Evaluation of the Osteogenic Activity of Injectable Bovine Lactoferrin Gel

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Statement of Purpose: Lactoferrin (LF), a 78 kDa iron-binding glycoprotein, possesses multifold functions, including iron homeostasis, angiogenesis and immuno-modulation [1]. Of the biological functions credited to LF, it is also recognized for its influential role in bone growth, thus implicating this glycoprotein as a potential target for skeletal regeneration. Thusfar, our lab has clearly demonstrated the bioactivity and osteogenic activity of LF *in vitro* and *in vivo*. LF's anabolic effect in bone morphogenesis is attributed to its potent dose-dependent proliferative and anti-apoptotic actions of osteoblastogenesis and inhibition of osteoclastogenesis [2]. This protein has also been shown to reduce bone resorption and increase bone mass *in vivo*.

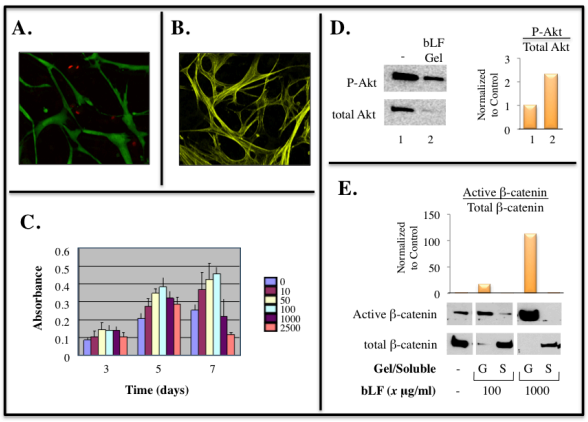
There is a critical need for better clinical treatments of bone tissue defects. Today, the synergistic use of biomaterials with cells and growth factors provides a great alternative treatment for bone regeneration. Injectable biomaterials that can favorably modulate the biological functions of the encapsulated cells have great potential to improve cell-based therapeutic strategies. To this end, our laboratory is the first to utilize the biological functions of LF for the development of a bioactive, injectable biomaterial [3]. Based on the reported biological actions and preliminary studies, we hypothesized that cell encapsulation in bovine LF (bLF) gel will favorably modulate the mitogenic, anti-apoptotic and osteogenic effects of encapsulated cells.

Methods: The synthesis of the *in situ*—forming, biodegradable bLF gel utilizes standard carboiimide chemistry and requires the chemical modification of bLF with the addition of tyramine moieties, which provides additional enzymatic crosslinking reactive sites. The bLF gels are obtained by enzymatic crosslinking of the modified rhLF under the presence of peroxidase, as the catalyst, and H_2O_2, as the oxidant.

Cell viability of the bLF gel – encapsulated human mesenchymal stem cells (HMSCs) was qualitatively assessed using Live/Dead stain, which is used to measure membrane integrity and cellular esterase activity. To image the cell spreading, the actin was stained using Alexa Fluor 488 phalloidin. Proliferation studies were performed using MTS proliferation assay.

Western blot analysis was used to investigate the activation of β catenin and phosphorylation levels of Akt by bLF in soluble and gel form relative to control (cell on tissue culture plate with no bLF treatment). Equal volumes of protein lysate were loaded on gel and immunoblot was conducted with the appropriate antibodies.

Results: Gelation time of the bLF gels was directly correlated with the concentration of the modified bLF. Encapsulation of HMSCs in bLF gel resulted in high cell viability after 10 days of culture (Figure A). Furthermore, our studies have demonstrated the ability of the bLF gel to support 3D cell organization of HMSCs in the *in situ*—forming bLF gel after 10 days of culture (Figure B). The soluble form of bLF induced significant proliferation of HMCSs. Greatest proliferation was induced by 100µg/ml bLF on day 7 (Figure C). The bioactivity of the bLF gel was further evidenced by western blot analysis of murine MC3T3-E1 preosteoblastic cells. MC3T3 cells encapsulated in bLF gel after 28 days demonstrated a significant stimulation of Akt phosphorylation, which is a known marker for cell survival (Figure D). MC3T3 cells encapsulated in 100µg/ml bLF gel demonstrated approximately a 2-fold increase in phosphorylation levels of Akt relative to control cells after 28 days.



After establishing the increased mitogenic and pro-survival effects of bLF on MC3T3 cells, we then investigated the osteogenic properties of bLF by analyzing the activation levels of β-catenin being the penultimate signaling factor in the Wnt osteoblast pathway. The encapsulation of MC3T3 cells in bLF gel significantly induced the activation of β-catenin (Figure E). When evaluating the effect of bLF in a cross-linked gel versus soluble form, there is a substantiated effect of the bLF gel. This is likely due to the localized bLF within the biomaterial providing a controlled and sustainable influence on cell behavior verses the transient effect of soluble bLF caused by the internalization of the ligand/receptor complex.

Conclusions: This study has successfully demonstrated the feasibility of LF gel formation from functionalized tyramine groups of LF and the use of HRP in the presence of H_2O_2. We have demonstrated the significant anti-apoptotic and osteogenic effects bLF gel. The present study increased our understanding of bLF in modulating skeletal cells and thus helps to further establish LF gel as a good candidate for a skeletal regenerative biomaterial.

References

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2. Cornish J et al. 2004. Endocrinology 145 (9): 4366-4374.
3. Nair LS, U.S. Patent Application No. 61/322,935.