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Postprint: Study on the Rapid Repair Process of Skin Wounds by Self-Assembling Short Peptide R2I4R2

Authors: Zhang Huinan, Li Mengmeng, Wen Jing, Wu Shuyi, Lan Shijian, Luo Zhongli

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Abstract

This study investigated the application efficacy of self-assembling short peptide R2I4R2 in three-dimensional in vitro culture of human skin fibroblasts and its role in the wound healing process. Circular dichroism spectroscopy was employed to analyze the effects of different physicochemical conditions on its secondary structure; Congo red staining was used for macroscopic detection of peptide self-assembly; human skin fibroblasts were cultured in vitro to explore the growth status and apoptosis of cells within the nanofiber network formed by R2I4R2; and a skin wound model in SD rats was established, with histopathological changes in skin wound repair examined via HE staining and immunohistochemistry. The results demonstrated that R2I4R2 could form a relatively stable secondary structure under various conditions; after 24 h of self-assembly, it could form a uniform and stable membranous sheet-like structure, providing a scaffold for three-dimensional cell culture; human skin fibroblasts could grow with good viability in the three-dimensional nanofiber network environment formed by R2I4R2; and animal experiments indicated that the short peptide R2I4R2 could reduce inflammation, promote neovascularization, and accelerate the skin wound healing process. As a novel nanoscaffold material, self-assembling short peptide R2I4R2 can be utilized for three-dimensional cell culture and skin wound repair.

Full Text

Self-Assembling Peptide R2I4R2 for Rapid Skin Wound Repair

Hui-Nan Zhang[1], Meng-Meng Li[1], Jing Wen[1], Shu-Yi Wu[1], Shi-Jian Lan[1], Zhong-Li Luo[1]* [1]College of Basic Medical Sciences, Molecular Medicine

and Cancer Research Center, Chongqing Medical University, Chongqing 400016, China
Corresponding author. Email: Zhongliluo@163.com

Abstract

This study investigated the application of self-assembling peptide R2I4R2 in three-dimensional (3D) culture of human skin fibroblasts in vitro and its role in wound healing. Circular dichroism spectroscopy was employed to analyze the effects of different time, temperature, and ionic conditions on its secondary structure. Congo red staining was used to macroscopically examine peptide self-assembly. Human skin fibroblasts were cultured in vitro to explore cell growth and apoptosis within the nanofiber network formed by R2I4R2. An SD rat skin wound model was established, and pathological changes during wound repair were examined via HE staining and immunohistochemistry. The results demonstrated that R2I4R2 could form a stable secondary structure under various conditions. After 24 hours of self-assembly, it formed a uniform and stable membrane-like structure suitable as a scaffold for 3D cell culture. Human skin fibroblasts grew well within the 3D nanofiber network environment formed by R2I4R2. Animal experiments revealed that the peptide R2I4R2 could reduce inflammation, promote neovascularization, and accelerate skin wound repair. As a novel nanoscaffold material, self-assembling peptide R2I4R2 shows promise for applications in 3D cell culture and skin wound repair.

Keywords: Self-assembling peptide; Skin wound repair; 3D cell culture

Introduction

The skin serves as the body' s primary immune barrier and plays a crucial role in protection and maintenance of the internal environment. However, due to its large surface area and direct exposure to the external environment, the skin is also one of the most vulnerable organs to trauma. In recent years, the number of skin wound cases has continued to increase, accompanied by a growing incidence of chronic non-healing wounds resulting from paraplegia, diabetes, and local radiation exposure. Abnormal wound healing not only affects physical appearance but can also cause structural and functional impairments to the skin and related organs.

During the wound repair process, various factors and the extracellular matrix (ECM) work synergistically to complete the three phases of healing: inflammatory phase, granulation tissue formation phase, and tissue remodeling phase. Key components include inflammatory cytokines (IL-6, IL-1, etc.), growth factors (TGF- , CD34, etc.), and signaling molecules (EGF, VEGF, etc.). The ECM connects and supports cells while also promoting and regulating cellular biological behaviors. During wound repair, the ECM protects regenerative cells, promotes collagen layer formation at the wound site, accelerates wound contraction, and repairs damaged tissue.

Self-assembling peptides represent a class of nanobiomedical materials that mimic the ECM scaffold. These peptides can simulate certain ECM functions and exhibit excellent biocompatibility. They degrade into amino acid residues without cytotoxicity and can recreate a 3D environment that simulates in vivo cell growth, supporting cell proliferation and differentiation. Building upon our previous research, our team has identified a class of short peptides with demonstrable effects in regeneration and repair.

This study selected the self-assembling peptide R2I4R2, an 8-amino-acid peptide, to investigate its self-assembly properties and influencing factors. We examined the culture conditions of human skin fibroblasts (HFF-1) in R2I4R2 hydrogel through in vitro cell culture and evaluated the repair efficacy of R2I4R2 on SD rat skin wounds through in vivo experiments. Compared with other biomaterials, this self-assembling peptide showed no toxic effects on cells or tissues, suggesting superior advantages for applications in wound repair and related fields.

Materials and Methods

1.1 Experimental Materials R2I4R2 peptide powder was kindly provided by Chengdu Scienebay Biological Technology Co., Ltd. PBS buffer powder, acridine orange/ethidium bromide (AO/EB) solution, and citrate antigen retrieval solution were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. 4% paraformaldehyde fixative and DAB chromogenic reagent kit were obtained from Shanghai Beyotime Biotechnology Co., Ltd. Fetal bovine serum was purchased from Sciencell (USA). Penicillin-streptomycin mixture was from Genview (USA). High-glucose DMEM medium and trypsin were from Hyclone (USA). Immunohistochemistry staining kits were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. Mouse anti-VEGF antibody and mouse anti-CD34 antibody were from Santa Cruz (USA).

1.2.1 Secondary Structure Analysis of the Peptide **Effect of Assembly Time:** R2I4R2 peptide was dissolved in PBS at a concentration of 0.1 mM. A 400 L sample was placed in a quartz cuvette, and CD spectra were measured at room temperature at 0 h, 4 h, 6 h, 8 h, and 22 h post-assembly (scanning wavelength 190–260 nm).

Effect of Temperature: R2I4R2 peptide was dissolved in PBS at 0.1 mM and measured at temperatures from 20–95°C with a step size of 2°C (scanning wavelength 190–260 nm).

Effect of Ions: R2I4R2 peptide was dissolved at 0.1 mM in PBS, 0.15 mM NaCl, or 15 mM MgCl . A 400 L sample was placed in a quartz cuvette for CD detection (scanning wavelength 190–260 nm).

Measurements were recorded and the [ ] value was calculated using the following formula, with wavelength plotted on the x-axis and [ ] on the y-axis:

$$[\theta] = \frac{[m(\text{deg})]}{10(\text{peptide})ln}$$

Note: m represents the instrument reading; *(peptide)* is the relative molecular mass of the peptide; l is the optical path length (0.2 cm); and n is the number of amino acids in the peptide.

1.2.2 Congo Red Staining for Macroscopic Observation of R2I4R2 Self-Assembly R2I4R2 peptide solution (5 mg/mL) was prepared in PBS or 0.15 mM NaCl and incubated at room temperature for 24 h. At 0 h, 4 h, and 24 h post-assembly, 10 L of peptide hydrogel was placed on a glass slide, stained with Congo red for 30 s, and observed under a light microscope to examine gel formation.

1.2.3 2D and 3D Culture Systems for HFF-1 Cells **2D Cell Culture:** HFF-1 cells were seeded in culture flasks with high-glucose DMEM medium containing 15% fetal bovine serum and 1% penicillin-streptomycin mixture. Cells were incubated at 37°C with 5% CO . Upon reaching approximately 90% confluence, cells were passaged with trypsin for subsequent experiments.

3D Cell Culture: When cells reached approximately 90% confluence, 3D culture was initiated. R2I4R2 peptide powder was dissolved in 1 mL enzyme-free water at 4°C to prepare a 10 mg/mL stock solution and stored at 4°C until use. Cells were trypsinized, centrifuged at 1000 r/min for 5 min, resuspended in 1 mL medium, and counted. The cell concentration was adjusted to 1×10 cells/mL. A 50 L cell suspension was mixed with 50 L peptide solution, and the mixture was immediately transferred to a 96-well plate. After incubating at 37°C with 5% CO for 10 min to allow self-assembly and 3D scaffold formation, 100 L of high-glucose DMEM medium containing 15% fetal bovine serum and 1% penicillin-streptomycin was gently added to each well, and cultures were maintained at 37°C with 5% CO .

1.2.4 AO/EB Staining of HFF-1 Cells in 3D Environment To assess the growth status and viability of human skin fibroblasts (HFF-1) in 2D and 3D environments, AO/EB staining was performed to observe morphological changes. Acridine orange and ethidium bromide (1 mg each) were dissolved separately in 10 mL PBS to prepare 0.1 mg/mL stock solutions, filtered, and stored at 4°C. The solutions were mixed in equal volumes immediately before use. Staining was performed on days 1, 3, 5, 7, and 9 of 3D culture. Cells were fixed with 4% paraformaldehyde for 10 min, stained with 10 L AO-EB dye per well for 10–15 min at room temperature in the dark, washed 2–3 times with PBS, and observed under a fluorescence microscope every 5 min.

1.2.5 CCK-8 Proliferation Assay of HFF-1 Cells in 3D Environment To evaluate HFF-1 cell proliferation in 2D and 3D environments, CCK-8 assays

were performed. HFF-1 cells were cultured in 96-well plates under both conditions. On days 1, 3, and 5, 10 L of CCK-8 solution was added to each well, incubated in the dark for 4 h, and absorbance at 450 nm was measured using a microplate reader.

1.2.6 SD Rat Skin Wound Model Adult male and female SD rats were anesthetized intraperitoneally, and two circular wounds (approximately 0.8 cm diameter) were created on the dorsal skin down to the subcutaneous fascia layer. Wounds were treated with either normal saline or R2I4R2 (5 mg/mL). Wound healing was observed and wound areas measured on days 1, 3, 5, 7, 15, and 21 post-surgery. Tissue samples were collected, fixed in 4% paraformaldehyde for 48 h, paraffin-embedded, and sectioned for HE staining and immunohistochemical analysis.

1.2.7 HE Staining Sections were deparaffinized in xylene I for 1 h and xylene II for 10 min, then rehydrated through graded alcohols (100%, 95%, 80%, 70%, 3 min each) and rinsed in tap water for 3 min. Samples were stained with hematoxylin for 5 min, rinsed, differentiated for 30 s, and stained with eosin for 10 min. After dehydration through 70%, 80%, 95%, and 100% alcohol (1 min each for 70–95%, 1 min for 100% I, and 3 min for 100% II), sections were cleared in xylene I and II (15 min each), mounted, and observed under an optical microscope.

1.2.8 Immunohistochemical Staining Sections were deparaffinized and rehydrated as described above. Antigen retrieval was performed by heating in 0.1 M citrate solution. Endogenous peroxidase was blocked with 3% H O for 15 min, followed by incubation with non-specific staining blocker at 37°C for 30 min. Primary antibodies were applied overnight at 4°C. After rewarming at 37°C for 30 min, sections were incubated with secondary antibody at 37°C for 30 min, followed by streptavidin-peroxidase at 37°C for 30 min. DAB chromogenic reaction was performed, nuclei were counterstained with hematoxylin, sections were mounted, and observed under an optical microscope.

Results

2.1 Circular Dichroism Analysis of Physicochemical Factors on R2I4R2 Secondary Structure **Effect of Assembly Time:** With increasing self-assembly time, the characteristic peak absorbance of R2I4R2 initially decreased then increased, indicating that the secondary structure gradually stabilized over time.

Effect of Temperature: The peptide remained stable between 25°C and 60°C. As temperature increased, characteristic peak absorbance gradually increased. At 95°C, qualitative analysis revealed a slight shift from -helix to -sheet structures, suggesting that excessive temperature affected the stability of R2I4R2 secondary structure.

Effect of Ions: Metal ions influenced peptide secondary structure. Using PBS as control, we examined the effects of Na and Mg² cations with the same anion (Cl). CD spectra showed that R2I4R2 exhibited stronger spectral signals at the same wavelength in 15 mM Mg² , indicating more stable secondary structure.

[Figure 1: see original paper] Self-assembling time influence to the secondary structure of R2I4R2 by PBS (peptide concentration: 0.1 mM) after 0 h, 4 h, 6 h, 8 h, 22 h.
[Figure 2: see original paper] Temperature influence to the secondary structure of R2I4R2. Peptide concentration was 0.2 mM dissolved in PBS.
[Figure 3: see original paper] Various ions influence to the secondary structure of R2I4R2 at 25°C cultured in different salt solutions: 0.15 mM NaCl, 15 mM MgCl , PBS (pH=7.2), peptide concentration 0.1 mM.

2.2 Congo Red Staining of R2I4R2 Self-Assembly Congo red staining revealed that at 0 h, the peptide structure was loose and sand-like. At 4 h, sparse membrane-like structures appeared. After 24 h of self-assembly, the peptide formed uniform, stable membrane-like structures, suggesting that R2I4R2 could serve as a matrix material for 3D cell culture under appropriate conditions.

[Figure 4: see original paper] Congo red images of self-assembling peptide R2I4R2.

2.3 Microscopic Observation of HFF-1 Cell Growth in 2D and 3D Environments In 2D culture, human skin fibroblasts (HFF-1) exhibited an adherent, spindle-shaped morphology. In 3D culture, cells appeared spherical with clear boundaries, maintained good viability, and grew in multiple layers. After 7 days, 2D-cultured cells showed contact inhibition and began to detach and die, whereas 3D-cultured cells remained viable and translucent, confirming that HFF-1 cells could grow well in the 3D environment formed by self-assembling peptide R2I4R2.

[Figure 5: see original paper] HFF-1 culture in 3D microenvironment of R2I4R2.

2.4 AO/EB Staining for HFF-1 Cell Viability AO/EB staining showed that viable cells exhibited green nuclear chromatin with normal structure, early apoptotic cells showed green chromatin with condensed or beaded morphology, dead cells displayed orange-red chromatin with normal structure, and late apoptotic cells showed orange-red chromatin with condensed or beaded morphology. In the 3D environment constructed by R2I4R2, HFF-1 cells stained green, indicating good viability, with only minimal apoptosis observed after 7 days.

[Figure 6: see original paper] Image of AO/EB staining of HFF-1 in 3D microenvironment of R2I4R2.

2.5 CCK-8 Proliferation Assay in 3D Environment CCK-8 proliferation assays showed that HFF-1 cells proliferated more slowly in the 3D envi-

ronment constructed by R2I4R2, but no cell number reduction was observed. This demonstrated that HFF-1 cells could grow in the R2I4R2 3D environment, confirming that the peptide scaffold had no toxic effects on cell growth and proliferation.

[Figure 7: see original paper] Image of cck-8 of HFF-1 in 3D microenvironment of R2I4R2.

2.6 SD Rat Skin Wound Healing Wound area measurements in the SD rat model showed that the experimental group healed significantly faster than the control group from days 1-21. By day 21, the wound area in the experimental group was only 12% of the original trauma area, compared to 17.7% in the control group. The wound closure rate in the experimental group was approximately 1.07 times faster than that of the control group.

[Figure 8: see original paper] Image of wound area recovery in SD rats skin wound model.

2.7 Histological Analysis by HE Staining HE staining results revealed that on day 5, the experimental group showed more inflammatory cells than the control group, with cellular aggregation and a trend toward capillary formation. On day 7, granulation tissue proliferation was more abundant and thicker in the experimental group, with greater capillary generation compared to controls. By day 15, the epidermal layer was significantly thicker in the experimental group, with new tissue beginning to transform into scar tissue.

[Figure 9: see original paper] Image of HE staining of skin wound healing in peptide R2I4R2.

2.8 Immunohistochemical Staining Immunohistochemical staining was performed for vascular endothelial growth factor (VEGF) and CD34, key proteins in tissue repair. Results showed minimal CD34 expression in both groups at day 7. VEGF expression was high in both groups at day 7, but significantly higher in the experimental group, with concentrated distribution at the wound front compared to the diffuse pattern in controls. By day 15, VEGF expression was nearly absent in the control group but remained high in the experimental group.

[Figure 10: see original paper] Image of immunohistochemical staining of skin wound healing in peptide R2I4R2 (100×).

Discussion

Skin trauma is a common yet serious condition in daily life. In severe cases, the cellular microenvironment at the wound surface is disrupted, making self-repair difficult. Current wound dressings on the market primarily focus on hemostasis and promoting blood circulation, which differ substantially from the natural

extracellular microenvironment. Some dressings may even increase wound debridement difficulty and trigger inflammatory responses. In contrast, nanofiber scaffolds formed by self-assembling peptides can better mimic the extracellular matrix and the natural cellular environment. Moreover, the degradation products of self-assembling peptides are amino acids, which are readily absorbed and eliminated by the body, offering advantages over traditional dressings.

The self-assembling peptide R2I4R2 selected in this study formed stable secondary structures at 37°C and self-assembled into nanofiber networks in salt solutions, maintaining structural stability under various conditions. The peptide hydrogel effectively simulated the ECM and served as a scaffold for 3D cell culture, providing a more physiologically relevant environment for HFF-1 cell growth and proliferation. HFF-1 cells grew well in the 3D system formed by R2I4R2 and remained viable for extended periods. In vivo wound healing experiments demonstrated that R2I4R2 as a dressing material reduced inflammation, promoted neovascularization, upregulated growth factor and cytokine levels, and accelerated the repair process.

This interdisciplinary study explored the effects of self-assembling peptide R2I4R2 on wound repair from three perspectives: peptide secondary structure, in vitro cell experiments, and in vivo animal models. These fundamental findings provide new strategies and insights for clinical treatment of skin trauma and regenerative repair, holding strong theoretical value and market application potential.

Acknowledgments

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