Molecular Technologies, Inc, Japan) was used to display the viable cell rate or dead cell rate in microtissues. Both live and dead cell micrographs were taken and merged by using CellSense Entry software.

II. RESULTS & DISCUSSIONS

The NGR peptide was synthesized by using the solid-phase peptide synthesis method. Their effectiveness at 0 mM, 1 mM, 2 mM, and 4 mM have been determined. Dimensional and viability analyzes of microtissues have been tested.

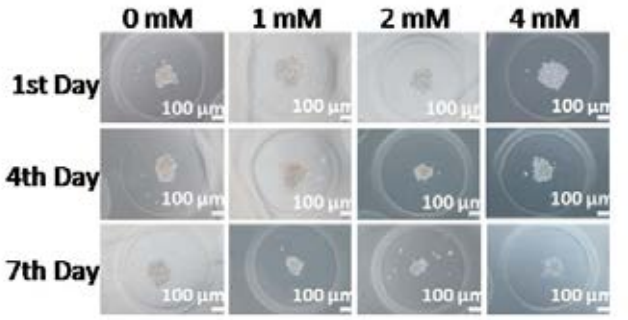

Fig. 1. *Effect of NGR on MCF-7 microtissueenlargement*

In line with the information obtained from the diameter analysis, on day 1, all microtissues were successfully formed. The size of the microtissues did not show a significant difference at 0 mM NGR concentration.However, a noticeable reduction in microtissue diameter was observed at 1 mM NGR concentration. The reduction in microtissue diameter was more recognizable at a concentration of 2 mM NGR compared to a concentration of 1 mM NGR. The most visible reduction in microtissue diameters was observed at a concentration of 2 mM NGR. While a decrease in diameter was observed in all NGR concentrations, the most significant size reduction was observed at 2 mM NGR.

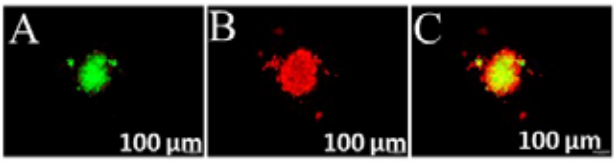

Fig. 2. *The viability of MCF-7 microtissues at 2mM NGR concentration*

Intensity ratios of green and red colors give information about vitality. The intense red color means that the number of dead cells is high. The opposite is also true. If the green color is more intense, it means that the number of living cells is high.

In line with the observations from the live&dead assay, in the 0 mM NGR group, the green color was more intense compared to the other groups, which means that the the highest number of living cells is in the 0 mM NGR group. The ratio between the intensities of colors has become less noticeable in the 1 mM NGR group. Based on this, it can be concluded that the number of dead cells and living cells are closer to each other than the 0 mM NGR group in the 1 mM NGR group. In the 2 mM NGR group, red color is more intense

than other groups. This indicates that the group with the highest number of dead cells is the 2 mM NGR group. At the 4 mM NGR concentration, the difference between the red and green color intensities was less noticeable than 2 mM, which indicates that the dead cell ratio is decreased when compared to 2 mM NGR.

Considering these results, it can be said that the most effective concentration was 2 mM which supports the result obtained in the dimensional analysis. With the NGR peptide used in the treatment, it was observed that the antitumor effect on breast cancer microtissue can be achieved.

The effect of the NGR peptide in cancer treatment has already been proven using monolayer cultures. Investigation of the anticancer effect of NGR peptide sequence under the scope of this study will be a valuable resource for NGR peptide, MCF-7 breast cancer cell line, and cancer-related research and will contribute significantly to future studies.

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