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Curcumin-functionalized silk materials for enhancing adipogenic differentiation of bone marrow-derived human mesenchymal stem cells

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ABSTRACT

Curcumin, a natural phenolic compound derived from the plant *Curcuma longa*, was physically entrapped and stabilized in silk hydrogel films, and its influence on human bone marrow-derived mesenchymal stem cells (hBMSC) was assessed related to adipogenic differentiation. The presence of curcumin significantly reduced the silk gelation time and changed the porous morphology of gel matrix, but did not change the formation of the silk beta-sheet structure. Based on spectrofluorimetric analysis, curcumin most likely interacted with hydrophobic residues in silk, interacting with the beta-sheet domains formed in the hydrogels. The antioxidant activity of silk film-associated curcumin remained functional over at least one month in both the dry and hydrated state. Negligible curcumin was released from silk hydrogel films over 48 h incubation in aqueous solution. For hBMSC cultured on silk films containing more than 0.25 mg ml^{-1} curcumin, cell proliferation was inhibited, while adipogenesis was significantly promoted based on transcripts as well as Oil Red O staining. When hBMSC were cultured in media containing free curcumin, both proliferation and adipogenesis of hBMSC were inhibited when curcumin concentrations exceeded 5 μM, which is more than 1000 times higher than the level of curcumin released from the films in aqueous solution. Thus, silk film-associated curcumin exhibited different effects on hBMSC proliferation and differentiation compared with curcumin in solution.

1. Introduction

Human mesenchymal stem cells (hMSC) are multipotent stromal cells, capable of differentiation into bone, cartilage, fat, ligament, muscle and other connective tissue lineages, offering versatility in regenerative medicine and tissue engineering [1]. In addition, they can be readily expanded ex vivo for several passages without losing their self-renewal capacity. These functions provide therapeutic potential for treating conditions where complex tissue regeneration and remodeling are major concerns, such as bone, cartilage and soft tissue engineering [2]. For these applications, hMSC are often seeded on biomaterial-based scaffolds, and their lineage differentiations are regulated by growth factors embedded in the scaffolds and released in temporally and spatially controllable manners. Other factors, including small molecule compounds that interact with specific signaling pathways (e.g. Wnt and/or TGF-β/BMP pathways) at different levels also have potentials to regulate hMSC differentiations [3].

Curcumin is the yellow pigment of *Curcuma longa* found in turmeric spice, which has been shown to have potent antioxidant, anti-inflammatory and anti-carcinogenic effects [4]. Curcumin positively affected both peroxisome proliferator-activated receptor-γ (PPAR-γ) [5–9] and p21 signaling pathways [10], which are intimately involved in adipogenesis. For example, curcumin dramatically induced the PPAR-γ expression and activated PPAR-γ in activated rat hepatic stellate cells [8]. Curcumin treatment produced a significant increase in PPAR-γ mRNA and protein levels in the liver of septic rats [6]. Moreover, curcumin moderately increased the p21 level in breast (MCF-7 and T47-D) and prostate (LNCap) cancer cell lines [10]. The ability of curcumin to affect these same pathways in hMSC has yet to be documented, but make curcumin an attractive candidate for enhancing adipogenesis in hMSC, which is of significance to soft tissue engineering. It has

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been found recently that the effect of curcumin on cell proliferation and differentiation is highly dose-dependent, and the solubility and stability of curcumin may also change in cell culture media under different conditions, such as the concentration of serum and frequency of medium replenishment [11,12].

In the studies described here, attention was focused on the role of curcumin in the adipogenic differentiation of hMSC, laying the basis for developing curcumin-functionalized biomaterials for hMSC-based soft tissue regeneration. Bone-marrow derived MSC (hBMSC) were used in the study, and the findings will be instructive for MSC from other sources. Recent studies have shown that a large portion of curcumin added to media directly bound to cell membranes and further stimulated intracellular signaling pathways either by interacting with molecular receptors on the membrane or by changing the membrane physical properties [12,13]. The present authors' hypothesis was that binding curcumin to the surface of the cell culture substrate may stimulate intracellular signaling pathways in hBMSC in a more efficient manner than free curcumin in the medium, thus promoting hBMSC differentiation. Surface-associated curcumin may interact with the binding sites (possibly receptors) on hBMSC cell membrane in a controlled and sustainable manner, resulting in the activation of related signaling pathways and promoting adipogenesis. Curcumin delivered in solution may transiently bind to cell membrane and lead to a different cell response.

To address the hypothesis, two curcumin delivery methods were compared: free curcumin in solution and surface-associated curcumin. The material chosen for immobilizing curcumin and supporting cell growth was silk fibroin, the structural protein isolated from *Bombyx mori* silkworm cocoons. Silk fibroin has been shown to be a versatile biomaterial, owing to its biocompatibility, mechanical properties, cell-dependent degradation profile and processibility [14–16]. Silk proteins can be readily fabricated into a variety of material formats through all-aqueous processing, such as porous sponge, particles, films and hydrogels. Recent work in the present authors' labs has produced mechanical [17] and chemical methods [18,19] to rapidly gel silk protein solutions by inducing β-sheet formation to generate physically crosslinked hydrogels. Small molecules or proteins can be encapsulated in these hydrogels, and the release profiles can be tailored, depending on the extent of β-sheet formation. In the present study, the present authors chose to physically encapsulate curcumin in silk hydrogel films rather than using chemical cross-linking, because hydrophobic curcumin molecules bound tightly to the silk hydrogel matrix with limited release in aqueous solution.

2. Materials and methods

2.1. Materials

Partially degummed silk fibers were purchased from Xiehe Silk Incorporation, Shengzhou, Zhejiang province, China. Curcumin (Cat#C7727, >80% pure), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). mRNA isolation kits were purchased from Qiagen Inc. (Valencia, CA). Cell medium ingredients and qPCR reagents were purchased from Life Technologies (Grand Island, NY).

2.2. Silk purification

The silk fibers had been pretreated to remove most of the sericin contaminants. Further degumming was performed in the laboratory at a scale of 10 g for each batch. The fibers were boiled in 0.02 M sodium carbonate solution for 30 min, rinsed with ultrapure water three times, drained and dried in a fume hood overnight. The dried fibers were dissolved in 9.3 M lithium bromide solution, dialyzed against pure water for 2 days to remove lithium bromide and centrifuged to remove insoluble fibrous debris, as reported in the literature [20]. The silk concentration was ~6% (w/v) after purification. The solution obtained was stored at 4 °C and further diluted to 4% (w/v) with water before being used to prepare the hydrogel films.

2.3. Silk–curcumin hydrogel film preparation

A stock solution was prepared by dissolving curcumin in absolute ethanol to obtain a concentration of 5 mg ml^{-1}. The solution was further diluted with ethanol to obtain working solutions at various curcumin concentrations. To prepare a large hydrogel film for material characterization, 2 ml of curcumin working solution was mixed with 8 ml of 4% silk solution and the mixed solution was poured into a 100 mm plastic Petri dish. The dish was covered and placed in a fume hood overnight until the solution formed a hydrogel that did not flow when inverted. The cover was then removed to allow the gel to dry in the air, forming a transparent yellow thin film at the bottom of dish. For curcumin release and stability testing, the film was peeled off and cut into small pieces weighing ~5 mg each. Ethanol alone was used to prepare the control sample of plain silk film with the same ethanol to silk ratio as used above. The film pieces were placed in Eppendorf tubes for storage or testing. To prepare small hydrogel films for cell culture, curcumin was mixed with silk in the same way, and 200 μl aliquots of the mixture were added to 24-well plates. The silk and curcumin solutions were filtered through 0.45 μm sterile membranes (Millipore, Billerica, MA) before mixing, and the mixing step as well as the subsequent film drying were performed in a laminar flow hood. After drying, the plates were wrapped with aluminum foil and stored at room temperature before use. Curcumin concentrations in silk hydrogels are listed in Table 1. The concentrations in milligrams per milliliter were used to distinguish them from free curcumin in the cell culture and release medium in molar concentrations (μM).

2.4. Fluorescence spectroscopy

Curcumin/DMSO stock solution (2.71 mM) was diluted in an aqueous solution containing silk. The final concentration of curcumin was kept constant at 25 μM, while the concentration of silk varied from 0 μM (pure water) to 12.5, 25 or 50 μM. After mixing, 1 ml of the mixture was added to a quartz cuvette, which was loaded into a spectrofluorimeter (FluoroMax-4, HORIBA Jobin Yvon Inc., Edison, NJ) for fluorescence measurements. The excitation

Table 1
Curcumin concentrations used and effects on hBMSC.

Concentration in hydrogel (mg ml^{-1})	Concentration in medium (μM)	Effect on hBMSC proliferation	Effect on hBMSC adipogenesis
Free curcumin in medium			
N.A.	0	No effect	No effect
N.A.	0.1	No effect	No effect
N.A.	1	No effect	No effect
N.A.	5	Inhibition	N.A.
N.A.	10	Inhibition	Inhibition
Film-associated curcumin			
0	0	No effect	No effect
0.025	<0.001	No effect	No effect
0.05	<0.005	No effect	No effect
0.125	<0.005	No effect	Promotion
0.25	<0.005	Inhibition	Promotion