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Differential effect of hypoxia on human mesenchymal stem cell chondrogenesis and hypertrophy in hyaluronic acid hydrogels

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ABSTRACT

Photocrosslinked hyaluronic acid (HA) hydrogels provide a conducive 3-D environment that supports the chondrogenesis of human mesenchymal stem cells (hMSCs). The HA macromer concentration in the hydrogels has a significant impact on the chondrogenesis of the encapsulated MSCs due to changes in the physical properties of the hydrogels. Meanwhile, hypoxia has been shown to promote MSC chondrogenesis and suppress subsequent hypertrophy. This study investigates the combinatorial effect of tuning HA macromer concentration (1.5–5% w/v) and hypoxia on MSC chondrogenesis and hypertrophy. To decouple the effect of HA concentration from that of crosslinking density, the HA hydrogel crosslinking density was adjusted by varying the extent of the reaction through the light exposure time while keeping the HA concentration constant (5% w/v at 5 or 15 min). It was found that hypoxia had no significant effect on the chondrogenesis and cartilaginous matrix synthesis of hMSCs under all hydrogel conditions. In contrast, the hypoxia-mediated positive or negative regulation of hMSC hypertrophy in HA hydrogels is dependent on the HA concentration but independent of the crosslinking density. Specifically, hypoxia significantly suppressed hMSC hypertrophy and neocartilage calcification in low HA concentration hydrogels, whereas hypoxia substantially enhanced hMSC hypertrophy, leading to elevated tissue calcification in high HA concentration hydrogels irrespective of their crosslinking density. In addition, at a constant high HA concentration, increasing hydrogel crosslinking density promoted hMSC hypertrophy and matrix calcification. To conclude, the findings from this study demonstrate that the effect of hypoxia on hMSC chondrogenesis and hypertrophy is differentially influenced by the encapsulating HA hydrogel properties.

1. Introduction

Human mesenchymal stem cells (hMSCs) are an ideal cell source for regenerative medicine, especially for cartilage repair, because of major beneficial features, such as easy availability and multipotency. However, inadequate cartilaginous matrix production by chondrogenically induced MSCs and the unstable chondrogenic phenotype of hMSCs following the initial induction are considered to be the two major hurdles to the successful application of hMSCs in cartilage repair and regeneration [1,2]. Photocrosslinked hyaluronic acid hydrogels have been proven to be an effective biomaterial that supports the chondrogenesis of hMSCs [3,4]. However, in addition to their ability to undergo chondrogenesis, hMSCs also exhibit the tendency to differentiate towards a hypertrophic phenotype after the initial chondrogenic induction, similar to that observed in the terminal differentiation of hypertrophic chondrocytes during endochondral ossification, leading to extensive calcification of the neocartilage matrix after ectopic transplantation in subcutaneous mouse models [2,5].

It is known that the components and structure of the extracellular and pericellular matrices play an important role in the regulation of chondrocyte hypertrophy and matrix calcification [6–8]. Chondrocytes entering terminal differentiation also substantially remodel their surrounding cartilage matrix to produce a template that facilitates calcification [9]. Therefore, the physical properties of the hydrogel scaffold, such as macromer concentration and crosslinking density, which controls the quality and distribution of the newly formed cartilage matrix, may influence the hypertrophic differentiation of chondrogenically induced MSCs and consequently calcification of the neocartilage matrix. Previously, we showed that changing the crosslinking density of HA hydrogels, by varying the HA macromer concentration or changing the ultra-

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violet light (UV) exposure time, influences neocartilage formation, hypertrophy and neocartilage calcification by encapsulated chondrocytes and MSCs [10–12].

Meanwhile, hypoxia, or low oxygen tension, which mimics the physiological avascular microenvironment of articular cartilage, has been shown to significantly influence cartilaginous matrix production by chondrocytes and the chondrogenesis of MSCs via factors such as hypoxia-inducible transcription factor (HIF-1α) [13–15]. HIF-1α plays a critical role in chondrogenesis and cartilage development during skeletogenesis by regulating Sox9, a chondrogenic marker gene required for the initiation of chondrogenesis [16,17]. Recent studies have also indicated that hypoxia suppresses hypertrophy differentiation of chondrocytes and multipotent stromal cells [15,18,19].

We have previously shown that, after chondrogenic induction (either with or without subsequent hypertrophy induction), hMSC hypertrophy and the resulting neocartilage calcification can be mitigated by co-culture of chondrocytes with hMSCs, as well as by mechanical loading [20,21]. However, further understanding of the regulation of hMSCs hypertrophy and tissue calcification is needed to ensure the successful clinical application of hMSCs for cartilage repair. With these issues in mind, we hypothesize that the hypoxia condition will regulate the chondrogenic and hypertrophic differentiation of hMSCs encapsulated in HA hydrogels. As shown previously by our group, the HA hydrogel macromer concentration and crosslinking density influence the initial chondrogenesis, neocartilage formation and subsequent hypertrophy and matrix calcification by encapsulated MSCs [12]. Hence, the second hypothesis is that there will be an interactional effect between hypoxia and the hydrogel macromer concentration or crosslinking density on hMSC hypertrophy and cartilage calcification. Therefore, the objective of this study was to investigate the effect of hypoxia on hMSC chondrogenesis and hypertrophy in HA hydrogels of varying HA macromer concentration and crosslinking density. To facilitate the investigation, the hypertrophy of hMSCs was studied using an in vitro hypertrophy model established by a previous study [22].

2. Material and methods

2.1. Macromer synthesis

Methacrylated HA (MeHA) was synthesized as previously reported [23]. Briefly, methacrylic anhydride (94%, FW 154.17, Sigma) was added to a solution of 1 wt.% HA (sodium hyaluronate powder, research grade, MW ~74 kDa, Lifecore) in deionized water, adjusted to pH 8 with 5 N NaOH, and reacted on ice for 24 h. The macromer solution was purified via dialysis (MW cutoff 6–8 k) against deionized water for a minimum of 48 h with repeated changes of water. The final product was obtained by lyophilization and stored at −20 °C in powder form prior to use. The final macromer products were confirmed by ^{1}H NMR to have a methacrylation level of ~29%. Lyophilized macromers were dissolved in phosphate-buffered saline containing 0.05 wt.% of the photoinitiator 2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (I2959, Ciba) to allow for UV-mediated polymerization.

2.2. Characterization of HA hydrogels

The Young's moduli of acellular HA hydrogels were evaluated under unconfined compression with a mechanical tester (TA Instruments) at a strain rate of 10% min^{-1} up to a compressive strain of 20%, and moduli were determined by fitting the stress vs. strain curve and calculating the slope. The effective diffusivity of macromolecules in acellular HA hydrogels was determined as described in a previous study [12]. Briefly, HA hydrogel disks were incubated in a fluorescein-labeled dextran solution (10 kDa, 10 mg ml^{-1}; Molecular Probes). At selected time points, the distribution of fluorescent intensity across the hydrogel cross-section was imaged. The average fluorescence intensity was fitted to a finite element simulation of diffusion to simulate dextran diffusion. An estimated value of the effective diffusivity was derived by fitting the simulation curve to the experimental findings using the least squares method.

2.3. Sample preparation and in vitro culture

Human MSCs (Lonza) were expanded to passage 3 under the condition of normoxia (21% oxygen tension) in growth medium consisting of α-minimum essential medium with 16.7% fetal bovine serum and 1% penicillin/streptomycin, to 20 million hMSCs ml^{-1}. The hMSCs were then photoencapsulated with UV light (wavelength 360 nm; intensity 1.2 mW cm^{-2}) in three different formulations of MeHA hydrogel disks of identical size (5 mm diameter, 2.5 mm thickness). The three formulations (*x*%–*y*m, where *x* is the MeHA concentration (w/v) and *y* is the UV exposure time (min)) were: low HA concentration and low crosslinking density hydrogels (1.5%–15m), high HA concentration and high crosslinking density hydrogels (1.5%–15m), and high HA concentration and low crosslinking density hydrogels (5%–5m) (Fig. 1A and B). The high MeHA concentration in the 5% (w/v) precursor solution (hence more crosslinkable methacrylate groups in the precursor solution) allows formation of hydrogels with differential degrees of crosslinking by varying the UV exposure time. The constructs formed were cultured in chondrogenic medium (Dulbecco-s modified Eagle's medium, 1% ITS + Premix, 50 μg ml^{-1} L-proline, 0.1 μM dexamethasone, 0.9 mM sodium pyruvate, 50 μg ml^{-1} ascorbate, antibiotics) supplemented with transforming growth factor-β3 (TGF-β3, 10 ng ml^{-1}), which was changed three times per week [24]. Normoxia groups were cultured at 21% atmospheric oxygen level and 5% carbon dioxide. The hypoxia groups were incubated in a hypoxia chamber supplied with 1% oxygen and 5% carbon dioxide. To induce hypertrophy, constructs were first

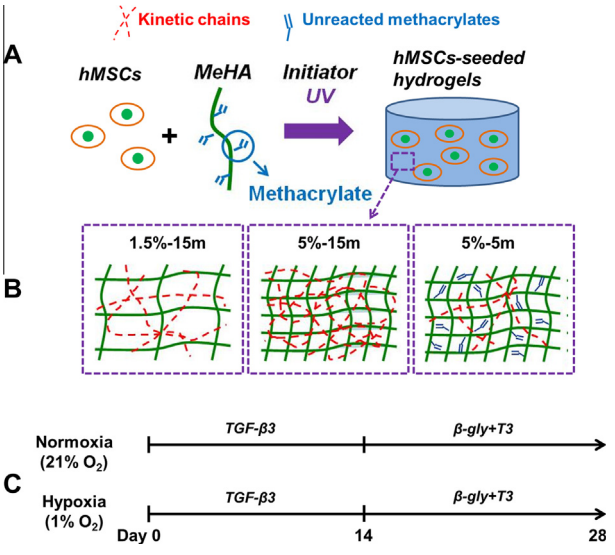

Fig. 1. Fabrication of MeHA hydrogels with varying crosslinking density by changing either the macromer concentration or the exposure time (A). 1.5%–15m: 1.5% MeHA solution crosslinked by 15 min of UV exposure; 5%–5/15m: 5% MeHA solution crosslinked by 5 or 15 min of UV exposure (B). Timeline, culture condition and media supplements of all groups. TGF-β3: transforming growth factor, T3: triiodothyronine, β-gly: β-glycerophosphate (C).