Multivalent Sonic Hedgehog as an Enhanced Potency Biomaterial Modification for Angiogenesis

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Introduction: The incorporation of growth factors into biomaterials to elicit tissue specific response is a recurring theme in tissue engineering. Here we investigate the role that the angiogenic protein Sonic Hedgehog (Shh) can play in tissue engineering applications when presented in a multivalent manner. Recombinant multivalent Shh was produced by biogconjugation of the protein to hyaluronic acid (HA). Bioactivity of the conjugates was substantially enhanced by increasing the stoichiometric ratio of Shh molecules to HA linear chains. In addition, these constructs also increased angiogenesis as evaluated by the chick chorioallantoic membrane (CAM) assay and can be incorporated into tissue engineering hydrogels to add angiogenic bioactivity.

Methods: The N-terminal signaling domain of Shh was recombinantly produced from full length rat cDNA as previously described,[1] with the addition of a cysteine residue on the c-terminus for sulfhydryl reactions. To create the bioconjugates, 10^6 Da MW HA (Genzyme, Cambridge MA) was first reacted with EDC, sulfo-NHS, and EMCH (Pierce Biotechnology, Rockford, Il) overnight in 0.1 M MES buffer, pH 5.0 to introduce maleimide functionality to the HA chains. This was then reacted overnight at 4°C in .1 M MES buffer (pH 6.5) with the Shh in stoichiometric amounts to produce conjugates of varying molecular substitution. The conjugates were assayed by gel electrophoresis to assay efficiency.

In order to test bioactivity, C3H10T1/2 cells (American Type Culture Collection, Manassas VA) were induced to differentiate by exposure to Shh.[2] The cells were plated at 5000 cells / well in 96 well plates in moral growth media (10% FBS). After 2 days, the media was replaced with a low FBS (2%) media supplemented with Shh and conjugates in the 1 – 100 nM range. After incubation for an additional 3 days, the cells were washed, lysed and assayed for differentiation by measuring alkaline phosphatase (ALP) activity using the fluorescent probe 9-H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate (DDAO, Molecular Probes, Eugene OR).

As Shh is a known angiogenic agent,[3] induced angiogenesis was assayed using the CAM assay. Fertile white leghorn eggs (Charles River, Franklin CT) were incubated until day 8, at which time a small window was made in the shell and test materials placed on the developing CAM. Angiogenesis was measured by photomicrography after an additional 3 days of incubation.

Results: Conjugates were efficiently produced in the 1:1 HA:Shh - 1:30 HA:Shh range. Bioactivity testing showed a substantial decrease in activity for the low ratio conjugates, from 1:1 up to 1:10, possibly due to steric inference of the large tethered HA molecule. However, as ratios increased past 1:10, there was a substantial increase in potency of the materials, with a 50 fold decrease in EC_{50} between the soluble Shh and the 1:30 HA:Shh conjugate (Figure 1).

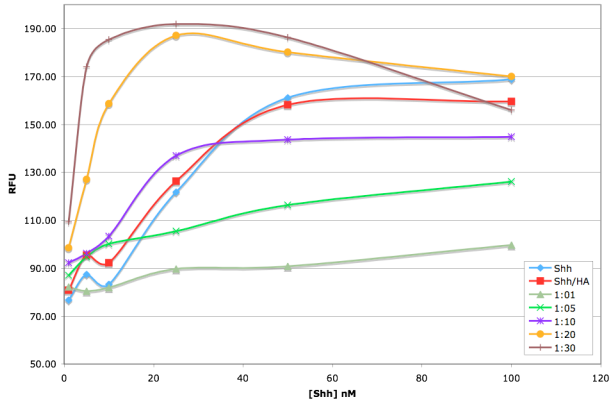

Figure 1. C3H10T1/2 cellular response to soluble Shh and HA : Shh conjugates as measured by AP DDAO cleavage.

CAM angiogenesis assays also demonstrated increased potency of the multivalent conjugates as compared to soluble forms.

Discussion: The produced Shh-conjugates show a clear trend of increased potency with increasing number of protein molecules attached to a long molecular backbone. Such results suggest that multiple binding events on a single cell increase the measured cellular response and that multivalent conjugates are able to bind more effectively to surface receptors due to multiple interactions across the cell surface.

This is an important finding for tissue engineering applications, as solid state addition of signals to a material may have dramatically different activities than soluble factors based on how they are presented to target cells and tissues mode of presentation. In addition, the use of multivalent protein signals offers a way of increasing the potency of soluble signals *in vivo*.

References:

1. Lai, K. *Nature Neurosci* **2003,** 6, (1), 21-27
2. Pepinsky, R. B. *JBC* **1998,** 273, (22), 14037-14045
3. Pola, R. *Nature Med* **2001,** 7, (6), 706