GHK-Modified Alginate Hydrogels Enhance VEGF Secretion by Mesenchymal Stem Cells

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Statement of Purpose: Angiogenesis is a critical process to support normal tissue growth and wound healing. The local delivery of potent angiogenic cues such as vascular endothelial growth factor (VEGF) is under investigation for speeding neovascularization, yet the efficacy of this approach is limited due to systemic toxicity, a lack of site-specific delivery, and short half-lives, requiring the administration of supraphysiological concentrations. Mesenchymal stem cells (MSC) secrete bioactive trophic factors, and thus, may provide an effective alternative to the delivery of recombinant angiogenic proteins. The human tripeptide GHK (Gly-His-Lys) is used extensively in various cosmetic products and has distinct biological actions such as increasing protein synthesis of collagen, elastin, and proangiogenic factors such as VEGF, suppression of inflammation, and enhancement of many other wound healing processes [1]. We hypothesized that GHK tripeptide could be effectively incorporated into alginate gels to drive production of these factors by entrapped human MSC.

Methods: Human MSC (D. Prokop, Texas A&M) were expanded from passage 2 in αMEM/10% FBS/1% antibiotics until use. Cells were cultured in growth media supplemented with increasing concentrations (1-500 ng/ml) of GHK peptide (G_4GHKSP, Commonwealth Biotechnologies) for 3 days, and the conditioned media was collected. Toxicity of GHK on MSC was assessed by changes in protein and DNA content, AlamarBlue, and caspase 3/7 activity. Studies were also performed with ammonium tetrathiomolybdate (TM) to bind endogenous copper from the media and test the necessity of the GHK-Cu complex to induce VEGF secretion. Conditioned media from GHK-stimulated MSC were placed on human endothelial colony forming cells (ECFC) to observe proangiogenic potential by measuring tubule formation, ECFC proliferation, and ECFC migration [2]. Alginate hydrogels (MVG, Pronova) were modified with GHK peptide (22 μmol/g alginate) using aqueous carbodiimide chemistry [3], and 2% alginate gels were cross-linked with $CaSO_4$ to entrap 1e6 MSC/ml for culture up to 7 days. Unmodified alginate and RGD-modified alginate gels served as control groups. The ability of MSC to adhere to each gel was measured by seeding cells on the surface and staining with calcein. MSC-secreted VEGF and other angiogenic cues were quantified in conditioned media by ELISA and angiogenic protein array.

Results: The addition of GHK to MSC was not cytotoxic at any concentration, as observed by consistent levels in protein concentration and DNA content. We detected small decreases in caspase activity with increasing GHK dose. MSC secreted VEGF in a dose-dependent manner when stimulated with GHK in the culture medium, and other proangiogenic trophic factors such as bFGF and angiogenin were significantly upregulated compared to untreated MSC. We observed a 70% increase in VEGF secretion at 100 and 500 ng/ml GHK after 3 days compared to control MSC. Conditioned media from stimulated MSC induced corresponding increases in ECFC proliferation, migration, and tubule formation on Matrigel (**Fig. 1**). The addition of a panVEGF antibody to ECFC cultures negated detectable differences in ECFC proliferation, demonstrating the bioactivity of secreted VEGF by GHK-stimulated MSC. GHK was successfully incorporated onto the backbone of alginate using identical protocols for RGD, used as a control reaction. MSC successfully adhered and spread on RGD-modified alginate when seeded on the surface, while MSC in GHK-modified and control gels did not adhere. When

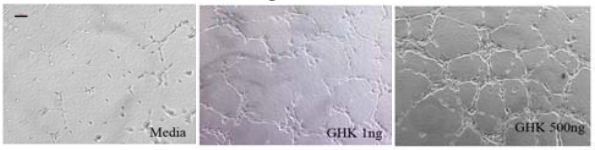

Figure 1. Tubule formation is increased with conditioned media from MSC stimulated with increasing GHK dose.

suspended in each gel, we measured increased VEGF secretion by MSC in GHK-modified gels compared to RGD- or control gels as early as 1 day and increasing over 7 days (**Fig. 2**). Trends in VEGF production were identical when TM was added to the media, yet VEGF

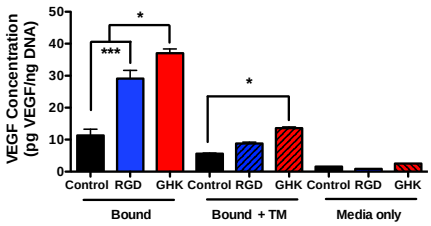

Figure 2. VEGF secretion is increased from MSC suspended in GHK-modified alginate gels.

levels were significantly lower, thereby demonstrating the importance of an available copper source to drive VEGF production. The addition of soluble RGD or GHK had little effect on VEGF secretion by MSC in control gels.

Conclusions: These data demonstrate the ability of the GHK peptide to enhance trophic factor secretion by human MSC. VEGF secretion and other angiogenic proteins are increased. The lack of toxicity observed in MSC stimulated with increasing GHK dose gives flexibility in translating its delivery to hydrogels where it is difficult to ascertain the true peptide concentration presented to entrapped cells. The availability of copper to enable the formation of the GHK-Cu complex appears necessary in 3D culture. Ongoing studies include assessing the ability of GHK-modified gels to promote angiogenesis and bone repair *in vivo*.

References:

1. Pickart L. J Biomat Sci 2008;19:969-88.
2. Decaris ML. Angiogenesis 2009;12:303-11.
3. Rowley JA. Biomaterials. 1999;20:45-53.