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Original Article

Functional peptides for cartilage repair and regeneration

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Abstract: Cartilage repair after degeneration or trauma continues to be a challenge both in the clinic and for scientific research due to the limited regenerative capacity of this tissue. Cartilage tissue engineering, involving a combination of cells, scaffolds, and growth factors, is increasingly used in cartilage regeneration. Due to their ease of synthesis, robustness, tunable size, availability of functional groups, and activity, peptides have emerged as the molecules with the most potential in drug development. A number of peptides have been engineered to regenerate cartilage by acting as scaffolds, functional molecules, or both. In this paper, we will summarize the application of peptides in cartilage tissue engineering and discuss additional possibilities for peptides in this field.

Keywords: Peptide, cartilage repair, cartilage tissue engineering, transforming growth factor-β, mesenchy-mal stem cells, peptide hydrogel

Introduction

Cartilage repair remains a challenge both in the clinic and for scientific research due to the limited regenerative capacity of this tissue [1]. Over the last two decades, the development of autologous chondrocyte implantation (ACI) has resulted in significant progress in this field [2, 3]. To date, ACI is the golden therapy for articular cartilage defects in the absence of osteoarthritis and other complications. However, propagation of chondrocytes in monolayers *in vitro* often leads to chondrocyte dedifferentiation, notably compromising the outcome of ACI and, thus, being a major obstacle for the wide application of ACI [4, 5].

Cartilage tissue engineering uses a combination of scaffolds, cells, and certain active molecules to produce functional human cartilage with which to repair tissue defects [6-8]. To attain this goal, biomimetic scaffold materials, different stem cell types, and optimized differentiation protocols have been developed [9-11].

Peptides have been widely used in drug development and biotechnology over the past decade [12]. Peptides can mimic the functions of proteins but with a lower production complexity and cost. In addition, peptides can target curtain "flat pockets" that are undruggable by small molecules [13]. Peptides are easily synthesized, robust, and have tunable sizes, functional groups, and activity, making peptides the molecules with the most potential for drug development.

A number of peptides are involved in orthopedic disorders [14]. Some of these have already been translated into the clinic, such as parathyroid hormone 1-34 peptides, which are used to treat osteoporosis in postmenopausal women [15]. Other peptides have been used in scientific research on cartilage tissue engineering, where they function as active molecules, in cell adsorption, as enrichment motifs, and as scaffolds (**Figure 1**). Therefore, the aim of this study was to identify peptides related to cartilage engineering and identify novel prospects for peptides in this field.

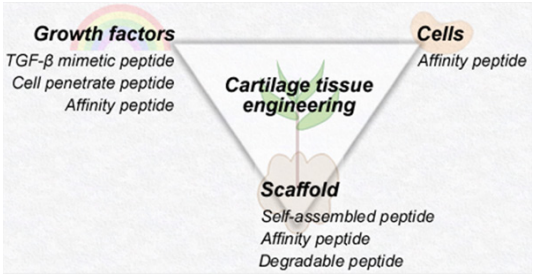

Figure 1. The use of peptides in cartilage tissue engineering, which utilizes a combination of growth factors, cells, and scaffolds. In terms of growth factors, TGF-β mimetic, cell penetrating, and growth factor affinity peptides work by mimicking, delivering, and recruiting growth factors, respectively. For cells, mesenchymal stem cell (MSC) affinity peptides work through MSC recruitment and *in situ* purification. For scaffolds, peptides work by self-assembling into hydrogel, thus mimicking the native environment of cartilage using affinity peptides and increasing degradation using degradable peptides.

Results

Transforming growth factor-β mimetic peptides

Transforming growth factor-β (TGF-β) has crucial roles in cell differentiation, collagen synthesis, and matrix deposition in cartilage tissue engineering [16]. However, its instability and dilution *in vivo* are two significant points of concern [17]. In addition, purification is expensive, making it even more difficult to use in the clinic. Therefore, peptides able to mimic TGF-β activity have a promising role in cartilage tissue engineering due to their low costs, stability, long shelf-life, and ease of administration and controlled release.

TGF-β mimetic peptides, i.e., cytomodulins (CMs), are oligopeptides developed to function similarly to TGF-β [18]. These peptides contain 4-6 amino acids with a β-bend secondary structure under physiologic conditions. Furthermore, the first three and last residues are limited to certain amino acids. Like TGF-β, CMs can enhance collagen I expression in fibroblasts and aid in wound healing *in vitro* [19, 20]. In addition, when conjugated to a poly (lactide-co-glycolide) scaffold, CM-1 can increase wound healing in a full-thickness wound mouse model [21]. However, unlike TGF-β, CMs in a soluble form are unable to effectively induce chondrogenic differentiation of stem cells [22]. These peptides can induce chondrogenic differentiation of bone marrow-derived stem cells (BMSCs) only when ligated to the surface of microspheres [23]. These differences may be due to immobilization of the peptides slowing their degradation and constraining their secondary structure to an active one.

To test this hypothesis, we synthesized several peptides and assessed their activity using human umbilical cord mesenchymal stem cells (hUC-MSCs) that had been isolated from human umbilical cords and identified by flow cytometry (**Figure 2A**, **2B**). Two peptides derived from CM-10 and designated CM-10-D1 and CM-10-D2 were synthesized. To generate CM-10-D1 (Leu-Ile-Ala-Asn-Aib-Lys), the alanine in CM-10 was substituted with the unnatural amino acid aminoisobutyric acid to increase peptide stability. However, CM-10-D1 was unable to increase collagen II expression in hUC-MSCs when used in combination with basic chondrogenic differentiation media (**Figure 2C**). Therefore, the secondary structure of these TGF-β mimetic peptides was considered next. As illustrated in the patent, these peptides have a β-bend secondary structure [18]. D-pro-L-pro is a well-known dipeptide fragment that can initiate anti-parallel strands [24]. This fragment was therefore used to constrain the secondary structure of the peptides and CM-10-D2 (Leo-Ile-Ala-Asn-Ala-Lys-L-pro-D-pro-Lys-Ala-Asn-Ala-Ile-Leo) was synthesized. CM-10-D2 was assumed to have both a β-bend structure and a two-fold higher concentration. However, when these peptides were used to induce chondrogenesis of mesenchymal stem cells (MSCs), the results remained negative (**Figure 2C**).

As a result of these experiments, it was concluded to be difficult to design an active TGF-β mimetic peptide as how the peptide was designed was absent from the patent. Furthermore, the peptides were too short to allow for certain motifs to be introduced to constrain the structure. CM-10 has a positive effect when ligated to scaffolds, so perhaps this is the optimal manner in which to use these peptides as free peptides may actually be unstable and easy diluted *in vivo.*

Cell-penetrating peptides

Cell-penetrating peptides are peptides that can penetrate the cell membrane and deliver their 'cargo', including proteins, small interfering

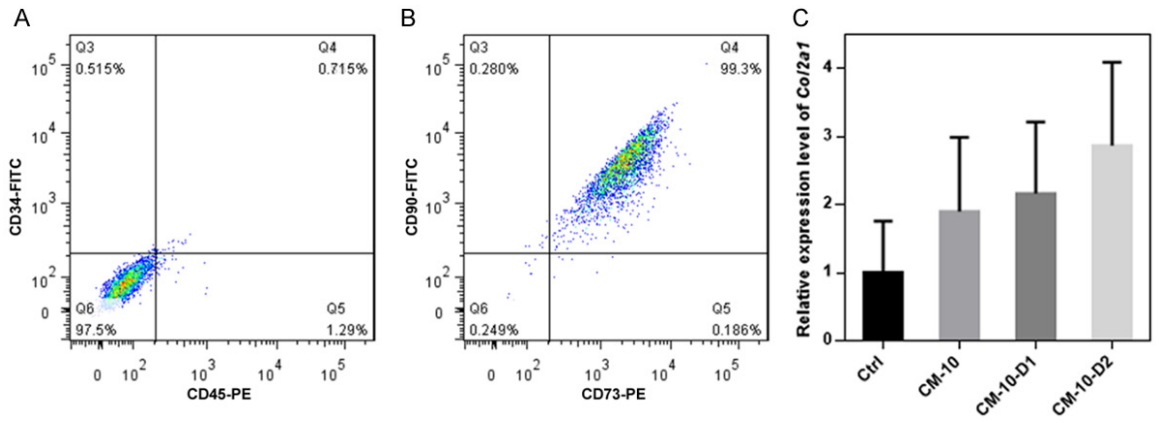

Figure 2. Chondrogenic potential of TGF-β mimetic peptide. A, B. Analysis of human umbilical cord-MSCs by flow cytometry, where cells are CD34 and CD45 negative (97.5%) and CD73 and CD90 positive (99.3%). C. *Col2a1* expression in human umbilical cord-MSCs induced with CM-10, CM-10-D1 and CM-10-D2 (10 μM) for 21 days as analyzed by quantitative real-time PCR (*GAPHD* house-keeping gene used as internal control).

RNAs, nanoparticles, oligonucleotides, and other peptides, into the cytoplasm [25]. Because TGF-β and other proteins regulate chondrogenic differentiation of stem cells, studies based on transfection of these genes into stem cells using adenoviral and lentiviral vectors have been conducted [26]. However, translation of these techniques incurs the risk of tumorigenesis and pathogenic contamination. Cell-penetrating peptides can transport certain genes into cells without incurring any of the risks mentioned above. You *et al.* used the NLS-TAT peptide to deliver the hTGF-β3 plasmid into precartilaginous stem cells, which promotes chondrogenesis of these cells [27]. Furthermore, they used this peptide as a carrier for the hTGF-β3 plasmid on a self-assembled peptide scaffold [28]. Some microRNAs, such as miR-140, play crucial roles in cartilage homeostasis and can also be delivered into cells by cell-penetrating peptides [29, 30].

Affinity peptides

Peptides with the ability to bind to specific cells, scaffolds, and cytokines are major contributors to the construction of engineered cartilage. Below, we will review the related studies in three parts.

Cells are recruited and adhere to the scaffolds when using cell-adhesive scaffolds. This method was first performed using a chondrocyte-specific antibody. Lin *et al.* later used anti-CD44 antibody and the biotin-avidin binding system to improve cell adhesion and cartilage repair [31]. However, the high cost and difficulty of antibody loading in this system make it impracticable. Cell affinity peptides are a better alternative due to being easier to synthesize and ligate onto the scaffold. Several CD-44 affinity peptides have been reported that could be used to replace this antibody-biotin-avidin system [32-34]. Baron *et al.* developed a chondrocyte affinity peptide using phage display [35]. When conjugating this peptide onto scaffolds, there was increased cell adhesion that potentially increased the repair of defects. Chondrocyte affinity peptides can also increase the integration of scaffolds and tissues due to their ability to bind cells at the sites of defects. In addition to chondrocyte affinity peptides, MSC affinity peptides can be used to construct cell-free scaffolds used to repair cartilage defects, where they recruit autologous MSCs. Ao *et al.* developed several MSC affinity peptides and used them to repair cartilage defects [36-39]. E7 is a peptide screened by phage display with a high affinity towards bone marrow-derived MSCs. E7 was covalently conjugated onto polycaprolactone electrospun meshes to construct an "MSC-homing device" [36]. This scaffold was implanted into a cartilage defect in a rat knee joint using a microfracture procedure. After 7 days, immunofluorescence staining revealed significantly more cells growing on this scaffold expressed MSC-specific surface markers than a RGD-conjugated scaffold. Furthermore, the percentage of CD68 positive cells in E7-conjugated scaffolds was much lower than that in the RGD peptide-conjugated scaffolds, suggesting the E7-conjugated scaffolds absorb

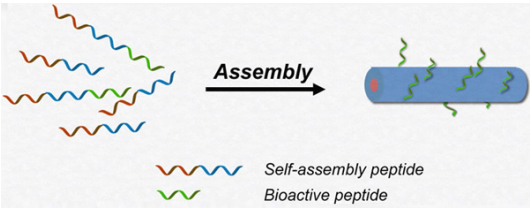

Figure 3. Schematic of the incorporation of bioactive peptides into self-assembly through peptide amphiphiles. The peptides form nanofibers through self-assembly with the hydrophobic terminal (red) as the interior and hydrophilic terminal (blue) as the exterior. Bioactive peptides (green) bind outside of nanofibers by ligating to the hydrophilic terminal.

fewer inflammatory cells. By selectively recruiting MSCs, MSC-affinity peptides on the scaffolds act as enrichment and purification tools *in vivo* [40].

In addition to MSCs, bone marrow also contains growth factors. TGF-β affinity peptide can recruit TGF-β to the impaired region following microfracture surgery [41, 42]. Stupp *et al.* developed a TGF-β1 affinity peptide and ligated it to peptide amphiphile nanofibers [42]. *In vitro* experiments indicate that this scaffold promotes the survival and chondrogenic differentiation of human MSCs. Furthermore, they confirmed the efficacy of this scaffold in repair of cartilage using a rabbit model. In addition to recruiting TGF-β *in vivo*, this peptide also overcomes the dilution problem of TGF-β, where a controlled release system can be built by reserving TGF-β in the scaffold using affinity peptides.

To recreate the biochemical and biomechanical functions of cartilage, different types of scaffolds have been developed. However, accurate recreation is still a significant challenge because the native environment of cartilage is complex and dynamic. Extracellular matrix (ECM) molecule affinity peptides can help mimic the native environment of chondrocytes and, thus, are frequently used as scaffold elements [43, 44]. Incorporating ECM molecules into a scaffold via affinity peptides avoids chemical modifications when constructing scaffolds, which may influence the degradation and biological functions of these molecules [45]. Bryant *et al.* demonstrated the promise of this strategy by successfully retaining chondrocyte-secreted proteoglycans in hyaluronic acid (HA)-binding peptide-modified poly (ethylene glycol) hydrogels using exogenous HA for 28 days [46]. In cartilage, ECM molecule concentrations increase gradually from the articulating surface towards the bone. Controlling the arrangement of these biomolecules is therefore of great interest when striving to mimic native tissues [47, 48]. Stevens *et al.* designed peptide-polycaprolactone conjugates with HA or chondroitin sulfate-binding sites in a specific spatial organization, which realized the biomimetic spatial distribution of HA and chondroitin sulfate [49]. This strategy successfully prepared a scaffold with the exceptional properties and functions of natural cartilage tissues. Besides the biochemical structure of cartilage, the biomechanical environment also needs to be considered. For affinity peptides, interactions with surrounding native ECM possibly mimic native ECM-like interactions, which may benefit cartilage regeneration. Stevens *et al.* reported that HA-binding and chondroitin sulfate-binding peptide-modified hydrogel significantly promote chondrogenesis of BMSCs [50]. Because the natural environment is complex, it is challenging to fully recaptiulate. However, using scaffold affinity peptides, we can synthesize versatile and well-designed biomimetic scaffolds.

Self-assembly peptides

Self-assembly peptides are composed of either alternating hydrophilic and hydrophobic amino acids or peptide amphiphiles [51-53]. These peptides can self-assemble into nanofibers and form nanofibrous hydrogels. Compared to hydrogels derived from biomacromolecules, peptide hydrogels are easier to synthesize and pose no risk of animal-derived pathogens.

PuraMatrixTM (3DM Inc., Cambridge, MA) is a commercially available hydrogel formed by RADA16-1 peptide that can support cartilage formation from chondrocytes and MSCs [54, 55]. Hydrogels formed from peptides with repeating KLD and RAD units can support cartilage formation comparably to agarose gels [56].

Peptide gels are amenable to incorporation of bioactive elements, especially bioactive peptides [57]. As shown in **Figure 3**, peptide gels can theoretically be created with specific properties or for specific cellular interactions by synthesizing scaffold peptide-functional peptide conjugates. For example, peptide gels with

Table 1. List of the peptides involved in cartilage tissue engineering

Name	Sequence	Function
Cytomodulins [18-23]	AA_1-AA_2-AA_3...AA_n (AA_1 = A, N, L; AA_2 = V, I; AA_3 = A; AA_n = Q, D, E, N)	TGF-β mimetic peptides
NLS-TAT [27, 28]	PKKKRKVKGRKKRRQRRRPPQ	Delivery of nucleic acid
E7 [36-38, 40]	EPLQLKM	Bone marrow mesenchymal stem cell affinity peptide
L7 [39]	LTHPRWP	Synovium-derived mesenchymal stem cell affinity peptide
TGFBP [42]	HSNGLPL	TGF-β affinity peptide
HA binding peptide [46, 47, 50]	RYPISRPRKRC	Binds specifically to Hyaluronan
Chondroitin sulphate binding peptide [49, 50]	YKTNFRRYYRF	Binds specifically to chondroitin sulphate
RADA16-1 [54, 55]	RADARADARADARADA	Forms hydrogel for scaffold
KLD-12 [56]	KLDLKLDLKLDL	Forms hydrogel for scaffold
RGD peptide [61-63]	Cyclic RGD	Aids cellular adhesion to scaffolds
MMP7-cleavable peptide [50]	CGGGPLELRAGGGC	Cleaved specifically by matrix metalloproteinase-7
MMP-degradable peptide [60]	KCGPQGIWGQCK	Cleaved by matrix metalloproteinase
N-cadherin mimic peptide [69]	HAVDIGGGC	Mimics the function of N-cadherin
Laminin-derived peptides [71]	CDPGYIGSR	Aids in the growth of bovine knee chondrocytes

binding sites for TGF-β1 have been prepared by synthesizing peptide amphiphile-TGF-β1 affinity peptide conjugates [42], resulting in controlled release systems that facilitate chondrogenic differentiation of encapsulated MSCs *in vitro* within 4 weeks.

Degradable peptides

Scaffolds provide three-dimensional structures for cells, enhance reservation of cells, and concurrently increase matrix synthesis. However, rates of degradation for many scaffolds are lower than the rates of matrix deposition by encapsulated cells [58]. Therefore, accelerating degradation of the scaffolds can make room for newly synthesized matrix.

Matrix metalloproteinases (MMPs) cleave collagen, gelatin, and other proteins in ECM [59]. Scaffolds linked through MMP-degradable peptides degrade faster in the presence of MMPs [50, 60]. Anseth *et al*. crosslinked poly (ethylene glycol) norbornene hydrogels with MMP-degradable peptides [60] and found this scaffold had significantly higher glycosaminoglycan and collagen deposition than control gels after 14 days of culture.

Other functional peptides

Other functional peptides, such as RGD peptides, are also used in cartilage tissue engineering to aid in cell adhesion [61-63]. As reported, a low RGD density is effective for chondrogenic differentiation, while a high RGD density often results in hypertrophy due to activation of integrins [64-66]. RGD can also increase adhesion of inflammatory cells and offset repair [36]. Therefore, RGD peptides are more useful in bone healing [67].

N-cadherin is a key factor mediating cell-cell interactions during mesenchymal condensation and chondrogenesis [68]. The introduction of N-cadherin mimetic peptides into scaffolds can promote both early chondrogenesis of MSCs and late cartilage-specific matrix production [69].

The use of laminin-derived peptides on chitosan/gelatin surfaces promotes attachment and neuronal differentiation of stem cells [70]. Kuo *et al*. found that laminin-related peptide on scaffolds increased adhesion of bovine knee chondrocytes and enhanced expression of glycosaminoglycans and collagen [71].

Discussion

Cartilage tissue engineering is a growing biomedical field. Meanwhile, peptides have been applied in a wide range of applications in medicine and biotechnology over the past decade. Therefore, combining cartilage tissue engineering and the use of peptides may create a number of opportunities. **Table 1** lists the peptides discussed in this review.

TGF-β is a key factor in chondrogenic differentiation, but several drawbacks, including high cost, delivery issues, a short shelf life, *in vivo* dilution, and potential immunogenicity, hinder its widespread use, including in the clinic.

Peptide mimetics can exert effects similar to factors such as TGF-β and overcome the problems mentioned above. However, free mimetic peptides fail to facilitate chondrogenesis, including in two unsuccessful avenues of optimization that were attempted in this study. Fortunately, peptides can promote cartilage formation when ligated onto scaffolds. Concurrently, TGF-β affinity peptide can recruit TGF-β to the defect from the bone marrow to assist cartilage regeneration. With these peptides in hand, we have some exogenous TGF-β alternatives available.

The variability of MSC preparations makes clinical translation difficult. Further *in situ* purification is realizable through the use of affinity peptides. In addition, exosomes secreted by MSCs have similar functions, but are less complex than MSCs, providing an alternative therapy for various inflammation-related diseases, including osteoarthritis [72-74]. As reported by Zhu *et al.*, exosomes derived from MSCs increase the proliferation and migration of chondrocytes *in vitro*, and exert notable therapeutic effects in a collagenase-induced osteoarthritis mouse model [75], while exosome-laden hydrogels can drive cartilage regeneration in a rabbit model of cartilage defect [76]. Peptides can be used in target delivery of exosomes and peptides that bind specifically to exosomes have been reported as well [77-80]. In the future, combining exosomes and peptides will likely open up new areas for cartilage tissue engineering.

Conclusion

Peptides have multiple functions in cartilage tissue engineering. Maturation of our knowledge in this field will give rise to novel applications for peptides in cartilage repair.

Materials and method

Cell culture

The collection of human umbilical cord was approved by Shenzhen Second People's Hospital. After the isolation of Wharton's jelly, we cut the jelly into 1-2 mm^3 cube and attached them on the plate, subsequently incubated with the medium (MesenGro medium, StemRD, MGro-500B; 10% FBS, Compass Biomedical, PLS6; 10 μg/L basic fibroblast growth factor, PeproTech, 100-31; 100 U/ml Penicillin-Streptomycin, Gibco, 15140-122) at 37°C with 5% CO_2. After 7-10 days' incubation, we digested the cells with 0.25% trypsin (Gibco, 25200-072) and cultured the cells for further use.

Flow cytometry analysis

hUC-MSCs were lifted by 0.25% trypsin and washed with PBS for three times. After wash, cells were suspended in PBS with a concentration of 5 × 10^5/100 μL. 2 μL of the antibody solution (PE Mouse Anti-Human CD45, BD Sciences, 555483; FITC Mouse Anti-Human CD34, BD Science, 555821; PE Mouse Anti-Human CD73, BD Science, 55257; FITC Mouse Anti-Human CD90, BD Science, 555595) were added to the cell suspension. After mixing with taping, the cells were incubated with the antibody for 1 hr at room temperature protected from light. Before loading to the machine (BD Sciences), the cells were washed with PBS once and suspended with 500 μL PBS.

Chondrogenic differentiation

hUC-MSCs P3 cells were seeded in 6-well plates with cell density of 50,000 cells/well. Cells differentiation were induced with 10 μM peptides, 0.1 mM dexamethasone (Sigma, D4902-100MG), and 50 mg/mL ascorbate (Sigma, A7631-25G) in DMEM High Glucose (Gibco, 11965-092) medium.

The media were changed every 3 days. After 21 days, RNA was purified with TRIzol (Invitrogen, 15596026). The expression level of *Ca2a1* was detected by real-time PCR (*GPADH*-Forward: 5'-GATCATCAGCAATGCCTCCT-3'; *GPADH*-Reverse: 5'-TGTGGTCATGAGTCCTTCCA-3'; *Col2a1*-Forward: 5'-CAACCAGGACCAAAGGG-ACA-3'; *Col2a1*-Reverse: 5'-ACCTTTGTCACCAC-GATCCC3').

Statistical analysis

Values were expressed as means ± SD. All statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA).

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Disclosure of conflict of interest

None.

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