A Modular, Hydroxyapatite-Binding Version of Vascular Endothelial Growth Factor

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Statement of Purpose: Hydroxyapatite (HAP) has been extensively used in orthopedics and dentistry since it promotes bone healing by improving bone integration. Although successful to a certain extent, HAP constructs often fail to complete proper bone regeneration. Therefore, inclusion of biologically active growth factors becomes highly desirable to more actively induce bone formation. Among them, pro-angiogenic vascular endothelial growth factor (VEGF) is particularly important in bone tissue engineering since vascular formation is imperative to facilitate the bone healing process. In this context, we present a modular, HAP-binding version of VEGF (mVEGF), which mimics the HAP-binding ability of protein osteocalcin (OCN) and the pro-angiogenic properties of a VEGF mimetic peptide. We chose 9 amino acid sequence from OCN, of which three γ-carboxylic glutamic acids (Gla, γE) are known to directly contribute to HAP binding.[1] The VEGF-mimetic sequence was adopted from previous work,[2] where it was shown to mimic VEGF's biological activity. We hypothesized that mVEGF would non-covalently bind to the HAP surface and promote pro-angiogenic activities including endothelial cell proliferation and migration.

Table 1. Amino acid sequence of synthetic peptide

Name	Amino acid sequence
mVEGF	KLTWQELYQLKYKGI-GGGAAAA-γEPRRγEVAγEL
VEGF-mimetic peptide	KLTWQELYQLKYKGI[1]
OCN-inspired sequence	γEPRRγEVAγEL

Methods: mVEGF and VEGF-mimetic peptide were prepared using Fmoc solid phase peptide synthesis. For fluorescent labeling, 5(6)-carboxyfluorescein was conjugated to the N-terminus of synthesized peptides. Peptide-HAP binding test was conducted by incubating HAP particles; (i) in different peptide concentration solutions for 30 min, or (ii) in 20 μM peptide solution for different periods. Biological activity of synthesized peptides was examined both in their soluble form and when immobilized on HAP substrate, and characterized by endothelial cell proliferation and migration (scratch wound healing) assays using C166-GFP endothelial cells. Endothelial cell proliferation assays were conducted by (i) treating the adhered cells with cell culture media containing soluble synthesized peptides, or (ii) culturing cells on peptide-immobilized HAP substrate. After 48 hour in culture, the cell numbers were quantified with CyQuant cell proliferation assay kit (invitrogen, Carlsbad, CA). In some experiments, cells were pre-treated with SU5416 (Sigma-Aldrich, St. Louis, MO), a pharmacological VEGF receptor inhibitor, to block VEGF receptor activation. For scratch wound healing assays, a scratch was made on endothelial cell monolayers formed on either cell culture plates or HAP slabs. After 24 hours, cell migration was assessed by counting cell numbers in the scratched area or by fluorescence imaging.

Results: The mVEGF peptide bound rapidly to HAP in a concentration-dependent manner (Figure 1a-b). The HAP

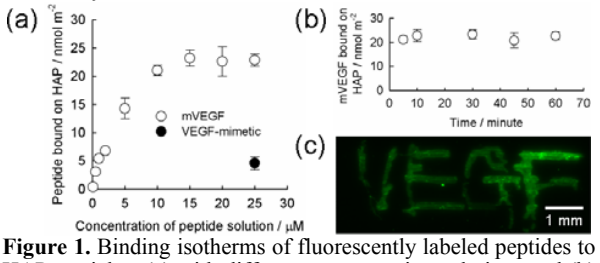

Figure 1. Binding isotherms of fluorescently labeled peptides to HAP particles; (a) with different concentration solutions and (b) with different incubation times. (c) Fluorescence micrographs of "VEGF" written with mVEGF solution on a HAP slab.

binding occurred more efficiently with mVEGF due to the presence of OCN-inspired portion. We could bind mVEGF locally, as demonstrated by writing "VEGF" with mVEGF solution on a HAP substrate (Figure 1c). The mVEGF and VEGF-mimetic peptides were each biologically active to a similar degree when added to cell culture media, confirmed by enhanced endothelial cell proliferation and migration compared with untreated control (data not shown). This result indicates that the presence of the OCN-inspired sequence did not negatively affect the bioactivity of VEGF-mimetic sequence. Importantly, the mVEGF retains pro-angiogenic activities when immobilized on HAP biomaterials (Figure 2). The mVEGF promoted endothelial cell proliferation and migration on a HAP surface, while the VEGF-mimetic peptide did not. The biological functions induced by mVEGF were not observed when the cells were pre-treated with the VEGF inhibitor SU5416, indicating that the cell response triggered by immobilized mVEGF is mediated via VEGF receptor activation.

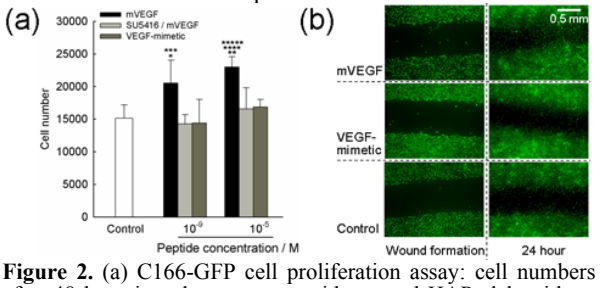

Figure 2. (a) C166-GFP cell proliferation assay: cell numbers after 48 hour in culture on a peptide-treated HAP slab with or without SU5416 treatment. (b) C166-GFP cell migration assay: Fluorescence micrographs after 24 hour in culture on a HAP slab after wound was created. F solution on a HAP slab.

Conclusions: The mVEGF peptide bound rapidly and efficiently to HAP, and induced early pro-angiogenic activities, including endothelial cell proliferation and migration both in the soluble form and when immobilized on a HAP biomaterial. The concept of the modular peptide growth factor may be generally applicable to a range of boimaterials by adopting amino acid sequences that bind to specific substrates.

References

[1] Hoang QQ. Nature. 2003;425:977-980.
[2] D'Andrea LD. Proc Natl Acad Sci USA. 2005;102:14215-14220.