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Copper nanoparticle cues for biomimetic cellular assembly of crosslinked elastin fibers

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Abstract

Elastin, a structural protein distributed in the extracellular matrix of vascular tissues, is critical to maintaining the elastic stability and mechanical properties of blood vessels, as well as regulating cell-signaling pathways involved in vascular injury response and morphogenesis. Pathological degradation of vascular elastin or its malformation within native vessels and the poor ability to tissue-engineer elastin-rich vascular replacements due to innately poor elastin synthesis by adult vascular cells can compromise vascular homeostasis, and must thus be addressed. Our recent studies attest to the utility of hyaluronan (HA) oligomers for elastin synthesis and organization by adult vascular smooth muscle cells (SMCs), though the elastin matrix yields in these cases were quite low relative to total elastin produced. Thus, in this study, we investigated the utility of copper (Cu^{2+}) ions to enhance cellular elastin deposition, crosslinking and maturation into structural fibers. Copper nanoparticles (CuNPs; 80–100 nm) in the dose range of 1–100 ng ml^{-1} were tested for Cu^{2+} ion release, and based on mathematical modeling of their release profiles, CuNPs (1, 10, and 400 ng ml^{-1}) were chosen for supplementation to adult SMC cultures. The 400 ng ml^{-1} dose of CuNPs cumulatively delivered Cu^{2+} doses in the range of 0.1 M, over the 21 day culture period. It was observed that while exogenous CuNP supplements do not up-regulate tropoelastin production by vascular SMCs, they promoted formation of crosslinked elastin matrices. The deposition of crosslinked matrix elastin was further improved by the additional presence of HA oligomers in these cultures. Immunofluorescence imaging and structural analysis of the isolated elastin matrices indicate that amorphous elastin clumps were formed within non-additive control cultures, while aggregating elastin fibrils were observed within SMC cultures treated with CuNPs (1–10 ng ml^{-1}) alone or together with HA oligomers. The presence of 400 ng ml^{-1} of CuNPs concurrent with HA oligomers furthered aggregation of these elastin fibrils into mature fibers with diameters ranging from 200 to 500 nm. Ultrastructural analysis of elastin matrix within cultures treated with HA oligomers and 400 ng ml^{-1} of CuNPs suggest that elastin matrix deposition as stimulated by Cu^{2+} ions proceeds via a fibrillin-mediated assembly process, with enhanced crosslinking occurring via stimulation of lysyl oxidase. Overall, the data suggest that CuNPs and HA oligomers are highly useful for regenerating crosslinked, fibrillar elastin matrices by adult vascular SMCs. These results have immense utility in tissue-engineering vascular replacements.

Keywords: Elastin fibers; Copper nanoparticles; Hyaluronan; Oligomers; ECM regeneration

1. Introduction

Elastic fibers, composed of amorphous elastin and associated microfibrillar proteins (e.g. fibrillin), are primarily responsible for the extensibility and resilience of connective tissues such as lungs, skin and blood vessels [1]. Elastic fibers and their components are also involved in signaling vascular cells via their surface receptors, to modulate their proliferation and phenotype [2,3]. Thus, the failure to reinstate a healthy elastin matrix, when these fibers are damaged by injury or disease, or when they are congenitally malformed or absent, can severely compromise vessel homeostasis [4]. In this context, active regeneration of elastin matrices in vivo and within tissue-engineered constructs provides an alternative promising approach [5].

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Current tissue-engineering strategies for elastin regeneration in situ are limited by very poor tropoelastin mRNA expression by adult vascular cells and the unavailability of scaffolds that can provide biomolecular cues necessary for cellular-mediated regeneration of native elastin mimics [6]. Although synthetic elastomers or elastin peptide assemblies can replicate the mechanics of native elastin [7,8], the absence of cell-signaling microfibrillar proteins (e.g. fibrillin) prevents the construct from eliciting native responses from vascular SMCs [9]. Based on earlier studies which suggested close association between several glycosaminoglycan (GAG) types (e.g. hyaluronic acid) and elastin within vascular tissues [10,11], we recently showed that the elastogenic effects of hyaluronan (HA) are highly fragment-specific, with HA fragments (<1 MDa) and shorter oligomers (<1 kDa) being more cell-interactive and elastogenic than the relatively bioinert long-chain HA (>1 MDa) [12–15]. We determined that HA oligomers, specifically, stimulate multifold increases in the production of soluble tropoelastin and crosslinked matrix elastin, and enhance elastin fiber assembly and the synthesis of lysyl oxidase (LOX, an elastin and collagen crosslinking enzyme) and desmosine crosslinks [13]. In follow-up studies, we showed that these "elastogenic" effects could be synergistically enhanced by stimulating cells with HA oligomers together with other growth factors (e.g. TGF-β1, IGF-1) [14,15]. These studies attested to the tremendous potential of HA oligomers, alone, or together with other growth factor cues, with respect to elastin matrix regeneration.

Although our studies demonstrated the utility of HA oligomers for increasing tropoelastin and total elastin (matrix elastin + tropoelastin) production on a per-cell basis, the net amount of crosslinked matrix elastin relative to the total elastin produced, defined as elastin matrix yield, remained low (~10–20%) [12–14]. This emphasizes the need to provide other exogenous "elastin maturation cues" to enhance cellular LOX production or LOX enzyme activity, to enhance elastin crosslinking or render it more efficient [16,17]. Since extracellular LOX availability and activity are dependent on the presence of copper ions (Cu^{2+}) [18], we hypothesize that the simultaneous delivery of HA oligomers and Cu^{2+} cues will enhance tropoelastin recruitment and crosslinking into mature elastin matrix. Thus, the objective of the current study is to evaluate the benefits of Cu^{2+} ion delivery concurrent with elastogenic cues (i.e. HA oligomers) on elastin crosslinking in a culture model of adult rat aortic smooth muscle cells (RASMCs). Since sudden exposure of vascular cells to Cu^{2+} ions provided at high doses, via exogenous supplementation of soluble copper salts, appears to induce some cytotoxicity and cell death [19–21], we now seek to determine if gradual release of Cu^{2+} ions from copper nanoparticles (CuNPs) concurrent with HA oligomeric cues can improve recruitment and crosslinking of soluble tropoelastin precursors, and facilitate their assembly into mature fibers. If shown to be beneficial, CuNP cues for elastin maturation will in the future be delivered together with other elastogenic cues (e.g. HA oligomers and growth factors) to simultaneously enhance elastin precursor and matrix synthesis, and maximize the yield of matrix elastin within tissue-engineered constructs.

2. Materials and methods

2.1. Copper ion release from CuNPs

To estimate the amounts of CuNPs (80–100 nm; Sigma–Aldrich, St. Louis, MO) necessary to generate Cu^{2+} ion concentrations of the order of 0.1 M, which in an earlier study [22] were shown to significantly enhance elastin crosslinking, but to be mildly cytotoxic, we fitted the Cu^{2+} ion release profiles experimentally generated with three randomly selected concentrations of (1, 10, and 100 ng ml^{-1}) of CuNPs, to a mathematical model. We quantified the concentration of Cu^{2+} ions in solution using an atomic absorption spectroscope (Perkin-Elmer Model 3030, Perkin-Elmer, Norwalk, CT), fitted with a copper lamp. Briefly, the nanoparticles were dispersed in 5 ml of distilled water (pH 7) at each of the above concentrations. The Cu^{2+} ion content in 1 ml aliquots of these solutions was measured at regular intervals over a 30 day period, and cumulative Cu^{2+} release calculated. The spent aliquots were replaced with 1 ml fresh distilled water, and the concentration of Cu^{2+} ions in the removed aliquots was accounted for in calculating the cumulative release of Cu^{2+} ions. All measurements were done in triplicate and the Cu^{2+} ion concentrations expressed in moles.

The experimental Cu^{2+} release profiles were then fit to a mathematical model, and that model was used to predict a CuNP dose that would cumulatively release approximately 0.1 M of Cu^{2+} ions over the 21 day period of culture. This process eliminated the innumerable "trial-and-error" experiments that would otherwise have been necessary to identify an "effective" CuNP dose. The dependence of Cu^{2+} ion release on CuNP concentration and time was fit using a hierarchical regression analysis, wherein a new predictor is added to or dropped from those used in the previous analysis based on the statistical significance of a particular model. The quadratic regression model which can accommodate linear, curvature and interdependence of the time (x) and CuNP concentration (y) used in this study was:

$$R = a + b^{*}x + c^{*}y + d^{*}x^{*}y + e^{*}x^{2} + f^{*}y^{2}, \quad (1)$$

where R represents the amount of Cu^{2+} ions released, and a–f are the estimated regression coefficients. After initialization with the full quadratic regression model given in Eq. (1), a backward elimination procedure was used to reduce the number of terms in the model until all the remaining terms were statistically significant ($P < 0.05$). Based on this modeling, it was estimated that 400 ng ml^{-1} of CuNPs would cumulatively release ~0.1 M of Cu^{2+} ions over the 21 day culture period, assuming no fouling occurs within the media.

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