Laminin Türevli Peptid Sekansı ile Modifiye Edilmiş Nanolif Tabanlı Mikrotüplerin Endotel Hücre Tutunmasına ve Çoğalmasına Etkisinin Belirlenmesi

Determination of Endothelial Cell Attachment and Proliferation on Nanofiber Based Microtubes Modified with Laminin-Derived Peptide

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***Özetçe*—Günümüzde doku mühendisliği çalışmalarında en sık yaşanan sorun hücrelere yetersiz besin ve oksijen taşınımından dolayı hücrelerin apoptoza uğramalıdır. Bu sorunun en büyük nedenlerinden biri de üretilen doku ve/veya organ yapılarındaki yetersiz damarlanmadır. Son günlerde, vaskülojenez ile damarlanma sorununu ortadan kaldırmak hedeflenmektedir. Bu çalışmada laminin-türevli IKVAV peptit sekansı ile modifiye edilmiş PLGA (Poli(laktik asit-ko-glikolik asit)) nanoliflerinden oluşan mikrotüplerin statik ve dinamik hücre kültürü ile hücre tutunumu ve çoğalması üzerindeki etkisi araştırılmaktadır. Bu amaçla elektro-eğirme yöntemi kullanılarak PLGA nanolifleri ile mikrotüpler oluşturulmuş ve IKVAV peptit sekansı PLGA nanoliflerine konjuge edilmiştir. Sonrasında statik ve dinamik ekim yöntemleri ile insan göbek kordonu damar endotel hücresi hücrelerinin (HUVEC) ekimi gerçekleştirilmiştir. Ayrıca ekilen hücreler statik ve dinamik kültür yöntemleri ile kültüre edilmiştir. Sonrasında her bir gruba hücre morfoloji ve MTT analizi uygulanmıştır. Sonuç olarak IKVAV konjuge edilmiş PLGA nanofiberlerinden oluşan mikrotüplerde daha fazla endotel hücre tutunumu, çoğalması ve görülmüştür. Ayrıca, dinamik ekim-dinamik kültür grubunun hücre tutunumu ve çoğalması üzerinde daha etkili olduğu gözlemlenmiştir.**

Anahtar Kelimeler: Vaskülojenez, statik kültür, dinamik kültür, biyoreaktör.

***Abstract*—Nowadays, the most common problem in tissue engineering studies is the apoptosis of cells due to insufficient nutrient and oxygen transport. One of the major reasons for this problem is the inadequate vascularization of tissue and/or organ structures. In this study, the effect of PLGA nanofiber based microtubes modified with laminin-derived IKVAV peptide on endothelial cell attachment and proliferation by static and dynamic cell culture were investigated. Microtubes were fabricated with Poly-lactide-co-glycolide (PLGA) nanofibers by using electrospinning technique. PLGA nanofibers on microtubes were conjugated with IKVAV sequence. Human umbilical vein endothelial cells (HUVEC) were seeded by using static and dynamic cell seeding techniques. Also, they were cultured with static and dynamic culture techniques. For dynamic cell culture experiments, a bioreactor system was developed. Then, morphology analysis and MTT assay were assesed for each group. It was observed that endothelial cell attachment and proliferation were higher on IKVAV conjugated microtubes compared to PLGA nanofiber. Also, it was observed that dynamic seeding-dynamic culture group was more effective on cell attachment and proliferation.**

Keywords: Vasculogenesis, static culture, dynamic culture, bioreactor.

I. INTRODUCTION

Primary roles of blood vessels are transportation of oxygen, metabolites, and nutrient, regulating homeostasis, and elimination of waste products. Also, they provide communication system between distant organs and tissues [1]. Construction of new vascular networks for therapeutic purposes is critical but also hard to understand for tissue engineering. A proper vascular network with anastomoses via host vasculature is prerequisite for viability of regenerating tissues [2].

There are two general processes responsible for new blood vessel development: vasculogenesis and angiogenesis. Formation of new blood vessels in embryo occurs via both vasculogenesis and angiogenesis. Same process occurs mainly through angiogenesis under certain physiological situations in adults [3]. Also, some researches showed that new vessel formation is combination of vasculogenesis and angiogenesis for adults [4]. Vasculogenesis is the new vascular structure formation via endothelial cells differentiated from progenitor

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endothelial cells in a region that was not previously vascular [5]. Hemangiogenic progenitor cells migrate and differentiate into hematopoietic progenitor cells to generate blood cells and into endothelial progenitor cells (EPCs) (angioblastic progenitor cells) to generate blood vessels. Here, growth factors play an important role in directing cell migration [6]. After formation of angioblasts, angioblast aggregation occurs. Angioblasts elongates into cord-like structures and vascular parts occur. The vascular parts become organized into capillary-like networks. Via endothelialisation and lumenization, blood vessels occur [7]. Angiogenesis is known as new blood vessel formation from pre-existing blood vessels. In angiogenesis, proliferative endothelial cells provide new vessel formation instead of angioblasts. [8, 9].

Laminin is the first protein of the ECM (Extracellular Matrix) to appear in embryo. It is essential for early embryonic development and organogenesis. Laminin has capacity for cell binding. It plays a key role in several cellular processes like differentiation, adhesion, and migration. Also, it mediates between cells and the basement membrane [10]. IKVAV (Ile-Lys-Val-Ala-Val) peptide sequence in laminin molecule promotes endothelial cell migration and invasion and encourages vascularization by inducing capillary-like structures in umbilical vein endothelial cells [11].

Biomaterial for scaffold fabrication is very important. The fabricated scaffold must stimulate specific cell response and trigger cell attachment, proliferation, differentiation, and ECM formation. Also, it must be biodegradable and bioresorbable to support new tissue formation without any side effect. The degradation products of the biomaterial should not cause any toxicity and must be removed from the body with metabolic pathway [12, 13]. PLGA is a copolymer of PLA and PGA. It is the most commonly used FDA approved synthetic polymer. Generally, it is used for drug delivery tools, sutures, and tissue engineering scaffolds. There are different PLGA copolymer types with different PLA:PGA ratios. Generally used PLGA copolymer types are 50:50 PLGA, 75:25 PLGA, and 85:15 PLGA [14-16].

Bioreactors are devices that is used to culture cells under monitored and controlled environmental parameters such as pH, temperature, mechanical stress, and biochemical gradients. These devices provide mimicking of native environment via stimulation of cells by chemical, electrical and mechanical signals to encourage cells to produce ECM [17]. They are used for cell proliferation on small and large scale, three-dimensional tissue formations, and direct organ supporting systems. They should allow control of environmental factors such as oxygen level, temperature, and nutrient and waste transfer [18]. The aim of this study is to investigate the effect of IKVAV conjugated PLGA microtubes on cell attachment and proliferation as a result of human umbilical vein endothelial cells (HUVEC) cultivation in static and dynamic environments.

II. MATERIALS AND METHODS

A. Solid Phase Peptide Synthesis

IKVAV(Ile-Lys-Val-Ala-Val) peptide sequence was synthesized manually on AAPPTEC Fmoc-protected Wang resin (0.67 mmol/g loading capacity) [19]. Kaiser test was applied to small amount of the resin solution to understand the presence of unreacted amine groups. After all aminoacid coupling reaction is completed, the obtained peptide was cleaved from resin by using 2.5% triisopropylsilane (TIPS), 2.5% distilled water, and 95% trifluoroacetic acid (TFA). Then, the solution was cast into cold-diethyl ether and precipitated at 20 °C for 24 hr. After that, the suspension was centrifuged, and supernatant part was removed. Finally, the obtained pellet was freeze-dried.

B. Fabrication of PLGA Nanofiber Based Microtubes

3 wt% PLGA (85:15) was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP; Matrix Scientific; Columbia). The prepared polymer solution was electrospun on a special collector which is developed by Izmir Katip Çelebi University Tissue Engineering and Regenerative Medicine Laboratory to obtain PLGA nanofiber based microtubes (Figure 1 and Figure 2). To obtain PLGA nanofibers on circular glass coverslips, the ejected nanofibers were collected by an aluminium rotating wheel, which is powered by a high-speed motor. The rotating wheel was covered with 13 mm circular glass coverslips to obtain nanofibers onto the glasses after electrospinning procedure.

Figure 1. Collector which is developed by Izmir Katip Çelebi University Tissue Engineering and Regenerative Medicine Laboratory.

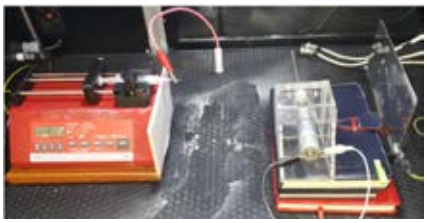

Figure 2. Electrospinning setup.

C. Scanning Electron Microscopy (SEM)

The characterization of PLGA nanofibers for determination of the nanofiber alignment, overall diameter and wall thickness of microtube was done by using scanning electron microscope (SEM; Carl Zeiss Microscopy, Germany) with 3 kV accelerating voltage.

D. Peptide Conjugation of Nanofibers

Nanofibers on circular glass coverslips and microtubes were washed with deionized water. Then, to obtain carboxyl rich surface, they were incubated with 5 mM NHS and 2 mM EDC in 0.1M MES solution for 40 min. Finally, nanofibers on the glasses and microtubes were conjugated with synthesized IKVAV peptide sequence in 1 mM sterile PBS for 24h at 4°C. Each of the conjugated glasses and microtubes were washed

with PBS before they were used. Experimental groups for this study are shown in Table 1.

Table 1. Experimental Groups

Static Seeding-Static Culture (Glass)	PLGA-IKVAV
Static Seeding-Static Culture (Graft)	PLGA-IKVAV
Static Seeding-Dynamic Culture (Graft)	PLGA-IKVAV
Dynamic Seeding-Dynamic Culture (Graft)	PLGA-IKVAV

E. Cell Culture

Human umbilical vein endothelial cells (HUVEC) were cultured with DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (fetal bovine serum), 100 mg / ml streptomycin, 100 U/ml penicillin and 4 ng/ml bFGF in cell culture flasks. It was then cultured in an incubator set to 5% CO_2 and 70-80% humidity at 37 °C. The medium was changed every 2 days, unless an opposite condition occurs for the cell line. When the cells have reached to 80% confluency, cells were passaged.

For static cell seeding-static culture of nanofibers on circular glass coverslips, glasses were placed into 24-well culture plate, one glass for each well. Each sample was seeded with HUVEC suspension. Fresh DMEM medium and also, EGM-2 (Endothelial Cell Growth Medium-2) medium were used to see vascularization during morphology analysis (three glasses for each PLGA and PLGA-IKVAV nanofiber groups). Medium was replaced in every 2 days.

For static cell seeding on microtubes, microtube was placed in a petri dish. HUVEC suspension was pipetted directly into the lumen of the microtube and incubated for 1 hour (Figure 3). After incubation, DMEM medium was added and the cells inside the microtube were incubated in incubator. Growth medium was replaced in every 2 days. For the dynamic culture, custom-made bioreactor system was used (Figure 4). For bioreactor system; two I.V. cannulas (BIÇAKCILAR, B-CAT2), two I.V. infusion sets (G.Z.T® , IS-12F), one 50 mL centrifuge tube, T-shaped tube connector (ISOLAB) and a peristaltic pump (Watson-Marlow, 120S) was used. The cap of the 50 mL centrifuge tube and the bottom end of the tube were drilled. The cannulas were connected to 18 the T-shaped tube connector. The infusion sets were cut from half and obtained pipes were connected to ends of each cannula. Ends of each pipe were connected to the cap and bottom and of the drilled centrifuge tube. Finally, the obtained system was connected to the peristaltic pump.

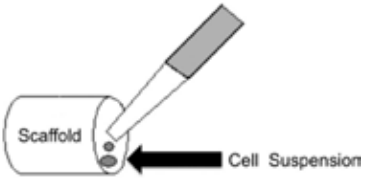

Figure 3. Visualization of cell seeding into lumen by pipetting [20].

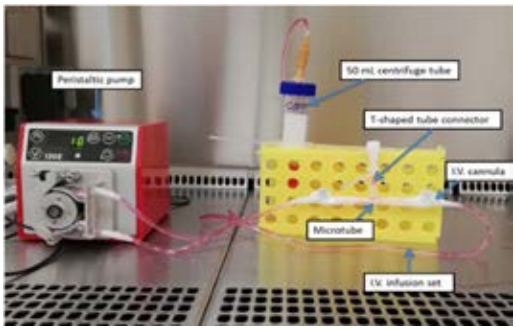

Figure 4. Bioreactor system.

F. MTT Assay and Morphology

MTT assay (Vybrant® MTT Cell Proliferation Assay Kit, Invitrogen, Waltham, MA, USA) was assessed on 1st, 4th and 7th days to evaluate cell proliferation. Briefly, the MTT solution was added on the each well and incubated for 2 hours at %5 CO2 37 °C. After 2 hours, the MTT solution was removed from the cells and 500 µl Dimethyl Sulfoxide (DMSO) was put on the cells. The cell numbers were obtained by absorbance reading at 570 nm by using plate reader.

Immunofluorescent staining was used to determine cell morphology. Since microtube structures are three dimensional, it was difficult to observe the stained cells with fluorescent microscopy. Therefore, immunofluorescent staining was applied only nanofibers on the glasses. Cell seeded nanofibers on glasses were incubated with DAPI and Alexa Fluor® 594 Phalloidin for staining of nucleus of the cells and actin filaments.

III. RESULTS AND DISCUSSION

A. Scanning Electron Microscopy (SEM) Characterization

SEM imaging technique was used for the investigation of alignment of the PLGA nanofibers and wall thickness of the microtubes. Microtubes were succesfully fabricated (Figure 5).

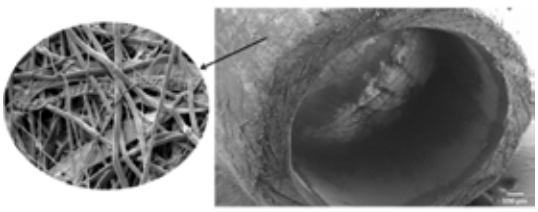

Figure 5. SEM image of fabricated microtube and PLGA nanofibers. Scale bar represents 100µm

B. Morphology

For the determination of cell morphology and vascularization, cytoskeleton and nucleus of the cells were stained with phalloidin and DAPI, respectively. As shown in Figure 7, HUVEC cells attach successfully to the surfaces for two different NF groups. In correlation with the MTT assay results, differences in cell proliferation for different NF groups were also observed. The highest cell density was observed in PLGA-IKVAV group.

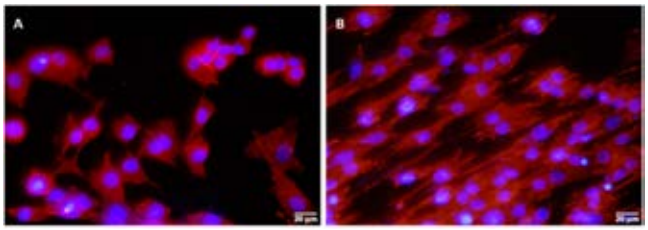

Figure 7. Cell morphology on (A) PLGA and (B) PLGA-IKVAV nanofibers. Phalloidin staining (red for cytoskeleton) and DAPI staining (blue for cell nucleus) images. Scale bar represents 20µm

C. MTT Assay

Cell proliferation and viability was evaluated by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide) assay on 1st, 4th and 7th days after cell seeding. PLGA-IKVAV nanofibers have higher viable cell number and better cell adhesion compared to the PLGA nanofibers on every 1st, 4th and 7th day. Therefore, IKVAV peptide sequence might be considered as an effective peptide sequence for endothelial cell attachment and proliferation.

On the other hand, dynamic seeding and culturing can be said to be more effective for endothelial cell attachment and proliferation when compared with other seeding and culturing methods ($p<0.001$).

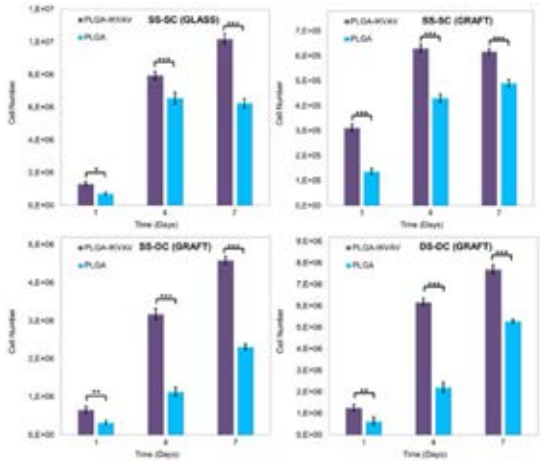

Figure 8. MTT assay results of nanofibers. (SS=Static Seeding, SC=Static Culture, DS=Dynamic Seeding, DC=Dynamic Culture)

IV. CONCLUSION

In summary, effects of PLGA nanofiber based microtubes conjugated with IKVAV peptide sequence with static and dynamic cell culture on endothelial cell attachment and proliferation were investigated. The results indicate that vascularization can be possible by using surface modification with IKVAV peptide sequence. Also, endothelial cell attachment and proliferation for vascularization can be supported with dynamic culturing technique via bioreactor.

V. ACKNOWLEDGEMENTS

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