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An anti-inflammatory cell-free collagen/resveratrol scaffold for repairing osteochondral defects in rabbits

Wei Wang, Liang Sun, Pengfei Zhang, Junfei Song, Wenguang Liu *

School of Materials Science and Engineering, Tianjin Key Laboratory of Composite and Functional Materials, Tianjin University, Tianjin 300072, PR China

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ABSTRACT

Inflammatory factor overexpression is the major cause of cartilage and osteochondral damage. Resveratrol (Res) is known for its anti-inflammatory, antioxidant and immunmodulatory properties. However, these effects are hampered by its water insolubility and rapid metabolism in vivo. To optimize its therapeutic efficacy in this study, Res was grafted to polyacrylic acid (PAA, 1000 Da) to obtain a macromolecular drug, PAA-Res, which was then incorporated into atelocollagen (Coll) hydrogels to fabricate anti-inflammatory cell-free (Coll/Res) scaffolds with improved mechanical strengths. The Coll/Res scaffolds demonstrated the ability to capture diphenylpicrylhydrazyl free radicals. Both pure Coll and Coll/Res scaffolds could maintain their original shape for 6 weeks in phosphate buffered saline. The scaffolds were degraded by collagenase over several days, and the degradation rate was slowed down by Res loading. The Coll and Coll/Res scaffolds with excellent cytocompatibility were shown to promote the proliferation and maintain the normal phenotype of the seeded chondrocytes and bone marrow stromal stem cells (BMSCs). In addition, the Coll/Res scaffold exhibited the capacity to protect the chondrocytes and BMSCs against reactive oxygen species. The acellular Coll/Res scaffolds were transplanted into the rabbit osteochondral defects. After implantation for 2, 4 and 6 weeks, the samples were retrieved for quantitative real-time polymerase chain reaction, and the inflammatory related genes interleukin-1β, matrix metalloproteinases-13, COX-2 and bone and cartilage related genes SOX-9, aggrecan, Coll II and Coll I were determined. Compared with the untreated defects, the inflammatory related genes were down-regulated and those bone and cartilage related genes were up-regulated by filling the defect with an anti-inflammatory scaffold. After 12 weeks, the osteochondral defects were completely repaired by the Coll/Res scaffold, and the neo-cartilage integrated well with its surrounding tissue and subchondral bone. Immunohistochemical and glycosaminoglycan staining confirmed the distribution of Coll II and glycosaminoglycans in the regenerated cartilage. The anti-inflammatory acellular Coll/Res scaffolds are convenient to administer in vivo, holding a greater potential for future clinical applications.

1. Introduction

Osteoarthritis (OA) involving full-thickness damage to the hyaline cartilage and underlying bone, is encountered frequently in the clinical setting. The therapy of OA is seriously hampered by the limited self-repair potential of hyaline cartilage due to the absence of vasculature and lymphatics [1–4]. Surgical treatments, such as microfracture, osteochondral grafting and autologous chondrocyte implantation (ACI), are employed to restore the cartilage and repair osteochondral damage. All of these treatments have shown success in relieving pain, but suffer from severe limitations [2–7]. Recent efforts have focused on exploring tissue engineering and regenerative medicine to repair osteochondral damage.

Bioabsorbable tissue engineering scaffolds have been used to repair osteochondral lesions. The optimal scaffold for articular cartilage repair should be biocompatible, sufficiently robust in initial strength, degradable and absorbable without being toxic and causing inflammation [3,8,9]. Naturally derived materials such as collagen, alginate, agarose, fibrin, hyaluronic acid (HA), gelatin, chitosan and chondroitin sulfate have been shown to be preferable materials for cartilage tissue engineering scaffolds because of their ability to elicit biological repair actions and stimulate an immune-mediated response. Collagen, the most important constituent of cartilage and bone, has already been used in the ACI procedure for years [10]. However, for clinical applications, safety and non-immunogenicity of the scaffold is essential. Atelocollagen as a scaffold material

* Corresponding author.
E-mail address: wgliu@tju.edu.cn (W. Liu).

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is considered to be safer than telocollagen because atelocollagen is devoid of terminal telopeptides (the antigenic determinants on the peptide chain of collagen type I). In a previous study, an atelocollagen hydrogel film was successfully used to regenerate a rabbit cornea without problems of immunogenicity or inflammation [11]. On the other hand, compared with telocollagen, a much higher concentration can be acquired by atelocollagen, allowing much easier fabrication of high-strength hydrogel for soft tissue engineering [11].

Pro-inflammatory cytokines, such as cytokine interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) mediate the catabolic degradation of extracellular matrix (ECM) in articular cartilage and play a key role in rheumatoid arthritis (RA) and OA pathogenesis. These cytokines induce chondrocytes and synoviocytes to synthesize proteolytic enzymes, such as matrix metalloproteinases (MMP) and inflammatory mediators. Overexpression of MMPs is implicated in cartilage loss. Additionally, these pro-inflammatory cytokines induce chondrocyte apoptosis [12–14]. Thus, it is critical to inhibit inflammation in the treatment of RA and OA. The clinical results showed that the inhibition of IL-1β could reduce pain and swelling of patients with RA [15]. Recently, resveratrol (*trans*-3,5,4′-trihydroxystilbene, Res), a natural polyphenolic compound, has been shown to have anti-inflammatory, antioxidant, antiaging, anticarcinogenic and immunmodulatory properties [16]. Res has demonstrated anti-inflammatory effects through inhibition of TNF-α, IL-1β and COX-2 by blocking NF-κB activation [17–19]; it can inhibit the IL-1β-induced degradation of mitochondria and apoptosis in chondrocytes and bone marrow stromal stem cell (BMSC)-derived chondrocytes [20,21]. Res can also promote osteogenic differentiation of BMSCs and adipose-derived stem cells (ADSCs), enhance DNA synthesis and alkaline phosphatase (ALP) activity in osteoblasts and prevent femoral bone loss in rats [22,23]. After intra-articular injection, Res can significantly decrease the expression of inflammatory factors and prevent the degradation of glycosaminoglycans (GAGs), and relieve the symptoms of arthritis remarkably [13,24]. Despite these extensive biological effects, Res is known to be quickly metabolized in the human body, resulting in an extremely short plasma half-life. The results of pharmacokinetic tests showed that the half-life of Res in the rabbit and rat blood was 14.4 and 10.3 min, respectively. Moreover, the high hydrophobicity of Res and its ability to be oxidized easily may cause serious problems during use [18,22]. Therefore, preventing the rapid metabolism of Res is essential for its application. Sheu et al. fabricated oxidized hyaluronic acid (Oxi-HA) hydrogel with chemically bonded Res, which was shown to protect the chondrocytes against lipopolysaccharide (LPS) [16]. Li et al. used Res to modify the surface of porous polycaprolactone (PCL). The Res-PCL led to a significant increase in osteogenesis [22]. Recently, many attempts to utilize the anti-inflammatory property of Res have been reported, but most of these research projects were carried out in vitro only [25–27]. To the best of our knowledge, there has been no report on a Res-incorporated scaffold to restore osteochondral defects in vivo.

In this study, Res was grafted to polyacrylic acid (PAA, 1000 Da) to achieve a macromolecular drug (PAA-Res) with much improved water solubility compared to Res alone (Fig. 1A). Then PAA-Res was incorporated into atelocollagen to construct an anti-inflammatory scaffold (Coll/Res) (Fig. 1B). The degradation property of this scaffold and the release behavior of Res were characterized in vitro. The ability of Res to capture diphenylpicrylhydrazyl (DPPH) free radicals and the protective effect of Coll/Res scaffold on the chondrocytes and BMSCs in the reactive oxygen species (ROS) environment caused by H_2O_2 were assessed. Finally, Coll/Res scaffolds were transplanted into osteochondral defects in rabbits (Fig. 1C). The inflammatory and chondrogenic gene expressions were evaluated by a quantitative real-time polymerase chain reaction (qRT-PCR) and the neo-tissue in the defect zone was examined macroscopically, histologically and immunohistologically after 12 weeks.

2. Materials and methods

2.1. Materials

Type I porcine atelocollagen was supplied by Nippon Meat Packers Inc. (Tokyo, Japan). Res was obtained by the Tianjin Jianfeng Natural Product R&D Co. Ltd (Tianjin, China). *N*,*N*′-Carbonyldiimidazole (CDI) and DPPH were obtained from the Shanghai Pepmed Co. Ltd (Shanghai, China). 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl tetrazolium bromide (MTT, 98%) and *N*-hydroxysuccinimide (NHS) were supplied by Fluka (Buchs, Switzerland). Collagenase type I and type II were purchased from Sigma-Aldrich. All the other reagents were of analytical grade and used without further purification.

2.2. Preparation and characterization of PAA-Res

Freeze-dried PAA was dissolved in dimethyl sulfoxide (DMSO), followed by the addition of CDI (10% w/v, in DMSO) with a CDI:COOH molar ratio = 3:10. This reaction was kept at 60 °C for 3 h. After cooling to room temperature, a 3-fold excess Res (molar ratio) was added to the resultant solution, which was kept in the dark at room temperature for 12 h. The product was dialyzed against pure water through a dialysis membrane (molecular weight cutoff = 1000) in the dark for 3 days. Finally, the solution was lyophilized to collect dry PAA-Res. The Res, PAA and PAA-Res were characterized by proton nuclear magnetic resonance (^{1}H-NMR, Varian INOVA 500MHZ). The formula below was used to calculate the Res grafting ratio:

$$\mathrm{Res}\% = \sum_{6}^{7.5}\frac{I_1}{9}\Big/\sum_{1}^{2.8}\frac{I_2}{3}$$

I_1 is the sum of the integral area of those peaks in 6–7.5 which are attributed to α-CH of the phenolic hydroxyl groups in Res, and I_2 is the sum of the integral area of those peaks in 1–2.8 which are attributed to —CH_2—CH— in PAA.

The water solubility of PAA-Res was evaluated by the absorption at 305 nm [28] on a TU-1810 UV-Vis spectrophotometer (Pgeneral, China).

DPPH, a stable and commercially available free radical, has been extensively applied in the study of antioxidant activity of natural compounds. The scavenging effect on DPPH radicals was determined in terms of the method described in the work of others [26,29]. Briefly, DPPH was dissolved in methanol to make a 25 μM stock solution. Then, 98 μl of DPPH solution was mixed with Res solution or PAA-Res solution. A control sample was prepared by mixing 98 μl of DPPH solution with 2 μl of Tris buffer. All the samples were stored at room temperature for 30 min in the dark. After that, the absorbance was measured at 517 nm by a spectrophotometer. The free radical scavenging effect was calculated according to the following formula:

$$\text{Scavenging effect}\ (\%) = \frac{(A_{\mathrm{DPPH}} - A_{\mathrm{sample}})}{A_{\mathrm{DPPH}}} \times 100$$

A_{DPPH} and A_{sample} are the absorbance of DPPH and the PAA-Res samples at 517 nm, respectively.

2.3. Preparation and characterization of Coll/Res scaffold

The Coll scaffold was fabricated according to a previously reported method [11]. Briefly, 0.3 g of Coll solution (13.7% w/w)