Hedgehog Signaling in Bone Fracture Healing
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Introduction: Bone repair is a stem/progenitor cell-driven process that requires the precise temporal and spatial presentation of factors to control their proliferation and differentiation at the injury sites. Periosteum plays a key role in bone repair via activation of residing stem/progenitor cells. The molecular signals regulating differentiation and expansion of periosteal stem cells during early repair are poorly understood. A thorough understanding of the spatial and temporal presentation of factors directing adult stem cell mediated bone repair is vital for the success of repair and regenerative therapies.

Hedgehog (Hh) signaling has been critically implicated in skeletal development. Recent studies further suggest that Hh proteins play important roles in mesenchymal stem cell differentiation as well as in intramembranous and endochondral bone formation. Deletion of Ihh in postnatal growth plate resulted in marked deformation of growth plate and shortened stature. It has been reported that Ihh pathway is induced during endochondral bone repair. However, the perinatal mortality of the knockout mouse severely limits our understanding of Hh signaling in adult bone repair. In addition, the detailed information about temporal and spatial presentation of Hh signals and signaling molecules during adult bone repair remain elusive. In our current study we used real time PCR and in situ hybridization to determine the expression of Hh ligands and the related signaling molecules during the course of bone fracture healing. We also utilized a heterozygote Ptc1-LacZ mouse model in which LacZ expression represents the endogenous Hh signaling during repair. Our data showed that Hh signaling was induced as early as day 5 during endochondral bone repair, primarily in periosteal progenitor cells including chondroprogenitors, osteoprogenitors and vascular progenitors surrounding the prehypertrophic chondrocytes. Furthermore, periosteal progenitor cells isolated from day 5 callus demonstrated enhanced osteogenesis when treated with Hh signaling peptides, and further synergized with BMP-2. Collectively, our data suggest that Hh signaling may play an important role in periosteal callus formation and morphogenesis.

Materials and Methods: Femur fracture healing model: Femur fracture was created in C57BL6 mice and in Ptc1-LacZ mice using standard method. A stainless steel 25G spinal needle was inserted into the intramedullary space of the femur, followed by three-point bending with an Einhorn device. Mice were sacrificed post fracture at 0, 3, 5, 7, 10, 14 days. Samples were pooled and real-time PCR was used to assess Ihh, Shh, Ptc1, Gli1, Gli2, Gli3, Smoothened, col2, colX, osteocalcin expressions. In situ hybridization was performed to determine Shh, Ihh, Gli1, Gli2, Gli3, col2 and colX expression during repair using standard protocol.

LacZ staining: LacZ staining was conducted in the frozen sections of the fracture callus harvested from Ptc1-LacZ mice using previously published protocol.

Periosteal cell preparation: Periosteal cells were isolated from day 5 autografted bone callus using a method established in our laboratory. Cells were seeded and cultured as monolayer in aMEM. Adherent cells were treated with BMP-2 and/or ShhN peptide for 7 days to examine osteogenesis. RNA was harvested and gene expression of Runx2 and OSX were examined.

Results: Real time PCR analyses showed that Ihh, primarily Ihh was upregulated as early as day 5 and peaked at day 10 post-fracture. Gli1, 2 and Ptc-1 were also markedly induced. Interestingly, Gli1 and Ptc-1 induction paralleled with Ihh expression predominantly in the chondrogenic phase of healing, whereas Gli2 expression remained elevated up to 14 days, spanning both the chondrogenic phase and bone formation phase. Smoothened, the receptor for all Hh signals was induced at day 3 and remained elevated until day 21. Notably, the induction of Gli2 and Smoothened also paralleled with that of Runx2, BMP-2 and 4 gene expression, indicating a potential role of Hh signaling in modulating the expression of these genes. Shh gene expression was low and not regulated during fracture repair.

In-situ hybridization was performed to determine the spatial and temporal regulation of Hh pathway in early repair. Shh was not detected in the callus or its surrounding tissues, whereas Ihh gene expression was localized to prehypertrophic and hypertrophic chondrocytes. Strong Ihh signal was detected as early as day 5 along periosteum in early prehypertrophic chondrocytes. Expression was increased at 7 and peaked at day 10.

Using heterozygote Ptc1-LacZ mice, we examined the endogenous Hh signaling during fracture healing. X-Gal staining showed that weak Ptc1 signal was detected in sporadic periosteal cells at day 3. At days 5 and 7, Ptc1 signals were markedly increased and were found in mesenchymal cells, early chondroprogenitors, proliferating chondrocytes and early vascular progenitor cells within or around hypertrophic chondrocytes (Ihh sending cells). Ptc1-LacZ was also found in early osteoblast progenitors in intramembranous new woven bone. By day 10, Ptc1 was found in the invading vessels as well as in hypertrophic chondrocytes associated with bone formation. At day 14, Ptc1 signals can be found in a few osteoblasts along newly formed trabeculae.

Periosteal progenitors cells were isolated from the day 5 callus and cultured as monolayer. Cells were treated with 250ng/ml ShhN peptide and/or 50ng/ml BMP-2. ShhN peptide markedly increased alkaline phosphatase activity in monolayer and further synergized with BMP-2. RNA analyses demonstrated that ShhN increased Runx2 and OSX mRNA expression and synergized with BMP-2 in stimulation of OSX gene expression.

Discussion: The role of Hh in bone repair and regeneration has been proposed. Studies using experimental rat demonstrated that Ihh expression was markedly reduced in aged rat, correlating with decreased BMP-2 expression. Our current study provide further evidence to demonstrated that Hh signaling, specifically Ihh signaling may play key role in the early periosteal bone healing. The Ptc1-LacZ staining corroborates in situ localization for Ihh, strongly suggesting a “morphogenetic” role for Ihh in adult repair. Localization of Hh signaling in early progenitors is particularly interesting due to the fundamental role of the Hh pathway in controlling proliferation and differentiation of progenitors throughout development. The marked effects of Hh and BMP-2 in synergy to induce osteoblastic differentiation of periosteal progenitor cells further suggest that stimulating Hh pathway may present a novel therapy to enhance repair and regeneration.


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