Journal of Cellular Biochemistry 99:450–461 (2006)

Roles of COX-2 and iNOS in the Bony Repair of the Injured Growth Plate Cartilage

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Abstract Growth plate injuries often lead to bone growth defects, which primarily occur due to bony repair at injury sites. Bony repair is preceded by an injury-induced inflammatory response, which could play a role in regulating the repair process. Here, roles of two inflammatory mediators, cyclo-oxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS), in the injury responses were analysed by examining their gene expression and effects of blocking their activities, respectively, with celecoxib and aminoguanidine during 2 days prior to and until 7 days after injury in a rat tibial growth plate injury model. Quantitative RT-PCR assays revealed upregulated expression of COX-2 on days 1 and 4 and iNOS on day 1. Histological analysis of injury sites revealed significant reductions in inflammatory infiltrate (particularly neutrophils) on day 1 in treated groups compared to saline control. While bony tissue proportions at injury sites were unaffected by either treatment, mesenchymal tissue proportions were larger but cartilaginous tissue proportions were smaller on day 8 (though statistically insignificant), and bone remodelling appeared delayed with a smaller bone marrow proportion on day 14 in both treatment groups. These findings suggest that COX-2 and iNOS mediate injury-induced inflammatory response, and may play a role in enhancing mesenchymal cell differentiation to cartilaginous cells and in promoting bone remodelling during bony repair of growth plate injury sites. Furthermore, increased expression of cartilage-related (collagen-2, collagen-10, SOX-9) and bone-related molecules (osteocalcin, cbfα-1) suggest involvement of both endochondral and direct bone formation mechanisms during bony repair. J. Cell. Biochem. 99: 450–461, 2006.

Key words: growth plate; bone bridge; inflammatory response; COX-2; iNOS

The cartilaginous growth plate, responsible for lengthening of children's long bones, consists of the resting, proliferating and hypertrophic zones [Ianotti, 1990; Kronenberg, 2003]. Bone growth occurs through endochondral ossification, where cells are activated at the resting zone and upon stimulation, synthesise collagen-2 and proliferate at the proliferation zone in a columnar manner. These chondrocytes mature and express collagen-10 at the hypertrophic zone, where they mineralise their surrounding matrix and undergo apoptosis at the chondro-osseal transitional zone. Blood vessels invade and vascularise the mineralised hypertrophic cartilage, bringing in osteoblasts that form bone on the mineralised cartilage and osteoclasts that remodel newly formed bone at the metaphysis.

Growth plates in children are fragile, injury-prone and often become functionally disrupted when injured [Foster and Johnstone, 2000]. In such injuries, undesired bony tissue repair at the injury site [Wattenbarger et al., 2002] leads to bone growth defects [Ogden, 2000]. Xian et al. [2004] described sequential events, namely initial inflammatory, fibrogenic, osteogenic and bone remodelling responses in a rat growth plate injury healing model. Cellular and histological evidence of this earlier study suggested that bony repair at the injured growth plate occurred mainly through a direct bone

Grant sponsor: Bone Growth foundation (BGF); Grant sponsor: Channel-7 Children's Research Foundation of South Australia; Grant sponsor: Australian National Health and Medical Research Council (NHMRC).

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Received 16 February 2006; Accepted 17 February 2006
DOI 10.1002/jcb.20905

formation process (intramembranous ossification). Marrow-derived mesenchymal cells were recruited to the injury site, and some cells differentiated to osteogenic cells that expressed osteogenic differentiation transcription factor core binding factor-α1 (cbf-α1). These cells formed bony tissue containing bone matrix protein osteocalcin at the injury site 7 days after injury. Associated temporally with cellular events described above, changes in expression of proinflammatory cytokines and growth factors after growth plate injury were observed by Zhou et al. [2004]. Of note, there were transient increases in expression of proinflammatory cytokines interleukin 1-beta (IL1β) and tumour necrosis factor-alpha (TNFα) during the inflammatory response and TNFα during bone remodelling. While these cytokines inhibit expression of cartilage-related genes, such as chondrogenic transcription factor SOX-9 [Murakami et al., 2000], they are important chemotactic factors for mesenchymal cells [Kovacs and DiPietro, 1994]. TNFα is also essential for intramembranous bone formation during bone fracture healing [Gerstenfeld et al., 2001]. Since the inflammatory response is the first cellular event at the injured growth plate, we proposed it could play a regulatory role in subsequent events leading to bony repair at the injury site.

Inflammatory mediators prostaglandins (PGs) are synthesised from arachidonic acid by two isoforms of the cyclo-oxygenase (COX) enzyme [Smith and Langenbach, 2001]. COX-1 is constitutively expressed, while COX-2 is induced during an inflammatory response. Increased PGs levels following COX-2 expression upregulates various inflammation mediators, such as proinflammatory cytokines, and together with PGs, these mediators increase inflammation and subsequently promote healing in injuries. Studies have revealed that COX-2 and PGs are important for bone fracture healing [Simon et al., 2002; Zhang et al., 2002a,b; Brown et al., 2004; Gerstenfeld and Einhorn, 2004] and are essential for processes downstream of the initial inflammatory response at fracture sites. Using mouse bone fracture models, Zhang et al. [2002b] demonstrated a reduction in bone formation during fracture healing in COX-2 null mice, compared to COX-1 null mice and wild-type mice. Mesenchymal cell differentiation to bone cells was critically affected in COX-2 null mice through reduction in expression levels of BMP-2, cbfα-1 and osterix, three bone formation regulatory proteins. Of note, prostaglandin PGE_2 restoration reversed COX-2 mutation effects and normalised bone formation [Zhang et al., 2002b]. Various studies have also explored COX-2 role in bone fracture healing by utilising COX-2 inhibitors during fracture repair [Harder and An, 2003]. Simon et al. [2002] reported impairments in bone fracture healing in rats treated with celecoxib and rofecoxib, two COX-2 selective nonsteroid anti-inflammatory drugs (NSAIDS). Similar to Zhang et al.'s [2002b] study, the group observed inhibition in fracture healing and defects in endochondral ossification pathway due to COX-2 deficiency. Gerstenfeld and Einhorn, [2004] and Brown et al. [2004], however, did not observe fracture healing impairments in their studies after COX-2 inhibition, but both witnessed slight delays in fracture healing in their models.

It is believed that COX-2 could play a role in formation of bone resorbing cells (osteoclasts) and bone remodelling [Zhang et al., 2001; Theoleyre et al., 2004; Liu et al., 2005]. This process is mediated by three important signalling molecules—receptor activator of NFκ-B (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) [Theoleyre et al., 2004]. Interactions between RANK (surface receptor on osteoclast progenitor cells) and RANKL (found on surface of osteoblasts) initiate osteoclast formation/activity, while OPG, a decoy receptor for RANKL, prevents osteoclast formation/activity and antagonises bone remodelling processes. Hence a ratio of RANKL:OPG expression is a measure of bone remodelling. Zhang et al. [2001] showed COX-2 was essential for bone resorption during wear debris-induced osteolysis and Liu et al. [2005] demonstrated COX-2 and PGs were required for osteoblast growth and osteoclast function, and osteoclastogenesis was enhanced through PGs action on RANK/RANKL interactions.

Similar to PGs, nitric oxide (NO) is an important injury-induced inflammatory response mediator. Functions of NO include vasodilation promotion during trauma injuries and upregulation of COX-2 expression during inflammation [Needleman and Manning, 1999; Pelletier et al., 2001; Perez-Sala and Lamas, 2001; Ling et al., 2005]. NO is synthesised from L-arginine by three isoforms of the nitric oxide synthase (NOS) enzyme [Griscavage et al., 1995]. Notably, the inducible NOS (iNOS) which

synthesises NO during acute inflammatory responses is commonly upregulated by various inflammation mediators such as proinflammatory cytokines. Diwan et al. [2000] first evaluated the role of NO in bone fracture healing and observed maximal iNOS activity at the fracture site 15 days after fracture. Fracture healing was also impaired when NO synthesis was inhibited with NOS inhibitor L-NAME, but impairment was reversed upon NO supplementation. Little is known about the mechanisms of NO action in fracture healing; however, it is believed that presence of NO may affect fracture healing by indirectly influencing COX-2 signalling pathways.

The above animal studies have thus suggested COX-2 and iNOS have roles in bone cell differentiation/osteogenesis and osteoclastogenesis/bone remodelling during bone fracture healing. While osteogenic differentiation, osteogenesis, osteoclastogenesis and bone remodelling drive bony repair at the injured growth plate [Xian et al., 2004], it is unknown if COX-2 and iNOS have similar roles in injured growth plate bony repair as in bone fracture healing. In this study, we hypothesised that iNOS and COX-2 play important roles in the bony repair of the injured growth plate since both COX-2 and iNOS are important in regulating inflammatory responses and bone fracture healing. Using a rat growth plate injury model, this study examined gene expression of COX-2 and iNOS and the effects of inhibiting either enzyme on the injury-induced inflammatory response and downstream responses leading to bony repair.

MATERIALS AND METHODS

Growth Plate Injury Time Course, Delivery of Inhibitors and Specimen Collection

Fifty-four 6-week-old male Sprague–Dawley rats (approximately 120 g) were randomly placed in three groups, receiving COX-2 inhibitor (celecoxib), iNOS inhibitor (aminoguanidine hemisulfate) or a vehicle (saline) once daily by oral gavage. Each group had three time points of tissue harvest—1, 8 and 14 days after injury ($n = 6$ per time point), which are suitable time points for observing the inflammatory, osteogenic and bone remodelling responses, respectively, during growth plate injury repair [Xian et al., 2004; Zhou et al., 2004]. In addition, one group of untreated/uninjured age-matched rats ($n = 6$) (referred to as normal rats) and two groups of rats ($n = 6$) with growth plate injury but without any drug treatments and sacrificed on days 4 and 10 after injury were also set up for gene expression studies. Celecoxib (Celebrex, Pfizer) at 5 mg/kg/rat [Guirguis et al., 2001] was gavaged in 0.5% methyl cellulose and aminoguanidine hemisulfate (Sigma, NSW, Australia) at 120 mg/kg/rat [Chwalisz et al., 1999] was gavaged in H_2O (pH 6). Drug administration commenced 2 days before injury and continued until surgery day (day 0) for day 1 rats, and until day 7 for day 8 and 14 rats. A drill-hole injury, to disrupt the central part of the proximal tibial growth plate, was inflicted surgically using a 1.5-mm dental drill under anaesthesia as described [Xian et al., 2004]. All procedures were approved the Animal Ethics Committee of the Women's and Children's Hospital, South Australia.

Rats were sacrificed on days 1, 8 and 14 post-surgery by CO_2 overdose. Growth plate samples from all rats were scraped from the right proximal tibiae and snap-frozen for RNA extraction as described [Zhou et al., 2004]. Left proximal tibiae were collected and fixed in 10% formalin for 24 h, decalcified for 4 days in Immunocal solution (Decal Corporation), bisected longitudinally and processed for paraffin-embedding. Sections were sliced and collected on SuperFrost Plus coated slides for immunohistochemical and histological analysis.

Gene Expression Quantitation Using Real-Time RT-PCR

To examine expression of genes important for cartilage/osteogenic tissue formation, total RNA from frozen samples was isolated using TRIZOL solution (Sigma). Extracted RNA was treated with DNAse (Promega, NSW, Australia) and its quality/quantity was determined by spectrophotometry. Due to the small amount of total RNA that can be obtained from each individual rat growth plate scraped sample and the large numbers of genes that need to be analysed in this study, purified RNA from six rats in each treatment time point group was pooled (equal amount from each rat) to make up a total amount of 5 μg. cDNA of the pooled RNA was synthesised using random decamers (Geneworks, SA, Australia) and Superscript-II RNAse H reverse transcriptase (Stratagene), according to manufacturers instructions.

A SYBR Green real-time PCR assay was used to analyse the expression of genes stated in Table I. Cyclophilin-A was used as the internal reference and PCR assays for each gene and cyclophilin were run in parallel using their specific primer pairs [Zhou et al., 2004]. cDNA amount equivalent to 50 ng of total pooled RNA was used for each analysis. Each gene was analysed in triplicate on a Rotorgene 72-well PCR machine (Corbett Research, NSW, Australia) as described previously [Zhou et al., 2004]. For iNOS, Cox-1 and Cox-2 real-time PCR, TaqMan chemistry was used due to its increased sensitivity and specificity for inducible genes and genes expressed at very low levels. Cyclophilin was again used as the internal reference gene. All reagents for TaqMan assays were purchased from Applied Biosystems on demand.

Relative gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method [Livak and Schmittgen, 2001], where threshold cycle (C_T) values from triplicate runs were averaged, and calibrated in relation to cyclophilin C_T values. Levels of gene expression (fold changes) in treated samples were presented in relation to expression levels in normal uninjured samples (normal).

Histological and Immunohistochemical Analysis

Sections of growth plate and adjacent tibial bone were stained with alcian blue to visualise cartilage, and haemotoxylin and eosin (H&E) to visualise tissue structure [Xian et al., 2004]. An image analysis program (Image-Pro Plus, Media Cybernetics, CA) was used to measure areas of mesenchymal tissue, cartilaginous tissue, bone trabeculae and bone marrow at injury sites in day 8 and day 14 sections, and these were then expressed as percentages of the total injury area [Xian et al., 2004]. To examine treatment effects on the inflammatory infiltrate, neutrophils, monocytes/macrophages and lymphocytes at growth plate injury sites were counted in day 1 sections. These different types of inflammatory cells were distinguished by their distinct morphological structures (e.g. cell nucleus, cytoplasm and size) on H&E sections. The cell counts of each cell type were expressed as the average number of cells/mm^2 of injury site area.

To confirm the presence of cartilage or bony repair at the injured growth plate, immunostaining of cartilage (collagen-2 and collagen-10) and bone (osteocalcin) associated proteins were conducted as described previously [Xian et al., 2004].

Statistical Analysis

Experimental results (except real-time RT-PCR results) were expressed as mean $\pm$ SEM and statistical significance of results between treated groups and time points were analysed by two-way analysis of variance (ANOVA) tests. Statistical significance was assumed when $P < 0.05$. Since the RT-PCR data were generated from pooled RNA samples, data are presented as means of individual pooled samples (without SEM) and thus no statistical analysis was carried out.

RESULTS

Growth Plate Injury Caused Upregulation of Inflammatory Mediator Genes

Cox-2 levels at the injured growth plate were upregulated 2- to 3-fold on days 1 and 4 after injury but returned to normal by day 10 (Fig. 1A). The housekeeping gene Cox-1 remained constant across all time points (Fig. 1A). Real-time RT-PCR on iNOS revealed a dramatic 16-fold increase on day 1 at the injured growth plate with levels returning to the basal levels from day 4 onwards (Fig. 1B).

TABLE I. Primer Sequences of Genes Used for Real-Time RT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
TNFα	ATGGCCCAGACCCTCACACTCAGA	CTCCGCTTGGTGGTTTGCTACGAC
IL1β	GTTTCCCTCCCTGCCTCTGACA	GACAATGCTGCCTCGTGACC
RANKL	TGGGCCAAGATCTCTAACATGAC	TCATGATGCCTGAAGCAAATG
OPG	AGCTGGCACACGAGTGATGAA	CACATTCGCACACTCGGTTGT
Collagen-2	GGGCTCCCAGAACATCACCTACCA	TCGGCCCTCATCTCCACATCATTG
Collagen-10	GGCAGCAGCACTATGACCCAA	ACAGGCCTACCCAAACGTGAGTCC
SOX-9	GGCCCTTCCTCTCCTCAACC	ACTGCCGTGGCCTTTTACA
Osteocalcin	GCTGGCCCTGACTGCATTCTG	ATTCACCACCTTACTGCCCTCCTG
Cbf-α1	TACGAAATGCCTCTGCTGTTATGG	GAGGATTTGTGAAGACCGTTATG
Cyclophilin-A	CGTTGGATGGCAAGCATGTG	TGCTGGTCTTGCCATTCCTG

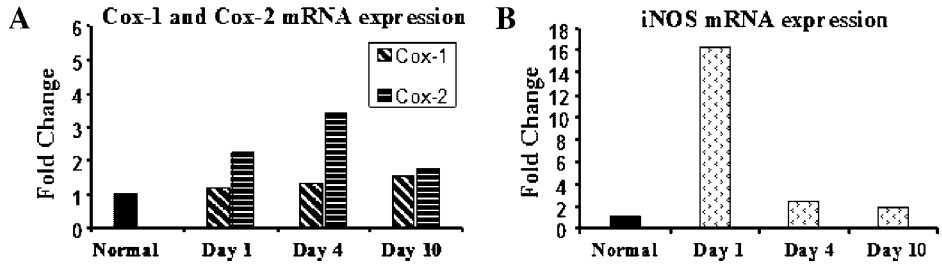

Fig. 1. Expression of inflammatory mediator genes COX-1, COX-2 and iNOS in the injured growth plate. Real-time RT-PCR relative quantitation analysis of (**A**) COX-1 and COX-2, and (**B**) iNOS was conducted on injured growth plate samples at days 1, 4 and 10 after injury in comparison to normal uninjured growth plate.

Inflammatory Infiltrate at Day 1 Growth Plate Injury Sites

Infiltrated inflammatory cells appeared densely populated at the growth plate injury sites in saline controls (Fig. 2A), while those in celecoxib (Fig. 2B) and aminoguanidine (Fig. 2C) treated samples appeared sparsely populated. Quantitative analysis revealed a significant reduction ($P < 0.05$) in inflammatory infiltration at day 1 growth plate injury sites of celecoxib (254 cells/mm^2) and aminoguanidine (261 cells/mm^2) treated samples, as compared to saline controls (1,712 cells/mm^2) (Fig. 2D). Interestingly, the significant reduction ($P < 0.05$) in inflammatory cell density was attributed to a reduction in neutrophils at injury sites of celecoxib and aminoguanidine treated samples as compared to saline controls ($P < 0.05$) (Fig. 2D). The reductions in monocytes/macrophages and lymphocytes in celecoxib and aminoguanidine treated samples were statistically insignificant.

Inhibitor Effects on Expression of Proinflammatory Cytokines IL1β and TNFα

Increased IL1β and TNFα mRNA expression levels were seen in all day 1 injured growth plate samples as compared to uninjured controls (Fig. 2E). IL1β was upregulated 30-fold in saline control samples while both treatment groups showed a smaller upregulation of approximately 20-fold. This trend was consistent with the inflammatory infiltration results (Fig. 2D). TNFα levels increased around 45-fold in both saline and celecoxib treated samples. The

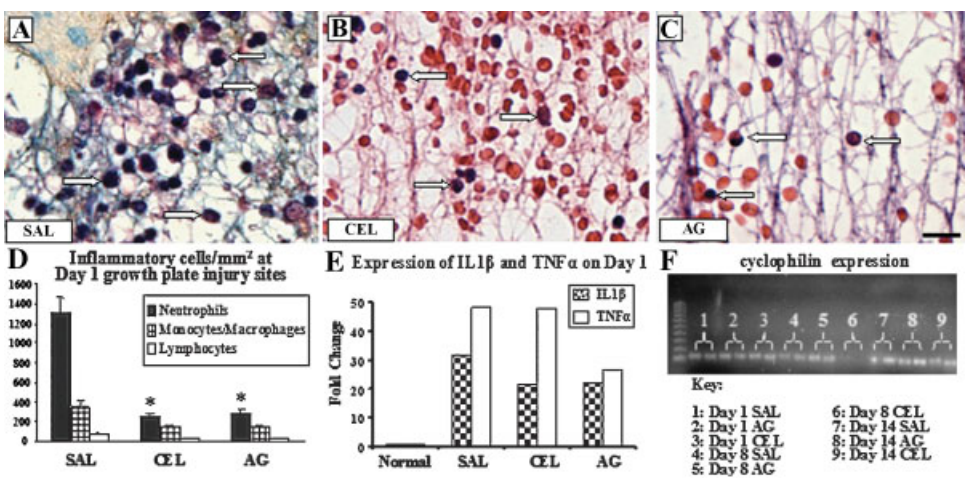

Fig. 2. Effects of COX-2 or iNOS inhibition on growth plate injury-induced inflammatory response. Inflammatory infiltrates (as indicated by block arrows), at day 1 growth plate injury sites of (**A**) saline (SAL), (**B**) celecoxib (CEL) and (**C**) aminoguanidine (AG) treated rats, were less abundant (particularly neutrophils) in the two treatment groups, as illustrated in (**D**) (*$P < 0.05$ vs. saline control) (cells without nuclei shown in (A–C) are red blood cells). **E**: Real-time RT-PCR expression level of proinflammatory cytokines IL1β and TNFα. **F**: Gel electrophoresis of RT-PCR products, showing effects of celecoxib treatment on expression of reference gene cyclophilin in day 8 samples (denoted 6). Bar in C = 50 μm (also applies to (A, B)). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

aminoguanidine treated samples showed a smaller increase of only 25-fold, and this may be possibly due to reduced NO levels after aminoguanidine treatment.

Treatment Effects on Internal Reference Gene Expression

Cyclophilin expression levels in all pooled samples were analysed by real-time RT-PCR to verify its suitability for use as a reference gene for subsequent real-time RT-PCR analysis (Fig. 2F). An unexpected reduction in cyclophilin expression in day 8 celecoxib treated pooled samples (Fig. 2F, sample 6) was observed while no anomalous expression was detected in the other pooled samples. An analysis on another reference control, 18S rRNA, also revealed a reduction in expression levels in day 8 celecoxib treated samples (results not shown). Repeated cDNA synthesis and PCR analysis with different RNA pools gave consistent results. Due to this unexpected celecoxib effect on housekeeping gene expression, day 8 celecoxib pooled samples were omitted from all subsequent real-time RT-PCR analysis.

Histological Analysis of Tissue Repair at Growth Plate Injury Sites

Histological analysis of day 8 and day 14 samples revealed four different repair tissue types at the injury sites, namely, mesenchymal tissue, cartilaginous tissue, bony trabeculae and bone marrow (Fig. 3A–F). While mesenchymal tissue was classified as area occupied by fibroblast-like cells, cartilaginous tissue was identified as area containing round cells or fibrous cartilage tissue stained prominently for alcian blue. The presence of these tissues at

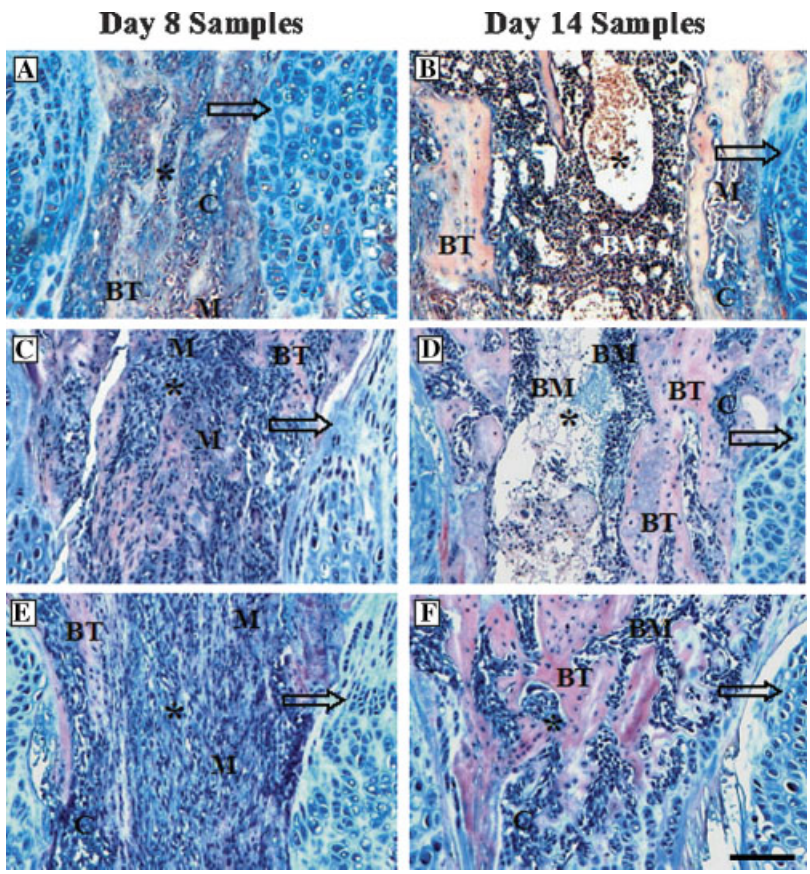

Fig. 3. Tissue repair at growth plate injury sites. Growth plate injury sites (*) in rats treated with (**A**, **B**) saline, (**C**, **D**) celecoxib or (**E**, **F**) aminoguanidine 8 and 14 days after injury. Cartilaginous tissue (denoted 'C') stained blue by alcian blue, mesenchymal tissue ('M') appeared as masses of fibrous cells, bone trabeculae ('BT') were smooth solid bony structure stained pink by eosin, and bone marrow ('BM') appeared as masses of marrow cells. Block arrows indicate growth plate adjacent to injury sites. Bar in (F) 150 μm, which applies to other photos. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

injury sites suggested the possibility of endochondral ossification during the bony repair process. Bony trabeculae were classified as bony structure with smooth surfaces that were stained pink at injury sites, and their presence in day 8 samples indicated bony tissue repair had commenced. The presence of bone marrow, seen as dark cell mass in day 14 samples only, suggested remodelling and maturation of bone trabeculae at injury sites. Bone marrow was more prominent in saline controls (Fig. 3B) as compared to celecoxib (Fig. 3D) and aminoguanidine (Fig. 3F) treated samples, suggesting a delay in bone bridge maturation after COX-2 or iNOS inhibition.

Tissue Proportions at Growth Plate Injury Sites

To further refine the differences observed in injury responses after blocking COX-2 or iNOS activity, the percentages of common tissue types present at the injury sites were scored and compared between day 8 and day 14 for all treatment groups. Mesenchymal tissue proportions were greater (though statistically insignificant) in day 8 celecoxib and aminoguanidine treated samples than in saline controls (Fig. 4A). In day 14 samples, mesenchymal tissue proportions were smaller compared to day 8, and proportion differences between treatment groups were also smaller. Differences in mesenchymal tissue amount between the two time points indicated that some mesenchymal cells had differentiated to either cartilage or bone cells at injury sites by day 14, while the smaller differences in mesenchymal tissue proportions between groups in day 14 suggested a possible 'catch-up' in mesenchymal cell differentiation after cessation of treatments on day 7.

Cartilaginous tissue proportions in day 8 samples (Fig. 4B) were greater (but statistically insignificant) in saline controls than in celecoxib and aminoguanidine treated samples. The larger proportion of cartilaginous tissue could have resulted from faster differentiation of mesenchymal cells to cartilage cells in saline controls. By day 14, cartilage tissue proportions were almost similar in saline and celecoxib treated groups, while a slightly larger proportion remained at injury sites of aminoguanidine treated samples.

Proportions of bone trabeculae in day 8 and day 14 samples (Fig. 4C) were similar for all treatment groups. Bone marrow was not present in any day 8 samples. On day 14, however, a slightly larger marrow proportion was seen in saline controls compared to celecoxib and aminoguanidine treated samples. The bone marrow in saline controls also appeared more mature (Fig. 3) compared to treated groups. The differences in bone marrow proportions may suggest a delayed bone remodelling process in celecoxib and aminoguanidine treated rats.

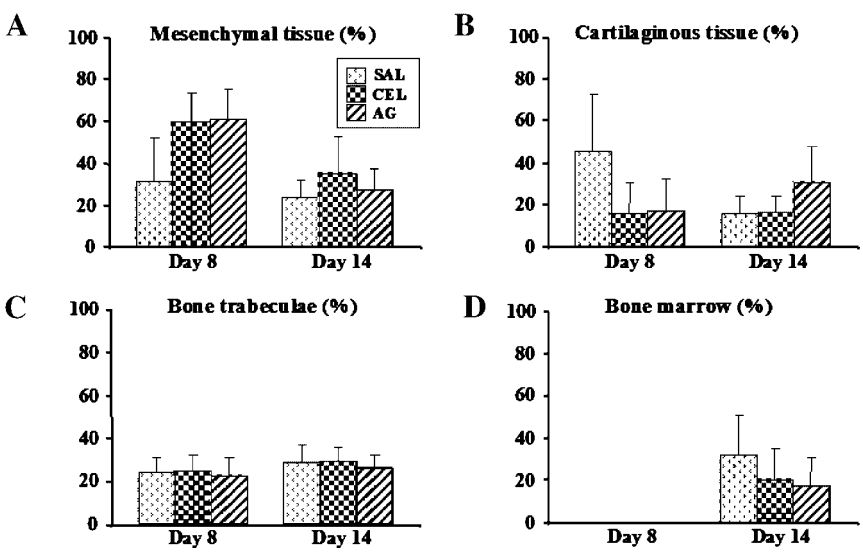

Fig. 4. Effects of COX-2 or iNOS inhibition on proportions of different repair tissues at growth plate injury sites. Mean proportions (percentage area of total injury area) of (**A**) mesenchymal tissue, (**B**) cartilaginous tissue, (**C**) bony trabeculae and (**D**) bone marrow formed at growth plate injury sites 8 and 14 days after injury.

Expression of Cartilage and Bone Related Genes During Bony Repair

To study molecular mechanisms during bony tissue repair, real-time RT-PCR analysis was carried out on genes involved in cartilage/bone formation and bone remodelling. An upregulation in mRNA levels of cartilage-related (collagen-2, collagen-10, SOX-9) and bone-related (cbf-α1, osteocalcin) genes was seen in saline and aminoguanidine treated day 8 samples (Fig. 5A–E). Of note, upregulation of cartilage matrix proteins collagen-2 and collagen-10 and chondrogenic transcriptional factor SOX-9 in all day 8 samples indicated cartilage formation and endochondral ossification mechanism involvement in bony repair at growth plate injury sites, which are also evident in the histology analysis (Figs. 3 and 4). Similarly, upregulated expressions of bone matrix protein osteocalcin (Fig. 5D) and bone cell differentiation transcription factor cbf-α1 (Fig. 5E) on day 8 also suggested presence of bone cell differentiation and bone formation at growth plate injury sites.

On day 14, mRNA expression levels of most genes analysed (Fig. 5A–D) were lower than the day 8 levels, suggesting that cartilage and bone cell differentiation during healing had slowed down on day 14 at this bone remodelling/maturation phase at growth plate injury sites. The presence of elevated SOX-9, cbf-α1 and collagen-2 levels compared to the uninjured controls, however, indicated that some chondrogenic and osteogenic differentiation processes may still be occurring during remodelling on day 14.

Apart from roles by bone-forming cells osteoblasts, bone remodelling is also carried out by resorptive cells osteoclasts. In all day 14 samples, expression of osteoclast differentiation factor RANKL and antagonist OPG was upregulated (Fig. 5E). RANKL expression was higher in saline and aminoguanidine treated samples as compared to celecoxib treated samples, while OPG expressions showed similar trends in upregulation as RANKL in all samples. While higher RANKL expression could have promoted a faster bone remodelling process in saline and aminoguanidine treated samples, the smaller RANKL:OPG ratio in celecoxib treated samples (1.8:1) compared to saline controls (2.7:1) could have contributed to the delayed bony maturation and bone marrow formation in day 14 celecoxib treated samples. However, similar RANKL:OPG ratios between saline (2.7:1) and aminoguanidine (2.6:1) treated samples suggested that delayed bone marrow formation in aminoguanidine treated rats may be due to other factors.

Immunostaining of Collagen-2, Collagen-10 and Osteocalcin at Growth Plate Injury Sites

Collagen-2 and collagen-10 immunostaining was carried out in day 8 samples and was seen in all treatment samples in both the adjacent growth plate proliferative (collagen-2, Fig. 6A) or hypertrophic zone (collagen-10, Fig. 6C) and some areas within the injury sites where chondrocytes were present (collagen-2, Fig. 6B; collagen-10, Fig. 6D). This immunohistochemical finding together with our histological data and

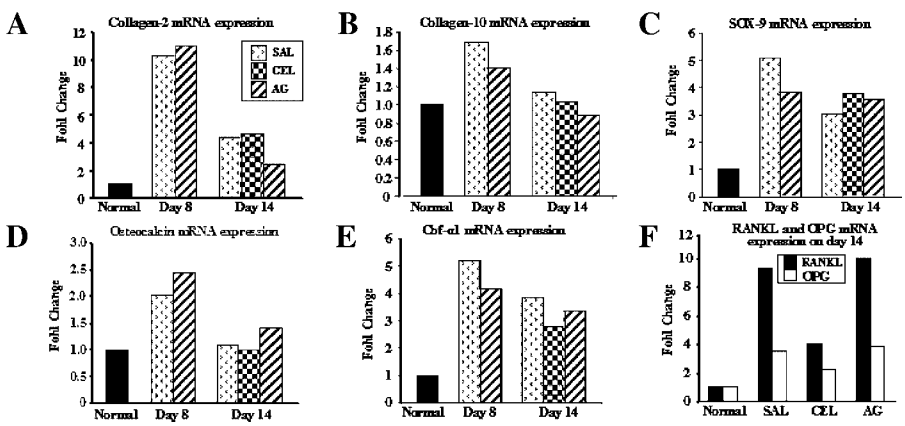

Fig. 5. Effects of COX-2 or iNOS inhibition on expression of genes associated with cartilage/bone formation and bone remodelling. Real-time RT-PCR analysis of (**A**) collagen-2, (**B**) collagen-10, (**C**) SOX-9, (**D**) osteocalcin, (**E**) cbf-α1 in day 8 and day 14 injured growth plate samples and (**F**) RANKL and OPG in day 14 injured growth plate samples.

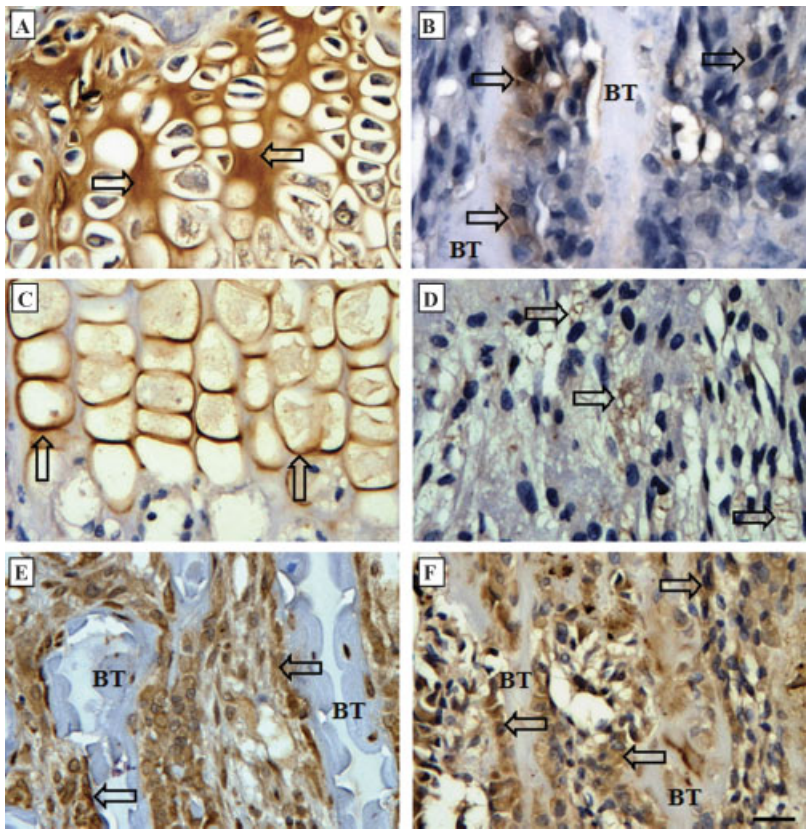

Fig. 6. Immunostaining of cartilage and bone matrix proteins at the day 8 growth plate injury sites. **A**: Collagen-2 immunostaining of chondrocytes within the proliferative zone of uninjured growth plate; (**B**) collagen-2 expression within the injury site; (**C**) collagen-10 immunostaining of hypertrophic chondrocytes within uninjured growth plate; (**D**) collagen-10 expression within the growth plate injury site; (**E**) osteocalcin immunostaining in bone cells lining normal bone trabeculae ('BT') or in some surrounding cells; (**F**) osteocalcin immunostaining in bone lining osteoblasts and bone matrix at growth plate injury sites. Bar in F = 50 μm, and applies to other photos. Block arrows indicate positive immunostaining. B, D, F were taken in the same sections and at the same magnification as the adjacent growth plate cartilage or metaphysis bone shown in (A, C, E), respectively.[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

gene expression results confirm that some mesenchymal cells were undergoing chondrogenic differentiation (expressing SOX-9 and collagen-2) and hypertrophy (expressing collagen-10), and suggest that endochondral ossification was involved in the bony repair of the injured growth plate. Immunostaining of osteocalcin in the bony trabeculae-lining cells or in cells close by (Fig. 6F), which is similar to the staining in normal metaphysis bone trabeculae on the same sections (Fig. 6E), confirms the presence of bone formation and involvement of intramembranous bone formation process occurring at growth plate cartilage injury sites, as we had observed previously [Xian et al., 2004].

DISCUSSION

The bony repair of the injured growth plate in children has some similarities to normal bone fracture healing. This study has verified Xian et al.'s [2004] and Zhou et al.'s [2004] findings that growth plate injury healing process begins with an initial inflammatory response and is followed by a fibrogenic healing response where mesenchymal cells migrate to the injury site and differentiate to bone cells that directly deposit bony tissue at the injury site. Through gene expression, histological and immunohistochemical analysis, the current study has additionally shown that some mesenchymal cells may differentiate to cartilage cells that undergo endochondral ossification contributing to bony repair at the injured growth plate. These processes have been witnessed in bone fracture healing in various bone fracture models [Simon et al., 2002; Zhang et al., 2002a,b; Brown et al., 2004; Gerstenfeld and Einhorn, 2004].

Since the initial inflammatory response is believed to be important in regulating

downstream events during bony repair, the current study examined potential roles of inflammatory mediators COX-2 and iNOS in the bony repair process. In the present study, we have chosen three time points of the key phases of injury responses in injured growth plate: inflammatory (day 1), osteogenic (day 8) and bone bridge maturation (day 14). Our gene expression analysis showed that COX-2 and iNOS were upregulated transiently within the injured growth plate, which occurs early in the inflammatory process on day 1 for iNOS and in the inflammatory (day 1) and fibrogenic processes (day 4) for Cox-2. This finding suggests that these inflammatory mediators may play a role in growth plate injury healing as they do within other injury models [Simon et al., 2002; Zhang et al., 2002a,b; Brown et al., 2004; Gerstenfeld and Einhorn, 2004]. Inhibition of these enzymes using celecoxib and aminoguanidine significantly reduced the inflammatory infiltrate, particularly neutrophils—the first inflammatory cells migrating to injury sites [Nussler et al., 1999]. However, since differences in repair tissue proportions at injury sites were statistically insignificant between treated rats and saline controls, inflammatory infiltration reduction after either inhibitor treatment did not significantly affect the overall tissue repair outcomes at growth plate injury sites.

However, an apparent delay in transformation of mesenchymal cells to cartilaginous tissue was seen in both inhibitor treated groups on day 8, which suggests a delay in the cartilage healing process at injury sites. Similar delays in bone fracture healing have been previously noted after COX-2 inhibition [Brown et al., 2004; Gerstenfeld and Einhorn, 2004]. On the other hand, a critical impairment in mesenchymal cell differentiation as seen in Zhang et al. [2002b] study was not seen in the current study. Since the delay was also seen in the iNOS-inhibited group, the inflammatory response in itself may play an important role in the healing processes at growth plate injury sites. The similar amounts of mesenchymal tissues, bone trabeculae and cartilaginous tissue (with the exception of cartilaginous tissue in aminoguanidine treated group) seen in day 14 samples could therefore indicate 'catch-up' healing in both treatment group samples after cessation of treatments. Despite these trends in treatment effects, large variations in healing outcomes seen among individual animals of the same group have prevented this study from drawing definite conclusions of effects of COX-2/iNOS inhibition on bony repair. Notably, variable results in bone fracture healing have been observed in many fracture models [Simon et al., 2002; Zhang et al., 2002a,b; Brown et al., 2004; Gerstenfeld and Einhorn, 2004]. In future studies, increasing animal numbers will assist in drawing more definitive conclusions to the trends observed.

While inhibiting the inflammatory response did not prevent bony tissue formation, histological analysis and RANKL and OPG expression studies suggest that bone remodelling processes might have been affected by the inhibitors. Ho et al. [1999] have previously demonstrated that bone remodelling could be affected following suppression of PGs synthesis through NSAID treatments. Thus, it would be interesting to determine if such delays in bone remodelling occurred in clinical situations after long-term administration of inflammatory inhibitors during treatment of growth plate injuries/bone fractures.

The reduction in endogenous reference gene expressions (cyclophilin and 18S rRNA) in day 8 celecoxib treated samples is an unexpected and yet interesting finding. This anomalous expression pattern could be a likely effect on global gene expression after 10 continuous days of celecoxib treatment. Interestingly, the reference gene expressions (both cyclophilin and 18S RNA) were not anomalous for any other samples (even day 1 and 14 celecoxib treated samples), which further suggests that 10 continuous days of celecoxib treatment could have affected global gene expression and that when the treatments cease, gene expressions return to normal. While long-term celecoxib treatment has recently been noted to reduce global gene expression in a preliminary clinical study [Dr I. Kirsch, personal communication], there is little information to date describing similar reductions in global gene expressions after celecoxib treatment. Further studies will thus be needed to determine if the delays seen in bone fracture healing after COX-2 inhibition in various bone fracture models [Simon et al., 2002; Zhang et al., 2002a,b; Brown et al., 2004; Gerstenfeld and Einhorn, 2004] and in this study could possibly have been a result of such effects on global gene expression.

In summary, this study has shown for the first time that COX-2 and iNOS appear to play

important roles in mediating the inflammatory response at growth plate injury sites. However, inhibiting COX-2 or iNOS did not prevent downstream bony tissue repair at the injured growth plate, which occurs through endochondral and intramembranous ossification mechanisms. However, inhibition of COX-2 or iNOS appeared to delay both mesenchymal tissue differentiation to cartilage tissue and bone remodelling processes. Finally, gene expression studies suggest that long-term celecoxib treatment may affect global gene expression, and this may possibly affect fracture healing.

ACKNOWLEDGMENTS

The authors wish to thank Mark Covino for some technical assistance, Lynn Scarman for expertise within the animal care facility, and Carmen Macsai for help in editing the manuscript. The project was funded by Bone Growth foundation (BGF) and in part by grants from Channel-7 Children's Research Foundation of South Australia, and from the Australian National Health and Medical Research Council (NHMRC).

REFERENCES

Brown K, Saunders M, Kirsch T, Donahue H, Reid J. 2004. Effect of COX-2-Specific inhibition on fracture-healing in the rat femur. J Bone Joint Surg 86-A(1):116–123.

Chwalisz K, Winterhager E, Thienel T, Garfield RE. 1999. Synergistic role of nitric oxide and progesterone during the establishment of pregnancy in the rat. Hum Reprod 14(2):542–552.

Diwan A, Wang M, Jang D, Zhu W, Murrell G. 2000. Nitric oxide modulates fracture healing. J Bone Miner Res 15:342–351.

Foster BK, Johnstone EW. 2000. Management of growth plate injuries. In: Benson M, Fixsen J, MacNicol M, Parsch K, editors. Paediatric orthopaedics and fractures. London: Harcourt Publishers.

Gerstenfeld LC, Einhorn TA. 2004. COX inhibitors and their effects on bone healing. Expert Opin Drug Saf 3(2):1–6.

Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Cruceta J, Graves BD, Einhorn TA. 2001. Impaired intramembranous bone formation during bone repair in the absence of tumour necrosis factor-alpha signalling. Cells Tissues Organs 169(3):285–294.

Griscavage JM, Hobbs AJ, Ignarro LJ. 1995. Negative modulation of nitric oxide synthase by nitric oxide and nitroso compounds. Adv Pharmacol 34:215–234.

Guirguis MS, Sattari S, Jamali F. 2001. Pharmacokinetics of celecoxib in the presence and absence of interferon-induced acute inflammation in the rat: Application of a novel HPLC assay. J Pharm Pharm Sci 4(1):1–6.

Harder AT, An YH. 2003. The mechanisms of the inhibition effects of nonsteroidal anti-inflammatory drugs on bone healing: A Concise Review. J Clin Pharmacol 43:807–815.

Ho ML, Chang JK, Chuang LY, Hsu HK, Wang GJ. 1999. Effects of nonsteroidal anti-inflammatory drugs and prostaglandins on osteoblastic functions. Biochem Pharmacol 58(6):983–990.

Ianotti JP. 1990. Growth plate physiology and pathology. Orthop Clin N Am 21:1–17.

Kovacs EJ, DiPietro LA. 1994. Fibrogenic cytokines and connective tissue production. FASEB J 8(11):854–861.

Kronenberg HM. 2003. Developmental regulation of the growth plate. Nature 423:332–336.

Ling JJ, Sun YJ, Zhu DY, Chen Q, Han X. 2005. Potential role of NO in modulation of COX-2 expression and PGE2 production in pancreatic β-cells. Acta Biochem Biophys Sin 37(2):139–146.

Liu X, Kirschenbaum A, Yao S, Levine A. 2005. Cross-talk between the interleukin-6 and prostaglandin E_2 signalling systems results in enhancement of osteoclastogenesis through effects on the osteoprotegerin/receptor activator of nuclear factor-κB (RANK) ligand/RANK system. Endocrinology 146(4):1991–1998.

Livak K, Schmittgen T. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25:402–408.

Murakami S, Lefebvre V, de Crombrugghe B. 2000. Potent inhibition of the master chondrogenic factor Sox9 gene by interleukin-1 and tumor necrosis factor-alpha. J Biol Chem 275(5):3687–3692.

Needleman P, Manning PT. 1999. Interactions between the inducible cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) pathways: Implications for therapeutic intervention in osteoarthritis. Osteoarthritis Cartilage 7:367–370.

Nussler AK, Wittel UA, Nussler NC, Beger HG. 1999. Leukocytes, the Janus cells in inflammatory disease. Langenbeck's Arch Surg 384:222–232.

Ogden JA. 2000. Skeletal injury in the child. 3rd edition. New York: Spring-Verlag.

Pelletier JP, Fernandes JC, Jovanovic DV, Reboul P, Martel-Pelletier J. 2001. Chondrocyte death in experimental osteoarthritis is mediated by MEK 1/2 and p38 pathways: Role of cyclooxygenase-2 and inducible nitric oxide synthase. J Rheumatol 28(11):2509–2519.

Perez-Sala D, Lamas S. 2001. Regulation of cyclooxygenase-2 expression by nitric oxide in cells. Antioxidants Redox Signaling 3(2):231–248.

Simon AM, Manigrasso MB, O'Connor JP. 2002. Cyclooxygenase 2 function is essential for bone fracture healing. J Bone Miner Res 17:963–976.

Smith W, Langenbach R. 2001. Why there are two cyclooxygenase isozymes. J Clin Invest 107(12):1491–1495.

Theoleyre S, Wittrant Y, Kwan Tat S, Fortun Y, Redini F, Heymann D. 2004. The molecular triad OPG/RANK/RANKL: Involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev 15:457–475.

Wattenbarger JM, Gruber HE, Phieffer LS. 2002. Physeal fractures, part 1: Histologic features of bone, cartilage, and bar formation in a small animal model. J Pediat Orthop 22(6):703–709.

Xian CJ, Zhou FH, McCarty RC, Foster BK. 2004. Intramembranous ossification mechanism for bone bridge formation at the growth plate cartilage injury site. J Orthop Res 22(2):417–426.

Zhang X, Morham SG, Langenbach R, Young DA, Xing L, Boyce BF, Puzas EJ, Rosier RN, O'Keefe RJ, Schwarz EM. 2001. Evidence for a direct role of cyclooxygenase-2 in implant wear debris induced osteolysis. J Bone Miner Res 16:660–669.

Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN, O'Keefe RJ. 2002a. Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. J Clin Invest 109(11): 1405–1415.

Zhang X, Xing L, Childs L, Boyce B, Puzas E, Rosier R, Schwarz E, O'Keefe R. 2002b. A critical role of COX-2 in intramembranous and endochondral bone repair. Trans Orthop Res Soc 27:202.

Zhou FH, Foster BK, Sander G, Xian CJ. 2004. Expression of proinflammatory cytokines and growth factors at the injured growth plate cartilage in young rats. Bone 35: 1307–1315.