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Roles of Wnt/β-catenin signalling pathway in the bony repair of injured growth plate cartilage in young rats

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ABSTRACT

Growth plate cartilage is responsible for longitudinal growth of the long bone in children, and its injury is often repaired by bony tissue, which can cause limb length discrepancy and/or bone angulation deformities. Whilst earlier studies with a rat growth plate injury repair model have identified inflammatory, mesenchymal infiltration, osteogenesis and remodeling responses, the molecular mechanisms involved in the bony repair remain unknown. Since our recent microarray study has strongly suggested involvement of Wnt–β-catenin signalling pathway in regulating the growth plate repair and the pathway is known to play a crucial role in the osteogenic differentiation of mesenchymal progenitor cells, the current study investigated the potential roles of Wnt–β-catenin signalling pathway in the bony repair of injured tibial growth plate in rats. Immunohistochemical analysis of the growth plate injury site revealed β-catenin immunopositive cells within the growth plate injury site. Treatment of the injured rats with the β-catenin inhibitor ICG-001 (oral gavage at 200 mg/kg/day for 8 days, commenced at day 2 post injury) enhanced COL2A1 gene expression (by qRT-PCR) and increased proportion of cartilage tissue (by histological analysis), but decreased level of osterix expression and amount of bone tissue, at the injury site by day 10 post-injury ($n=8$, $P<0.01$ compared to vehicle controls). Consistently, in vitro studies with bone marrow stromal cells from normal rats showed that β-catenin inhibitor ICG-001 dose dependently inhibited expression of Wnt target genes Cyclin D1 and survivin ($P<0.01$). At 25 mM, ICG-001 suppressed osteogenic (by CFU-f-ALP assay) but enhanced chondrogenic (by pellet culture) differentiation. These results suggest that Wnt/β-catenin signalling pathway is involved in regulating growth plate injury repair by promoting osteoblastogenesis, and that intervention of this signalling could represent a potential approach in enhancing cartilage repair after growth plate injury.

Introduction

The growth plate is responsible for achieving the longitudinal growth of all immature long bones via the process of endochondral ossification through which calcified hypertrophic cartilage is firstly produced and then converted to trabecular bone [1]. However, with its fragile cartilaginous nature, the growth plate is a common site of injury. Due to the poor regenerative ability of cartilage and the dynamic nature of an immature skeleton, growth plate injuries often result in significant life-long orthopedic problems such as limb length discrepancy and bone angulation deformity. Clinical and experimental studies have shown that the cause of these orthopedic problems is the bony repair tissue within the growth plate injury site as observed clinically in approximately 30% of child and adolescent patients with growth plate injuries particularly with the Salter-Harris types 3, 4 and 5 injuries [2]. Current methods for correcting these problems resulting from growth plate injuries are surgically based and hence invasive, prone to infections and with variable success rates. With the absence of a biological treatment, gaining a greater understanding of the molecular and cellular events mediating the unwanted bony repair of the injured growth plate is imperative.

Previous studies in a rat growth plate injury model have identified four main phases of repair responses following growth plate injury, namely the inflammatory, fibrogenic, osteogenic and remodeling phases at days 1–4, 5–8, 8–14, and 14 onwards post-injury, respectively [3]. In particular, the earlier fibrogenic phase involves an influx of mesenchymal cells containing potential mesenchymal stromal progenitor cells (MSCs) and/or osteoprogenitor cells, pre-osteoblasts and pre-chondrogenic cell types that have been shown to have the capability of differentiating into both (bone-forming) osteoblasts and chondrocytes [3–6]. Previous studies have reported direct bone formation (via intramembranous

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ossification) and to a lesser extent the indirect bone formation (via endochondral ossification) as mechanisms for osteogenic repair outcome from these infiltrated progenitor cells [3,5–7]. However, the exact molecular and cellular events leading towards this unwanted bony repair formation are yet to be clarified.

More recently, a micro-array study reported some differentially expressed genes and highlighted some potentially involved pathways with growth plate injury site specimens at various stages of injury repair [8]. During the late inflammatory to earlier fibrogenic phase, Wnt/β-catenin signalling pathway (a key pathway known critical for osteogenesis) was found to be one most significantly involved pathway following growth plate injury [8]. In particular, a 2-fold increase of β-catenin together with a 3.3- to 3.5-fold decrease of inhibitors or antagonists of Wnt signalling including sFRP-1 were seen on day 4 post-injury in comparison to day 8 post-injury [8]. This finding suggests a potential significant role for Wnt/β-catenin signalling during the early mesenchymal response in comparison to a later time point day 8 where early bone formation is taking place. The Wnt/β-catenin signalling pathway has been demonstrated as critical in the positive regulation of osteoblastogenesis of mesenchymal progenitor cells and bone mass [9–14]. The Wnt/β-catenin signalling pathway is also known to be crucial for osteoblast differentiation during bone fracture repair [15]. Skeletal injuries have been reported to activate endogenous Wnt signalling, triggering a cascade of events leading to the expansion of the skeletal stem/progenitor pool, bone formation and hence fracture repair [13,16].

However, it still remains unknown whether the Wnt/β-catenin signalling plays a role during the bony repair of injured growth plate. Hence as a step to investigate the potential role of Wnt/β-catenin in regulating growth plate injury repair, the current study examined treatment effects with a known Wnt/β-catenin inhibitor, ICG-001 [17], in a well-established rat growth plate injury model [3] as well as in osteogenic and chondrogenic differentiation assays with isolated bone marrow stromal cells. Previously, the low molecular-weight inhibitor, ICG-001, has been shown to successfully and selectively inhibit β-catenin/TCF mediated transcription by directly competing with β-catenin to bind to the CREB-binding protein (CBP) [17].

Methods and materials

Growth plate injury and treatment trial

Forty 6-week-old male *Sprague Dawley* rats were randomly placed in three different groups, namely the treatment group receiving β-catenin inhibitor ICG-001, vehicle control group and a non-treated non-injured control group. All the rats from the treatment and vehicle groups were subjected to experimental growth plate injuries in the proximal tibia of both hind legs. Injury was inflicted using the drill-hole method, disrupting the central part of the proximal tibial growth plate cartilage under anesthesia as described by Zhou et al. [18] and with approval from the Animal Ethics Committee of the Institute of Medical and Veterinary Sciences (IMVS), South Australia. Briefly, under halothane anesthesia, an incision to the anterior-medial aspect of the proximal tibial and growth plate was made. A 2 mm dental drill was then used to create a cortical window in the metaphyseal bone and a central disruption to the growth plate was made by drilling through the window and perpendicular to the growth plate cartilage. The injury site and tract were then thoroughly rinsed with saline for irrigation before wound closure. Rats were gavaged with vehicle (olive oil) or ICG-001 (synthesized by Drs A Piergentili, F Del Bello, and W Quaglia, Universita di Camerino, Italy) at 200 mg/kg/rat dissolved in olive oil (gavaged at 0.5 mL/100 g body weight). ICG-001 or vehicle administration commenced at day 1 post-injury and continued until day 4 (for specimen collection at day 5) or until day 9 (for specimen collection at day 10).

Rats ($n = 8$ per group and per time point) were sacrificed for specimen collection at days 5 and 10 post-surgery, two time-points found suitable to observe the growth plate injury repair responses during fibrogenic and osteogenic phases respectively. Both tibias were dissected and cleared of soft tissues. The right proximal tibia was manually snap-opened gently at the metaphyseal/growth plate border to expose the growth plate cartilage on either or both sides as well as the repair tissue or bone bridge at injury site. The repair tissue is usually exposed well separated from the adjacent growth plate as the repair tissue has not yet integrated well into the surrounding adjacent growth plate at these time points. The growth plate injury site was removed carefully using a small sterile surgical blade by scraping gently along the growth plate contour on both the metaphysis and the epiphysis sides. Immediately after collection, the samples were snap-frozen and stored in −80 °C until future use. The left proximal tibia was fixed in 10% formalin for 24 h at 4 °C and decalcified for 4 days in Immunocal solution (Decal Corporation, Tallman, NY). Decalcified bone was then bisected longitudinally through the growth plate injury site and processed for paraffin-embedding. Sections of 4 μm thick were cut from paraffin tissue blocks and mounted on SuperFrost Plus™ glass slides for immunohistochemical and histology staining.

H&E alcian blue staining and image analysis of tissue repair

In order to visualize the mesenchymal infiltrate, cartilage or bone formed within the injury site, deparaffinized tissue sections were stained with alcian blue, haematoxylin and eosin (H&E). The proportions of each of the different types of repair tissues (mesenchymal infiltrate, cartilaginous tissue, bone trabeculae, and bone marrow) within the injury site were analysed using an image analysis program (Image-Pro Plus, Media Cybernetics, Silver Spring, MD) [4]. The measurements on four separate sections for each tissue sample were then averaged and expressed as percentages of the total injury site area [7].

Real-time qualitative RT-PCR expression analysis of cartilage and bone related genes

To examine the expression of genes involved during growth plate bony repair, total RNA from frozen growth plate injury site samples was extracted using RNAqueous®-Micro extraction kit (Ambion, Life Technologies, Vic, Australia). Due to the small amount of total RNA that can be obtained from each individual rat growth plate injury site, and large numbers of genes which need to be analyzed in this study, purified RNA samples from two rats in each treatment time-point group were pooled. The quantity and quality of all RNA samples were measured using the NanoDrop 2000, samples only with 260/280 ratios of between 1.8 and 2.0 were used for the analyses. Complementary strand of DNA (cDNA) of the pooled RNA was then synthesized using random decamers (Geneworks, SA, Australia) and Superscript-II RNase H Reverse Transcriptase (Stratagene, La Jolla, CA). Relative real-time PCR was carried out as described by Chung et al. (2006) using gene specific primers detailed in Table 1 [7]. Cyclophilin-A (CycA) was used as an internal control [18], and gene expression data was expressed relative to cyclophillin.

Immunohistochemical analysis

Immunohistochemistry was performed to examine and localize the protein expression of β-catenin and Runx2 at the growth plate injury site. Briefly, deparaffinized tissue sections were quenched in 3% H_2O_2 and incubated in an antigen retrieval solution, either 0.01 M citrate buffer (pH 6.0) (for β-catenin) or DAKO retrieval solution (pH 6.0) (DAKO, Vic, Australia) (for Runx2). The sections were then incubated with rabbit primary antibodies against β-catenin (1:100) or Runx2 (1:400) and detected with biotinylated secondary antibodies