Post-printing Osteogenic Effect of siRNA-Loaded Collagen/Bioactive Glass Composite

Authors: Chen Yanling, Chen Liangjiao, Li Zhengmao, Lan Zedong

Date: 2018-06-15T21:41:26+00:00

Abstract

Objective: To investigate the osteogenic effect of siRNA-loaded collagen/bioactive glass composite materials. Methods: Three groups of composite materials were prepared by freeze-drying: collagen/bioactive glass, collagen/bioactive glass loaded with negative control siRNA, and collagen/bioactive glass loaded with noggin siRNA. The CCK8 assay was used to detect the effects of extraction solutions from the three different scaffold materials on cell proliferation in the blank control group, while ALP activity assay, q-PCR, and Alizarin Red staining were employed to evaluate the effects of the three different scaffolds on MC3T3 cell mineralization. Results: After co-culturing MC3T3 cells with material extraction solutions for 3 and 5 days, cell proliferation numbers in the collagen/bioactive glass composite group, the negative control siRNA-loaded collagen/bioactive glass composite group, and the noggin siRNA-loaded collagen/bioactive glass scaffold group were all significantly higher than those in the blank control group ($P<0.05$). After 14 days of culture, the ALP activity in the siRNA-loaded collagen/bioactive glass scaffold group was significantly higher than that in the pure scaffold group ($P<0.05$). After 14 days of culture, the expression levels of ALP, Runx2, and BSP in the siRNA-loaded collagen/bioactive glass scaffold group were significantly higher than those in the pure scaffold group ($P<0.05$). Alizarin Red staining results demonstrated that more mineralized nodules were formed in the siRNA-loaded collagen/bioactive glass scaffold group compared with the other groups ($P<0.05$). Conclusion: The siRNA-loaded collagen/bioactive glass composite scaffold exhibits good biocompatibility, and the collagen/bioactive glass composite material and noggin siRNA can synergistically enhance osteogenesis.

Full Text

Preamble

Osteogenic Effect of Collagen/Bioglass Composites Carrying Noggin siRNA

CHEN Yanling[1], CHEN Liangjiao[2], LI Zhengmao[2], LAN Zedong[3]
[1]Department of Orthodontics, Stomatological Hospital Affiliated to Fujian Medical University, Fuzhou 350004, China;
[2]Key Laboratory of Oral Medicine, Guangzhou Institute of Oral Disease, Stomatological Hospital of Guangzhou Medical University, Guangzhou 510140, China;
[3]Shenzhen Stomatological Hospital Affiliated to Southern Medical University, Shenzhen 518000, China

Abstract

Objective: To investigate the osteogenic effect of collagen/bioglass composites loaded with a small interfering RNA (siRNA) targeting noggin. **Methods:** Three types of composites were prepared by freeze-drying: collagen/bioglass alone, collagen/bioglass loaded with negative control siRNA, and collagen/bioglass loaded with noggin siRNA. CCK-8 assay was used to evaluate the proliferation of MC3T3 cells exposed to aqueous extracts of these composites. ALP activity assay, quantitative real-time PCR, and Alizarin Red staining were employed to assess the effects of the three composites on mineralization in MC3T3 cells. **Results:** After 3 and 5 days of co-culture with material extracts, all three composite groups showed significantly higher cell proliferation compared to the blank control group ($P<0.05$). At 14 days, the noggin siRNA-loaded collagen/bioglass scaffold group exhibited significantly higher ALP activity than the scaffold-only group ($P<0.05$). After 14 days of culture, the siRNA-loaded collagen/bioglass scaffold group showed significantly higher expression levels of ALP, Runx2, and BSP compared to the scaffold-only group ($P<0.05$). Alizarin Red staining revealed significantly more mineralized nodules in the siRNA-loaded collagen/bioglass scaffold group than in the other groups ($P<0.05$). **Conclusion:** The collagen/bioglass composite scaffold loaded with siRNA exhibits good biocompatibility, and the combination of collagen/bioglass composite with noggin siRNA synergistically promotes osteogenesis.

Keywords: siRNA; collagen; bioglass; osteogenesis

Introduction

The rapid repair of bone defects has long been a focal point in medical research. Since the discovery of RNA interference (RNAi) in the late 1990s, siRNA has been widely investigated for local therapeutic applications due to its ability to specifically induce gene silencing. However, the susceptibility of siRNA to degradation necessitates the development of effective local sustained-release systems, which represent a critical component of siRNA-based local therapy.

Concurrently, various osteogenic materials have emerged, among which collagen/bioglass composites have attracted considerable attention by combining the excellent osteogenic activity of bioglass with the superior biological properties of collagen. Both collagen and bioglass have been reported as siRNA delivery vehicles, yet their osteogenic capacity remains limited.

Noggin protein, a member of the transforming growth factor superfamily, acts as an antagonist to bone morphogenetic proteins BMP-2, -4, -5, -6, and -7 by binding these proteins and preventing their interaction with receptors. High noggin expression can inhibit osteogenic differentiation of pre-osteoblastic MC3T3 cells and reduce calvarial defect repair in mice. While siRNA noggin and collagen/bioglass composites have been applied separately in osteogenesis, whether this delivery system itself possesses local osteogenic promoting capabilities when combined remains unreported. This study aims to integrate the osteogenic effects of siRNA noggin and collagen/bioglass to design a collagen/bioglass composite loaded with siRNA, thereby achieving local sustained release of siRNA while enabling synergistic enhancement of osteogenesis.

Materials and Methods

1.1 Main Materials

The main materials included α-MEM medium, fetal bovine serum, and trypsin (Gibco, USA); ethyl-3-(3-dimethylaminopropyl) carbodiimide, N-hydroxysuccinimide, and chitosan (Sigma, USA); siRNA(noggin) and StealthTM RNAi Negative Control Duplexes (Invitrogen, USA); RT-PCR reverse transcription kit, PCR primers, and PCR kit (Takara, Japan); Triton-100 (Boster, China); alkaline phosphatase kit (Nanjing Jiancheng, China); CCK-8 kit, BCA protein quantification kit, and alizarin red staining solution (Beibo, China); collagen (KaoLisen, China); and bioactive glass (self-prepared).

1.2.1 Synthesis of siRNA-Loaded Collagen/Bioglass Composites

Chitosan powder was dissolved in 0.2 mol/L acetic acid to obtain a 4% chitosan solution. Bioactive glass powder was then dispersed in this solution to prepare a bioglass-chitosan solution at a mass ratio of 8:1 (bioglass:chitosan). Collagen was dissolved in 2% acetic acid to prepare a 70 mg/mL collagen solution. The bioglass/chitosan solution was slowly added to the collagen solution at a collagen/bioglass ratio of 3:2, with appropriate amounts of ethanesulfonic acid added to maintain the pH at approximately 4.8. After 24 hours of stirring, a slurry-like solution was obtained. A solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) at 4:1 ratio was prepared at 2.5 mg/mL, and the slurry was added to this solution and maintained at 4°C for 24 hours. The pH was adjusted to 7.2 with 1 mol/L NaOH solution, and the slurry concentration was adjusted with ddH_2O to a final collagen concentration of 30 mg/mL. The slurry was then aliquoted into plastic molds (48-well plates) at a height of 3 mm. siRNA was mixed with branched PEI (12,000 Da,

Sigma) at an N/P ratio of 15 in pH 7.4 nuclease-free PBS to form polyplexes. After 30 minutes, 4.5 L of siRNA polyplex solution was added to each well, immediately frozen, and freeze-dried for 24 hours to obtain the siRNA-loaded collagen/bioglass scaffolds.

1.2.2 CCK-8 Cytotoxicity Test of Scaffold Extracts

Scaffolds with a height of 1 mm and diameter of 10 mm were prepared. For noggin-loaded collagen/bioglass scaffolds, 3 L of 20 nmol/L noggin was added. Three samples each of collagen/bioglass scaffolds, noggin-loaded collagen/bioglass scaffolds, and negative control siRNA-loaded collagen/bioglass scaffolds were placed in 10 mL centrifuge tubes with 5 mL of α-MEM complete medium containing 10% FBS and 1% penicillin-streptomycin. Extracts were collected after 3 days of incubation at 37°C in a humidified 5% CO_2 cell culture incubator and stored at 4°C. Logarithmic-phase cells were digested, resuspended, and seeded in 96-well plates at 1$×10^{4}$ cells/cm^2. After overnight culture, the old medium was removed, and each well received 0.1 mL of different sample extracts (blank group received 0.1 mL serum-containing medium) with six replicate wells per group. At 1, 3, and 5 days, CCK-8 reagent was diluted 1:10 with serum-free medium, and 100 L of the mixture was added to each well. After 1-2 hours of incubation, absorbance at 450 nm was measured using a microplate reader, with results averaged across six wells.

1.2.3 ALP Activity Assay

Scaffolds with a height of 1 mm and diameter of 10 mm were prepared. For noggin-loaded collagen/bioglass scaffolds, 12 L of 20 nmol/L noggin was added. Each scaffold was divided into four equal parts for experiments. Cells (1.0$×10^{5}$ per scaffold) were seeded onto scaffolds and incubated at 37°C with 5% CO_2. After 4 hours, 2 mL of osteogenic medium was added. Additionally, 1.0$×10^{5}$ cells were directly seeded in 6-well plates as a reference. Medium was changed every 3 days with three replicate wells per sample. Experimental groups included: collagen/bioglass scaffold, noggin-loaded collagen/bioglass scaffold, and negative control siRNA-loaded collagen/bioglass scaffold. After 14 days of culture, cells were washed with cold PBS, treated with 0.5% Triton X-100 for 30 minutes on ice to increase membrane permeability. Protein concentration was determined using BCA protein quantification kit according to the manufacturer's instructions.

1.2.4 q-PCR Detection of Osteogenesis-Related Gene Expression

Cell seeding and culture methods followed the same protocol as described in section 1.2.3. Quantitative real-time PCR was used to detect expression of endogenous ALP, Runx2, and BSP genes. Total RNA was extracted using TRIzol reagent, reverse-transcribed into cDNA using Takara PrimeScript® RT reagent kit, and subjected to RT-PCR using Takara SYBR® Prime Ex Taq™ kit to detect noggin gene expression levels. Primer sequences are listed in .

1.2.5 Alizarin Red Staining

Cell seeding and culture methods were identical to those described above. Experimental groups included: blank control, collagen/bioglass scaffold, noggin-loaded collagen/bioglass scaffold, and negative control siRNA-loaded collagen/bioglass scaffold, with cells directly seeded on 6-well plates serving as reference. After 14 days of culture, cells were fixed with 4% paraformaldehyde for 10 minutes, stained with 10 mg/mL Alizarin Red for 30 minutes, and washed three times with PBS. Mineralized nodules were observed under an inverted phase-contrast microscope, and mineralization area was calculated using IPP 6.0 software (Image-Pro Plus 6.0).

1.3 Statistical Methods

Statistical analysis was performed using SPSS 22.0 software. Unpaired t-tests were used for comparison of means between two groups, with $P<0.05$ considered statistically significant.

Results

2.1 CCK-8 Cytotoxicity Test of Scaffold Extracts

The CCK-8 cytotoxicity test results are shown in [Figure 1: see original paper]. On day 1 of culture, no significant differences in cell proliferation were observed among groups. However, after 3 and 5 days of culture, the three composite groups showed significantly higher cell proliferation compared to the blank control group ($P<0.05$). No significant difference in proliferation was detected between the negative control siRNA-loaded collagen/bioglass scaffold group and the noggin-loaded collagen/bioglass scaffold group.

2.2 ALP Activity Detection

ALP activity assay results revealed no significant differences among groups after 7 days of culture. However, at 14 days, the noggin-loaded collagen/bioglass scaffold group exhibited significantly higher ALP activity compared to both the negative control siRNA-loaded collagen/bioglass scaffold group and the collagen/bioglass scaffold group ($P<0.05$).

2.3 q-PCR Detection of Osteogenesis-Related Gene Expression

Quantitative analysis of osteogenesis-related genes at the RNA level by q-PCR is presented in [Figure 3: see original paper]. After 14 days of culture, the noggin-loaded collagen/bioglass scaffold group showed significantly higher ALP expression compared to the negative control siRNA-loaded collagen/bioglass scaffold group and the collagen/bioglass scaffold group ($P<0.05$). Additionally, Runx2 and BSP expression levels in the noggin-loaded collagen/bioglass scaffold group were significantly higher than those in the other two groups ($P<0.05$).

2.4 Alizarin Red Staining

After 14 days of osteogenic induction, Alizarin Red staining revealed minimal scattered red spots in the cell-only group [Figure 4A: see original paper]. Both the negative control siRNA-loaded collagen/bioglass scaffold group and the collagen/bioglass scaffold group showed dense red nodules in scaffold-dispersed areas [FIGURE:4B, C]. The noggin-loaded collagen/bioglass scaffold group exhibited numerous mineralized nodules with orange-red coloration, with extensive distribution of red nodules in scaffold-dispersed areas forming confluent sheets [Figure 4D: see original paper]. Quantification of mineralized nodule area using IPP 6.0 software demonstrated that the noggin-loaded collagen/bioglass scaffold group produced significantly more mineralized nodules than the other three groups ($P<0.05$). Both the negative control siRNA-loaded collagen/bioglass scaffold group and the collagen/bioglass scaffold group showed higher mineralized nodule formation than the blank control group ($P<0.05$) [Figure 4E: see original paper].

Discussion

The discovery of RNAi in the late 1990s opened a new therapeutic frontier through siRNA-mediated specific gene silencing. Compared to other RNAi methods, siRNA offers significant advantages by directly interacting with the RNA-induced silencing complex (RISC) machinery, enabling simple dose control and obviating the need for nuclear entry. However, widespread clinical application of siRNA therapy remains challenging, with the primary obstacle being overcoming anatomical and physiological barriers to deliver siRNA into target tissue cells. Local siRNA therapy offers distinct advantages over systemic administration by ensuring effective local concentrations and reducing off-target effects. Numerous clinical trials have employed local injection and topical application of siRNA, achieving spatiotemporal control through sustained local release systems. In this study, the collagen/bioglass material demonstrated excellent local osteogenic effects when used as a siRNA delivery system, suggesting its potential as a novel bone tissue engineering material.

Our findings indicate that extracts from collagen/bioglass composites promote osteoblast proliferation. Collagen, an enzymatically degradable fibrous extracellular matrix component, interacts with cells to influence morphology, cytoskeletal assembly, and proliferation. Calcium and phosphorus ions released from bioactive glass are essential components of bone tissue that accelerate osteoblast mineralization and proliferation. Polyethylenimine (PEI), widely used as a non-viral gene transfection vector, complexes with siRNA through electrostatic interactions and protects siRNA from nuclease degradation via its proton sponge properties. PEI has successfully delivered DNA and siRNA to brain, lung, abdominal, and tumor tissues in numerous in vivo experiments. In this study, PEI served as a transfection reagent to successfully deliver noggin siRNA into cells. The low PEI dosage used showed no significant cytotoxicity, consistent with previous reports. Notably, noggin inhibition did not significantly

promote osteoblast proliferation in our study, contrasting with previous findings that BMP-2 promotes osteoblast proliferation and that noggin inhibition enhances this effect. This discrepancy may be attributed to the relatively low effective concentration of noggin in our experimental system, resulting in minimal proliferative effects.

Research on the mechanisms regulating bone tissue growth and mineralization has identified two distinct phases: proliferation and mineralization. The transition from early osteoblast proliferation to final mineralization results from sequential gene regulation. Studies have shown that FGF-2, which induces dedifferentiation, acts during the initial proliferation phase, whereas BMP-2 promotes osteoblast differentiation and mineralization with minimal effects on proliferation. Our results align with these findings, as did Betz et al.'s observation that delayed BMP-2 administration yields greater bone defect repair with enhanced mineralization and mechanical strength.

To evaluate the osteogenic effects of different materials on MC3T3 cells, we first examined ALP activity. ALP, an important extracellular matrix protein secreted by osteoblasts, participates in regulating bone maturation and calcification, with its activity serving as a key indicator of mineralization capacity and osteogenic transformation potential. While no significant differences were observed among groups at 7 days, the noggin-loaded collagen/bioglass scaffold group showed significantly higher ALP activity at 14 days compared to the other groups. Previous studies have demonstrated that local sustained release of noggin from polyethylene glycol (PEG) hydrogels enhances stem cell ALP activity, consistent with our findings.

Beyond ALP, we examined Runx2 and BSP gene expression as markers of early and late osteogenic differentiation, respectively. After 14 days of culture on the materials, the noggin-loaded collagen/bioglass scaffold group showed significantly higher ALP expression than the control groups, corroborating our ALP activity results. Moreover, Runx2 and BSP expression, particularly Runx2, was significantly elevated in the siRNA-loaded group. Downregulation of noggin led to increased expression of ALP, Runx2, BSP, and other osteogenesis-related genes, especially Runx2, consistent with Nguyen et al.'s findings. Fan et al. similarly reported that noggin downregulation increased expression of osteogenic genes including ALP, Runx2, and BSP, though they observed no significant difference in Runx2 compared to other genes. Since the BMP-Smad signaling pathway plays a crucial role in osteogenesis, they further demonstrated that noggin inhibition enhances BMP-Smad signaling, increasing Smad 1/5/8 phosphorylation and upregulating Runx2 and OCN expression. As Runx2 is directly regulated by the BMP signaling pathway, noggin downregulation directly increases Runx2 expression, explaining our results.

Mineralized nodule formation is critical in osteogenesis research. Our Alizarin Red staining after 14 days of osteogenic induction revealed numerous orange-red mineralized nodules in the noggin-loaded collagen/bioglass scaffold group, with extensive distribution forming confluent sheets. Quantitative analysis confirmed

significantly higher mineralized nodule formation in this group compared to the other three groups, consistent with previous studies. Both the negative control siRNA-loaded collagen/bioglass scaffold group and the collagen/bioglass scaffold group showed greater mineralized nodule formation than the blank control group. These results demonstrate that collagen/bioglass composites possess inherent osteogenic capacity, which is further enhanced by noggin siRNA loading.

In summary, this study confirms that extracts from siRNA-loaded collagen/bioglass composites promote cell proliferation, indicating good biocompatibility. Furthermore, we validated the excellent osteogenic effects of siRNA-loaded collagen/bioglass materials, suggesting their potential as a novel bone tissue engineering material to enhance local osteogenic capacity in bone defect regions.

References

[1] Tang D, Tare RS, Yang LY, et al. Biofabrication of bone tissue: approaches, challenges and translation for bone regeneration[J]. Biomaterials, 2016, 83: 363-82.
[2] Shin J, Cho JH, Jin Y, et al. Mussel Adhesion-Inspired reverse transfection platform enhances osteogenic differentiation and bone formation of human adipose-derived stem cells[J]. Small, 2016, 12(45): 6266-78.
[3] Nelson CE, Kim AJ, Adolph EJ, et al. Tunable delivery of siRNA from a biodegradable scaffold to Promote angiogenesis in vivo[J]. Adv Mater, 2014, 26(4): 607-14.
[4] Germershaus O, Nultsch K. Localized non-viral delivery of nucleic acids: Opportunities, challenges and current strategies[J]. Asian J Pharm Sci, 2014, 10(3): 159-75.
[5] Pon-On W, Charoenphandhu N, Teerapornpuntakit J, et al. Mechanical properties, biological activity and protein controlled release by poly(vinyl alcohol)-bioglass/chitosan-collagen composite scaffolds: A bone tissue engineering applications[J]. Mater Sci Eng C Mater Biol Appl, 2014, 38(38): 63-72.
[6] He P, Duan L. Combined application of collagen membrane and bioactive glass guided alveolar bone regeneration: X-ray energy spectrum analysis[J]. Guangdong Dental Disease Prevention, 2005, 13(3): 210-1.
[7] Vinas-Castells R, Holladay C, Di Luca AA, et al. Snail1 Down-regulation using small interfering RNA complexes delivered through collagen scaffolds[J]. Bioconjug Chem, 2009, 20(12): 2269-76.
[8] Zimmerman LB, De Jesús-Escobar JM, Harland RM. The spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4[J]. Cell, 1996, 86: 599-606.
[9] Aspenberg P, Jeppsson C, Economides A. The bone morphogenetic proteins antagonist noggin inhibits membranous ossification[J]. J Bone Miner Res, 2001, 16: 497-500.
[10] Devlin RD, Du Z, Pereira RC, et al. Skeletal overexpression of noggin results in osteopenia and reduced bone formation[J]. Endocrinology, 2003, 144:

1972-8.
[11] Kim TH, Singh RK, Kang MS, et al. Inhibition of osteoclastogenesis through siRNA delivery with tunable mesoporous bioactive nanocarriers[J]. Acta Biomater, 2016, 29: 352-64.
[12] Sliva K, Schnierle BS. Selective gene silencing by viral delivery of short hairpin RNA[J]. Virol J, 2010, 7(1): 248-58.
[13] Bartlett DW, Davis ME. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging[J]. Nucleic Acids Res, 2006, 34(1): 322-33.
[14] Dykxhoorn DM, Palliser D, Lieberman J. The silent treatment: siRNAs as small molecule drugs[J]. Gene Ther, 2006, 13(6): 541-52.
[15] Ma ZW, Yang CX, Song W, et al. Chitosan hydrogel as siRNA vector for prolonged gene silencing[J]. J Nanobiotechnol, 2014, 12(1): 1-11.
[16] Kim YM, Park MR, Song SC. Injectable polyplex hydrogel for localized and long-term delivery of siRNA[J]. ACS Nano, 2012, 6(7): 5757-66.
[17] Place ES, Evans ND, Stevens MM. Complexity in biomaterials for tissue engineering[J]. Nat Mater, 2009, 8(6): 457-70.
[18] Ferraz EP, Oliveira FS, De Oliveira PT, et al. Bioactive glass-based surfaces induce differential gene expression profiling of osteoblasts[J]. J Biomed Mater Res A, 2017, 105(2): 419-23.
[19] Benjaminsen RV, Mattebjerg MA, Henriksen JR, et al. The possible "proton sponge" effect of polyethylenimine (PEI) does not include change in lysosomal pH[J]. Mol Ther, 2013, 21(1): 149-57.
[20] Grabowska AM, Kircheis R, Kumari R, et al. Systemic in vivo delivery siRNA to tumours using combination polyethyleneimine and transferrin-polyethyleneimine conjugates[J]. Biomater Sci, 2015, 3(11): 1439-48.
[21] Laroui H, Geem D, Xiao B, et al. Targeting intestinal inflammation with CD98 siRNA/PEI-loaded nanoparticles[J]. Mol Ther, 2014, 22(1): 69-80.
[22] Cao H, Ke Y, Zhang Y, et al. Icariin stimulates MC3T3-E1 cell proliferation and differentiation through up-regulation of bone morphogenetic protein-2[J]. Int J Mol Med, 2012, 29(3): 435-9.
[23] Hughes-Fulford M, Li CF. The role of FGF-2 and BMP-2 in regulation of gene induction, cell proliferation and mineralization[J]. J Orthop Surg Res, 2011, 6(1): 240-7.
[24] Betz OB, Betz VM, Nazarian A, et al. Delayed administration of adenoviral BMP-2 vector improves the formation of bone in osseous defects[J]. Gene Ther, 2007, 14(13): 1039-44.
[25] Whyte MP. Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization[J]. Endocr Rev, 1994, 15(4): 439-61.
[26] Zheng L, Tu Q, Meng S, et al. Runx2/DICER/miRNA pathway in regulating osteogenesis[J]. J Cell Physiol, 2017, 232(1): 182-91.
[27] Scanlon V, Walia B, Yu J, et al. Loss of Cbl-PI3K interaction modulates the periosteal response to fracture by enhancing osteogenic commitment and differentiation[J]. Bone, 2017, 95: 124-34.
[28] Nguyen MK, Jeon O, Krebs MD, et al. Sustained localized presentation of RNA interfering molecules from in situ forming hydrogels to guide stem cell

osteogenic differentiation[J]. Biomaterials, 2014, 35(24): 6278-86.
[29] Fan JB, Im CS, Guo M, et al. Enhanced osteogenesis of adipose-derived stem cells by regulating bone morphogenetic protein signaling antagonists and agonists[J]. Stem Cells Transl Med, 2016, 5(4): 539-51.
[30] Cui ZK, Fan JB, Kim S, et al. Delivery of siRNA via cationic sterosomes to enhance osteogenic differentiation of mesenchymal stem cells[J]. J Control Release, 2015, 217: 42-52.

Note: Figure translations are in progress. See original paper for figures.