Reconstitution of Sonic Hedgehog Signaling Accelerate Fracture Healing via Recruitment of Endothelial Progenitor Cells

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Introduction: Hedgehog signals are crucial regulators of organ and skeletal development during embryogenesis. Recent data suggest that skeletal regeneration of adult is similar to fetal skeletal development, and the regenerative capacity of adult tissue may depend on the re-induction of the embryonic signals (1). On the other hand, fracture healing process is reported to be delayed based on the failure to express genes adequately regulating osteoblast differentiation and neovascularization in patients with diabetes mellitus (DM) (2). We have recently reported that mobilization and incorporation of bone marrow (BM)-derived endothelial progenitor cells (EPCs) contributed to fracture healing (3). In this study, we therefore tested the hypothesis that gene therapy of Sonic Hedgehog (Shh), which promotes bone marrow-derived EPC proliferation, migration and VEGF production (4), may have a therapeutic potential for fracture healing in type 1 DM mice.

Materials and Methods: Type 1 DM mice: We induced diabetes by 250mg/kg Streptozotosin intraperitoneal injection and confirmed DM by over 300mg/dl for 4 weeks of blood glucose level.

Preparation of pCS2-Shh plasmid: We prepared pCS2-Shh naked plasmid and checked its expression level.

Femoral fracture model and gene delivery: Femoral fracture was created in non-DM and DM mice (C57BL6J, 8-week-old). Then we delivered pCS2-Shh plasmid or pCS2-empty plasmid to the fracture site in DM mice. We established 4 groups: non-DM mice (nonDM), DM pCS2 empty plasmid injected mice (DM-Emp), DM pCS2-Shh 25ug injected mice (DM-Shh25) and DM pCS2-Shh 50ug injected mice (DM-Shh50).

Histology and osteoblast differentiation and mineralization: We demonstrated mouse calvarial osteoblast culture assay to evaluate the effect of Shh peptide to the osteoblast differentiation and mineralization. Osteoblasts were cultured for 21 days in the osteogenic condition medium supplemented with Shh peptide 0, 0.5, 1 and 5ug/ml.

Results: DM mice exhibited less expression of Shh at fracture site: Quantitative RT-PCR (qPCR) of tissue around the fracture site demonstrated that expression of Shh was significantly higher at week 1 in nonDM mice than DM mice (Shh gene expression level/GAPDH: nonDM, 449.2±91.3; DM, 98.9±64.4; p<0.05).

Radiography and histological examination of fracture healing: In animals receiving Shh plasmid, fracture radiographically healed with bridging callus formation about 1 week earlier than DM-Emp group (Fig. 1). In histological evaluation, the degree of fracture healing assessed by Allen's classification (5) were significantly greater in DM-Shh25 and DM-Shh50 groups than DM-Emp group at week 1, 2 and 3.

Shh accelerates neovascularization: Vascular staining with isolectin B4 at week 1 showed significantly higher in DM mice receiving Shh as well as nonDM group compared with DM-Emp group. Shh up-regulates osteo- and angiogenesis related gene: The gene expression of BMP-2 and VEGF around the fracture sites by qPCR were significantly higher in the DM-Shh50 group than DM-Emp group, reaching to equivalent level of nonDM group.

Shh recruits EPCs to the fracture site: We used BMT model to obtain direct evidence of enhanced BM-derived EPCs incorporation into foci of neovascularization at the fracture site. Immunohistochemical staining for β-gal and Isolectin B4 demonstrated that tissue samples 1 week post-fracture showed a significant increase in cells expressing β-gal/Tie-2 in fracture site of DM-Shh25 and 50 group as well as nonDM group compared with those of DM-Emp group (nonDM, 103.5±1.2; DM-Emp, 61.0±1.1; DM-Shh25, 92.3±3.2 respectively. P<0.05 for nonDM vs. DM-Emp and DM-Emp vs. DM-Shh25).

Shh promotes mineralization in osteoblasts: ALP activity was significantly higher dose dependently in Shh peptide containing groups. Arizain red staining demonstrated more amount of calcium deposits in culture with Shh peptide. Moreover, qPCR analysis demonstrated that gene expression of osteonectin and collagen 1 A1 were also significantly higher dose dependently in culture with Shh peptide than without Shh.

Discussion: Sonic Hedgehog gene therapy may have a therapeutic potential for fracture healing regulating osteoblast differentiation, maturation and neovascularization, especially via BMP-2 upregulation and BM-derived EPC recruitment, in type 1 DM mice.

References:

Fig. 1: Radiographs at week 2