Determination of the Effect of Glycosaminoglycan Mimetic Peptide Hydrogels on Cell Viability for Cartilage Tissue Engineering Applications

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***Abstract*—Cartilage is a tissue type that doesn't have blood vessels, neural networks and lymphatic vessels. Regeneration of the cartilage tissue is limited due to the small number of cells in the articular cartilage, low vascularization and low cell migration to the damage site. Biomaterial scaffolds are used for regeneration of cartilage since the cartilage needs structural and metabolic support in case of any damage. Mimicking the network structure of the natural cartilage is extremely important and hydrogels are good candidates for cartilage tissue engineering due to 3-D structure and the high-water holding capacity similar to the natural tissue. Also, biomimetic self-assembling peptides (SAP) can self-assemble with physiological conditions and form SAP hydrogels. Glycosaminoglycan (GAG) is crucial components of natural cartilage matrix, they are negatively charged chains, and they maintain the mechanical properties of tissue. In this study, it was develop GAG mimetic SAP hydrogels that can mimic original cartilage, and to determine the effect of these peptide hydrogels on cell viability and cell proliferation. SAP hydrogel structures were successfully produced by functionalization of SAP with GAG mimetic peptide epitope, and effect of these hydrogels on cell viability was evaluated by cell culture methods in this work.**

Keywords—peptide; cartilage tissue engineering (CTE); glycosaminoglycan (GAG); self-assembly; self-assembling peptide (SAP) hydrogel.

I. INTRODUCTION

Cartilage can be defined as a hard connective tissue that has less metabolic activity than muscle and bone tissue, and does not contain blood vessels, neural networks and lymph vessels [1]. Cartilage is exposed to high levels of stress, strain and mechanical loads throughout life in the human body [2]. The ability of cartilage tissue to repair and renew itself is limited due to lack of vascularity, inability to produce clot, and consequently the inability of related cells to migrate to the damaged area [3]. Since its capability to heal and regenerate itself is limited, when a damage or injury occurs, cartilage needs structural and metabolic support. Damages of articular cartilage with full thickness can fractionally repair, while partial thickness damages cannot restore or heal spontaneously [4]. Also, it is known that there is a possibility for another second degenerative disorder after an injury to cartilage due to restricted healing capability and not completely repair of cartilage tissue. For treating the defects in articular cartilage and osteochondral defects, there are some current clinical methods. However the cartilage repaired as a result of the existing treatments may not have all the features of the original cartilage, and cartilage cannot maintain its structural entirety and all functionality [5, 6]. Also these treatments have difficulties like donor region discomfort, material refusal and infection hazard [6]. Many techniques cause fibrocartilaginous tissue generation, cannot renew tissue completely, and this new tissue has lower characteristics and biochemical features when compared to original hyaline cartilage [7].

New regenerative strategies are needed to improve the effectiveness of cartilage repair and regeneration. Mimicking the biological and chemical composition and structure of the natural cartilage tissue is extremely critical for complete healing and regeneration of tissue. At this point, tissue engineering can lead to new approaches. Cartilage tissue engineering (CTE) concerns about to trigger cartilage repair and regeneration, producing structures that can provide the structure, components and functions of natural cartilage. For this purpose, scaffolds produced from biomaterials are used. Biomaterials are the synthetic or natural origin materials used to partially or completely repair, support, strengthen or change organs, tissues, body parts or body functions [8]. From these materials hydrogels can be defined as hydrophilic, 3-dimensional (3D) crosslinked polymer networks that are capable of swelling in water and holding large volumes of liquid within this swollen state [9]. Hydrogels can absorb large amounts of water and biological fluid [10], can also mimic the 3D environment of tissues [9]. Self-assembly is a process in which disorganized molecules organize themselves into some larger and ordered structures which have hierarchic arrangement and complication [11]. Peptides and proteins act as structural components for self-assembly mechanisms due to their unique 3D organizations and properties [12]. Biomimetic self-assembling peptides (SAP) can self-assemble under physiological conditions and can form biocompatible and biodegradable, 3D network hydrogels that can mimic the original extracellular matrix (ECM) structure [13].

ECM is responsible for the characteristics of cartilage tissue, such as flexibility, strength, endurance, resistance [10]. Also the components and molecules found in ECM give mechanical characteristics needed to provide functions of cartilage [6].

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There are three main components in ECM of cartilage as collagen, proteoglycans and other non-collagenous proteins [10]. Glycosaminoglycans (GAG) are negatively charged chains located in the natural cartilage matrix, covalently attached to the protein nuclei of proteoglycans. GAGs create a swelling pressure by contacting with ionic tissue fluid, they maintain the mechanical properties of the tissue [14].

In the current study, it was aimed to develop biomimetic SAP hydrogels that can mimic the natural cartilage, and to assess the effect of these structures on cell viability. SAP hydrogel structures were produced by adding GAG mimetic VVAGEDK (V:Valine, A:Alanine, G:Glycine, E:Glutamic acid, D:Aspartic acid, K:Lysine) epitope to KLD peptide (KLDLKLDLKLDL-NH_2, K:Lysine, L:Leucine, D:Aspartic acid) to functionalize the KLD peptide with a biomimetic peptide motif. Mesenchymal stem cells (MSCs) were encapsulated in the SAP hydrogels and cultured. Afterwards, MTT test and Live and Dead assay were performed to observe the viability and proliferation of the cells encapsulated in the hydrogels. As a result of the tests and analyzes performed, it was observed that GAG mimetic SAP hydrogels had a positive effect on cell adhesion, viability and proliferation and provided a suitable microenvironment for cells. In conclusion, it is believed that the produced GAG mimetic SAP hydrogels might be the potential and promising method in the future.

II. METHOD

A. Peptide Synthesis

KLD (Ac-Lys-Leu-Asp-Leu-Lys-Leu-Asp-Leu-Lys-Leu-Asp-Leu-NH_2) and KLD-VVAGEDK (Ac-Lys-Leu-Asp-Leu-Lys-Leu-Asp-Leu-Lys-Leu-Asp-Leu-Val-Val-Ala-Gly-Glu-Asp-Lys-NH_2) were synthesized on MBHA resin (0.67 mmol/g loading capacity) with peptide synthesis device (AAPPTEC Focus Xi, Louisville, KY, USA) [15]. Coupling of all amino acids was done with Fmoc-protected amino acids (2 equivalents), O-Benzotriazole-N, N,N',N'tetramethyluronium-hexafluoro-phosphate (HBTU; 2 equivalents), hydroxybenzotriazole (HOBt; 2 equivalents) and N,N-diisopropylethylamine (DIEA; 4 equivalents) in N,N Dimethylformamide (DMF) for period of 3 hours. 20% piperidine in DMF solution was utilized to remove the Fmoc-protecting group for 30 minutes. 10% acetic anhydride/DMF solution was utilized for acetylation of unreacted amine groups for each coupling stage during peptide synthesis. In all of the amino acid coupling and deprotection steps, Ninhydrin test was conducted to check each coupling and deprotection reaction. When all amino acids were coupled, to separate the synthesized peptide sequence from the resin, a solution of trifluoroacetic acid (TFA): triisopropylsilane (TIPS): water (H_2O) at ratio of 95:2.5:2.5 was utilized. Solution was poured into cold diethyl ether and peptide molecule was washed with this cold diethyl ether for 3 times in total. After that step, suspension was centrifuged, in that way supernatant was discarded and remaining ether was removed with vacuum evaporator. Lastly, peptide was lyophilized by freeze drying method at -80 °C (Biobase Biodustry BkFD10P, Shandong, China).

B. Peptide Hydrogel Production

In order to fabricate peptide hydrogels, KLD and KLD-VVAGEDK peptide powders were individually dissolved inside the deionized water that was sterilized (2%, 2% w/v) and exposed to sonication for a time period of 30 minutes before usage. Both of these solutions were prepared at a concentration of 2% for gelation of peptide solutions.. For producing KLD hydrogels, KLD solution was combined with sterilized Dulbecco's Modified Eagle's Medium (DMEM) cell culture medium with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer in the absence of Fetal Bovine Serum (FBS). In the biological safety cabinet, pre-gel solutions were filtered by using syringe filter with 0.22µm pore size and sterilized in that way. Then they were centrifuged and lastly the pre-gel solutions were put in the incubator for self-assembly.

C. Cell Culture and Encapsulation Inside The Peptide Hydrogels

For cell cultivation, human bone marrow derived MSCs (HMSC-AD-500, CLS cell lines Service, Lot#102, Eppelheim, Germany) were seeded in standard polystyrene cell culture flasks and cultured inside the basal medium (DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamicin, and 250 ng/mL fungizone). The culture medium were changed every two days and when the cells reach 80-90% density, they were passaged at the appropriate passage rate, by using a 0.25% trypsin/EDTA solution to remove the cells from the flask surface, and cells were transferred to a new flask containing fresh medium. In order to create adequate stocks in the relevant passage, human MSCs were frozen in a controlled manner in the freezing media at each passage stage, and stored at -196 °C in the liquid nitrogen tank (ThermoScientific, Bio-cane 47) [16-18]. In all experiments for seeding, cells at passage 3 were utilized. 600 µL of KLD peptide solution (2%) was put in a falcon. Then, 600 µL of KLD-VVAGEDK peptide solution (2%) was put in another falcon. 300 µL cell suspension was separately added to each of peptide solutions found in two falcons, and mixed. In that way human MSCs suspension was encapsulated inside the hydrogel structures ($5x10^6$ cells/mL) in basal medium, and then these solutions were placed into the wells of 96 well-plate. Finally the well-plate was placed into incubator, and for adhesion of cells they were incubated with appropriate conditions at 37 °C, %5 CO_2 for 24 hours, and cultured.

D. Cell Proliferation Analysis

To assess the proliferation analysis of MSCs that were encapsulated into the hydrogels, 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Vybrant® MTT Cell Proliferation Assay Kit, Invitrogen, Waltham, MA, USA) assay was utilized according to the manufacturer's instructions at 1., 4. and 7. days of the culture [19]. Firstly, cell medium was removed from hydrogel structures containing MSCs. 10% MTT dye in culture medium was given to each well in which encapsulated cells were found. The procedures with MTT were carried out in the dark. Hydrogel structures were incubated for 4 hours at 37°C and 5% CO_2. Then again the medium was removed from hydrogels, and 500 µL dimethyl sulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany) was added for

dissolving formazan crystals. After waiting the 10-15 minutes, hydrogel structures were smashed by pipetting and transferred to another 96 well plate. Finally by utilizing a microplate reader (Biotek Synergy HTX, Winooski, VT, USA), absorbance values of each well was read at 570 nm and recorded.

E. Cell Viability Analysis

Live and Dead assay was carried out for determining the viability of MSCs encapsulated within the hydrogels. Cell viability was evaluated by using Viability/Cytotoxicity Assay Kit for Animal Live and Dead Cells (Biotium, Inc. Hayward, MN, USA). Cell medium was removed from hydrogels found in 96 well-plate. MSCs found in 96-well plate were washed with PBS two times. After that, viability/cytotoxicity test solution that includes 5 µL of 4 mM Calcein acetoxymethyl ester (Calcein AM) and 20 µL of 2 mM Ethidium homodimer III (EthD-III) in the PBS (10 mL), was given to cells found in wells, and then incubated at room temperature for a time period of 20-30 minutes. After incubation period, for creating fluorescent pictures of cells, fluorescent microscope (Olympus CKX41, Tokyo, Japan) was utilized. Stained cells were visualized under microscope, and images of live cells (green) stained with Calcein AM and images of dead cells (red) stained with EthD-III were recorded.

III. Results

KLD and KLD-GAG peptides were synthesized and SAP hydrogel formation was achieved as shown Fig 1.

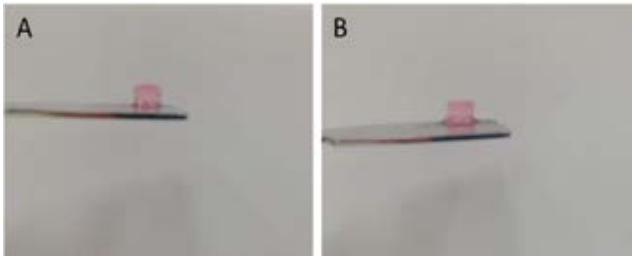

Fig. 1. Self-assembling peptide hydrogel KLD (A), KLD-GAG (B)

At 1, 4, and 7 of incubations of MSCs within developed hydrogel structures, cell proliferation analysis was conducted with MTT test for both KLD hydrogels and KLD-GAG hydrogels and graph in Fig. 2. shows the results. Proliferation of cells was assessed in this way. According to results of MTT test, as it was also shown in Fig. 2., number of cells enhanced with incubation time in both KLD hydrogels and KLD-GAG hydrogels. But cell numbers determined for KLD-GAG hydrogels was higher than the cell numbers determined for KLD hydrogels at 1, 4 and 7. days. This means that significant difference was observed between the values obtained for KLD and KLD-GAG hydrogels.

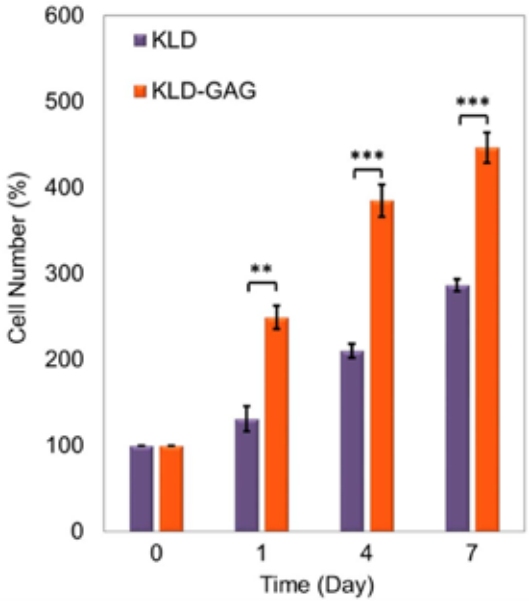

Fig. 2. Cell numbers of MSCs that were encapsulated inside KLD and KLD-GAG hydrogels. [Significant differences were determined by one-way ANOVA Newman-Keuls multiple comparison test (*p\0.05, **p\0.01, ***p\0.001).]

At day 7, Live and Dead analysis was conducted for determining the viability of cells encapsulated inside the control group KLD hydrogels and cells encapsulated inside the experimental group KLD-GAG hydrogels. At the end of the Live and Dead analysis, images that were obtained with fluorescent microscope were demonstrated in Fig. 3. In pictures, green color represents live cells while red color represents dead cells. As it can be clearly seen in Fig. 3. , KLD-GAG hydrogel constructs have higher green color intensity than KLD hydrogels, and red color intensity is greater in KLD hydrogels.

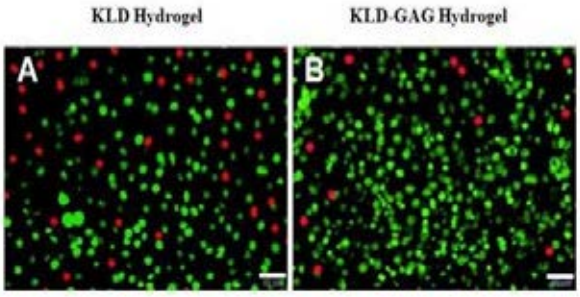

Fig. 3. Combined pictures of cells at the end of Live and Dead assay; image of cells encapsulated inside the control group KLD hydrogels (A) and image of cells encapsulated inside the experimental group KLD-GAG hydrogels(B).

IV. Discussion

In the current study, two types of SAP hydrogels were produced and their impacts on cell viability and cell proliferation were investigated. The effect of peptide hydrogels on cells proliferation was analyzed with MTT assay. According to results of proliferation analysis, for both KLD hydrogels and KLD-GAG hydrogels, the number of cells enhanced with incubation time. The increased number of cells in both control group and experimental group hydrogels recommends that both

of these SAP hydrogel structures didn't show a toxical impact on MSCs. Also, this result indicates that encapsulated cells were able to attach to peptide hydrogels well and they possessed sufficient amount of area for their proliferation. However, the cell numbers obtained for KLD-GAG peptide hydrogels were higher than the cell numbers obtained for KLD hydrogels at days of 1, 4 and 7 of culture. This finding reveals that KLD-GAG hydrogels were better at allowing and assisting the proliferation of cells, and providing a space more suitable for cell proliferation. Also, this shows the notable positive effect of KLD-GAG hydrogels and thus of the bioactive epitope as GAG mimetic peptide on cell proliferation. From literature in a study, researchers studied the impact of GAG mimetic SAP nanofiber system with diverse chemical groups on chondrogenic differentiation of ATDC5 cells. They found that the number of adhered cells on produced nanofibers was higher than the number of cells adhered on glass surface, and it was written that materials didn't lead to toxicity. They wrote that developed peptide nanofiber constructs ensure a suitable microenvironment for these cells [20]. And also these results are consistent with our study's results.

Results of Live and Dead assay indicated that there are both live and dead cells in both KLD hydrogels and KLD-GAG hydrogels. However, as a distinction, green color intensity was higher for KLD-GAG peptide hydrogels as compared to KLD hydrogels, and red color intensity was higher in KLD hydrogels. These mean that in KLD-GAG hydrogels, there were more number of viable cells than that of in KLD hydrogels and cell viability was superior. Similarly, it is observed that there were lower number of viable cells in KLD hydrogels, thus less cell viability. These also suggest that cell viability analysis results correlate with MTT results. All those findings indicate that KLD-GAG hydrogel structures were able to provide more suitable environment for encapsulated cells to live and remain alive, and were better at maintaining cell viability.

Getting better and more meaningful results from KLD-GAG hydrogels in terms of cell viability and cell proliferation may be due to the fact that cells liken and associate the environment in KLD-GAG hydrogels to the natural cartilage ECM due to the GAG mimetic epitope and thus survive better. Also, these positive outcomes show that there was a good interaction between cells and produced hydrogel. Therefore, these important outcomes overall suggest that GAG mimetic hydrogels might be utilized for regeneration of cartilage tissue. Nevertheless, in further investigations, some extra characterization methods such as hydrogel biodegradation test, chondrogenic differentiation analysis of MSCs in peptide hydrogels and GAG deposition analysis must be carried out in more details.

V. Conclusion

In this study, developed GAG mimetic SAP hydrogels successfully provided a microenvironment for MSCs under in vitro conditions. So, using GAG mimetic SAP hydrogels to create an optimal microenvironment for cell viability can be considered as a hopeful approach.

As a result, GAG mimetic SAP hydrogels were found to show positive effect on cell adhesion, viability and proliferation. When these peptide hydrogels are used in CTE applications as biomaterial scaffolds, they might have promising potential to show properties similar to original cartilage tissue and mimic the structure of cartilage.

In summary, it is thought that produced GAG mimetic SAP hydrogels might be a potential method in CTE in the future and the current study will provide an important infrastructure and direction for future studies on cartilage repair and regeneration.

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