Fibronectin Conjugated TGF-β1 via a PEG Linker: Bioactivity of the Surface-Bound Conjugate

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Statement of Purpose:

Previous work tethered transforming growth factor-β1 (TGF-β) to fibronectin (FN) using a heterobifunctional maleimide-N-hyrdroxysuccinimide poly(ethylene glycol) (MAL-NHS-PEG) chain. Activity of soluble TGF-β-PEG-FN was confirmed using a Mv1Lu growth inhibition assay, but activity of the surface-bound conjugate could not be verified, presumably due to low product yield and purification methods.

The current study focuses on validating the activity of surface-bound TGF-β-PEG-NHS conjugate using a MC3T3-E1 alkaline phosphatase (ALP) inhibition assay.[1] The conjugate was purified using dialysis and immunoprecipitation techniques, which would lead to a higher product yield and increased activity of the surface-bound conjugate.

Methods:

A 500 µg/ml human TGF-β (R&D Systems) solution in PBS, pH 7.4 was reacted with a heterobifunctional MAL-NHS-PEG, 3400 MW (Nektar Therapeutics) in a 1:2 molar ratio. The reaction occurred over 1 hour at room temperature, and was quenched with glycine in 5-fold excess over MAL-NHS-PEG. Controls consisted of non-derivatized 3400 MW PEG (Sigma-Aldrich) in lieu of the MAL-NHS-PEG. Concurrently, FN (0.1% solution, Sigma-Aldrich) was denatured in an 8 M guanidine hydrochloride solution for 1 hour. The sample solution was immediately added to the denatured FN solution at a molar ratio of TGF-β to FN of 1:5. The reaction proceeded at room temperature and pH 7.4 overnight with subsequent cystein quenching in 5-fold excess of PEG. Unreacted TGF-β and PEG were removed by overnight dialysis against PBS using a 100,000 MW membrane, with the dialysate changed after 2 and 4 hours. The remaining conjugate was filter sterilized using a 0.22 µm filter.

Immunoprecipitation was utilized to further isolate the conjugate using a protein G immunoprecipitation kit (Sigma-Aldrich). Briefly, samples were incubated with 1 µg/ml anti-hTGF-β (R&D Systems) in a spin column for 1 hour at 4 ºC. Protein G-Agarose beads were incubated with the sample for 1 hour at 4 ºC and then centrifuged in an ultracentrifuge at 12,000 g for 30 seconds. The supernatant was discarded and the precipitant was resuspended in PBS. The conjugate was then seeded onto a crosslinked fibrin gel for 3 hours at room temperature whereby the FN would interact with the fibrin gel.

MC3T3-E1 cells (ATCC) were cultured in α-MEM (Gibco) with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% FBS (HyClone), and 1% penicillin/streptomycin (Invitrogen). After 48 hours, MC3T3-E1 cells were enzymatically lifted, plated onto fibrin coated plates with either the growth factor conjugate or fibronectin alone, and cultured in α-MEM (Gibco) with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.2% FBS (HyClone), and 1% penicillin/streptomycin (Invitrogen) for 48 hours. ALP activity was assessed using a purchased ALP ELISA kit (AnaSpec) at day 0 and 2 of TGF-β conjugate culture.

Results / Discussion:

SDS-PAGE analysis shown in Figure 1 indicates that TGF-β was tethered to FN through the PEG linker prior to immunoprecipitation. The TGF-β-PEG-FN conjugate corresponds to the 250 kDa band while the other two bands could correspond to FN or FN-PEG fragments. Controls consisted of non-derivatized PEG carried through the same reaction steps.

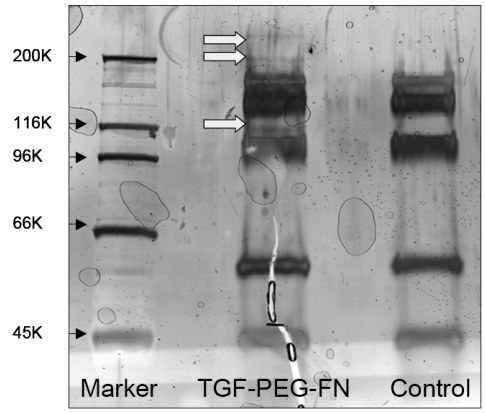

Figure 1: SDS-PAGE image of tethered PEG

Soluble TGF-β-PEG-FN was able to inhibit Mv1Lu growth in a dose-dependent manner as measured by calcein dye (see Figure 2). Controls consisted of non-derivatized PEG carried through the same reactions steps while standards were TGF-β added into the culture media. Similar amount of inhibition were observed between the tethered TGF-β and soluble TGF-β.

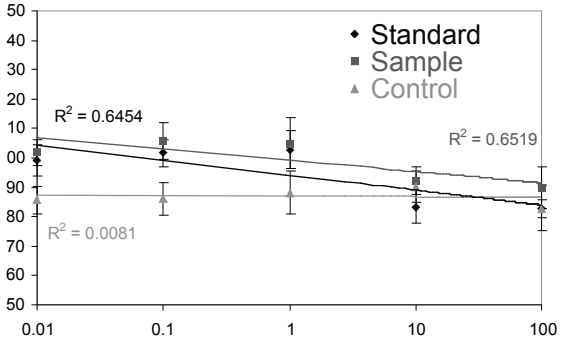

Figure 2: Mv1Lu inhibition by soluble TGF-β-PEG-FN

Conclusions:

TGβ- can be tethered to FN through a heterobifunctional MAL-PEG-NHS linker and retain its activity. Further investigation is needed to increase the product yield of the conjugate to ensure preservation of the surface-bound TGF-β-PEG-FN conjugate activity.

References:

1. Iu MF, et al. Journal of Endocrinology 2005;185(1).