Introduction

Inhibitors of cyclooxygenase (COX) have been widely used as anti-inflammatory and pain relief medications in clinical practice. These drugs inhibit both COX-1 and COX-2 at different ratios. While agents specific for the inhibition of COX-2 have recently been introduced, and have an improved safety profile, it has become apparent that cyclooxygenase activity is important in the skeletal reparative processes. Both animal and human studies demonstrate inhibition of fracture healing with multiple NSAIDs, including ibuprofen, indomethacin, and ketorolac. However, the relative role of COX-1 and COX-2 isoforms, and the target cells and tissues involved in this process have not been clearly defined.

Fracture healing is initiated by a trauma induced inflammatory response that involves various cytokines and growth factors, such as IL-6, TNF-α, IL-1, TGF-β and FGFs, many of which increase COX-2 expression in vitro. These cytokines and growth factors can initiate a cascade of events that will induce new woven bone formation through both endochondral and intramembranous pathways. Woven bone is further resorbed and replaced by lamellar bone through a process of bone remodeling.

To elucidate the role of COX-2 in the bone healing process, we created a stabilized mid-diaphyseal tibia fracture using an Einhorn devise in COX-1-/-, COX-2 -/- and wild type mice. We demonstrate that COX-2 but not COX-1 is required for efficient endochondral bone repair. We also injected growth factor FGF-1 onto mouse calvaria to examine growth factor induced intramembranous bone formation. We observed a significant reduction of new bone formation in COX-2 -/- mice.

Methods

Fracture healing model: Wild type, COX-1-/- and COX-2 -/- mice were anesthetized with Ketamine/Xylazine i.p. The skin was cut open to expose the left knee. A 0.25mm metal pin was inserted through the patellar tendon into narrow space of tibia. The incision was closed and a mid-diaphyseal fracture was created using three point bending by an Einhorn device. The healing was monitored weekly by radiographs. The mice were sacrificed on days 7, 14, 21 and tibias were harvested, fixed in 10% formalin and embedded in paraffin. Sections were stained with alcin blue and hematoxylin. Histomorphometry of fracture callus was performed using Osteometrics®.

Local FGF injection: Recombinant human FGF-1 was obtained from Rhone Poulenc Rorer (Vitry, France). Local injections were made subcutaneously on both sides of the mouse calvaria. They were injected 4 times a day for 3 days in 10ul vesicle containing 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.4). The mice were euthanized by CO2 on day 14 after the injection. Mouse calvaria were harvested and processed. New bone in decalcified calvarial sections was identified under polarized light illustration by its differential staining with eosin on the woven collagen structure. (Dunstan, 1999).

Results

COX-2 but not COX-1 activity is required for fracture healing: Radiographic analyses demonstrated a significant delay of hard callus formation in COX-2 -/- but not COX-1 -/- mice when compared with their wild type littermate controls. Histological analyses are consistent with the findings from X-ray. At day 7, both wild type and COX-2 -/- animals had abundant mesenchymal cell proliferation and little or no bone formation. By 14 days, 70% of the callus in wild type animals was occupied by woven bone, compared to only 30% in COX-2 -/- animals (n=7, p<0.001), while mesenchymal cells predominated in the COX-2 -/- callus (60% in COX-2 -/- and 13% in wild type; p< 0.001). By 21 days, cartilage was completely replaced by bone in wild type mice. In contrast, COX-2 -/- mice had significantly more cartilage, as well as three times more mesenchymal tissue at the fracture site (n=8, p<0.001). Osteoclast numbers were also reduced by 57% (n=7, p<0.001), indicating a deficiency in osteoclastogenesis in COX-2 -/- mice. In contrast to COX-2 -/-, COX-1-/- mice of the same age showed a similar healing pattern and woven bone formation as their wild type controls (n=6). No significant deficiency in osteoclastogenesis was observed in COX-1 -/- mice.

COX-2 is required for intramembranous bone formation induced by FGF-1: Recombinant human fibroblast growth factor-1 (FGF-1) was injected subcutaneously (1ug/day x 3 days) onto the calvaria to examine growth factor induced intra-membranous bone formation. The COX-2 -/- mice had 60% less (n=5, p<0.0001) new bone formation, as well as markedly reduced in situ osteocalcin expression compared to wild type animals.

Discussion

NSAIDs, which inhibit cyclooxygenase activity, are one of the most frequently used drugs in our society. Due to the improved safety profile of the selective COX-2 inhibitors, these drugs are likely to be used frequently and for long periods in older individuals. COX-2 inhibitors may also be useful in prevention of diseases such as colon cancer and Alzheimer’s disease. Hence, it is important to evaluate the effects of inhibition of COX-2 on bone, in which the metabolites of cyclooxygenase are the most abundant. In this study we used knockout mice to demonstrate that COX-2 but not COX-1 plays an important role in bone repair in vivo. This study confirms the long suspected role of COX-2 in bone healing and these findings have important clinical relevance given the widespread use of the NSAIDs in our society and controversy regarding their effects on bone reparative processes.

Both endochondral and intramembranous bone formation pathways play indispensable roles in bone reparative processes. We demonstrate that COX-2 activity is required for both pathways. Although the regulatory mechanisms responsible for reparative bone formation are poorly understood, several growth-promoting substances have been identified at the site of skeletal injury and appear to play a physiologic role in bone healing. FGFs are among those factors that are shown to play a role in this repair process. The reduction of FGF-1 induced bone formation on COX-2 -/- mouse suggesting an important role for COX-2 in mediating some of the effects of FGFs in reparative processes of bone in vivo.

The delayed endochondral bone formation in COX-2 -/- mice suggests that COX-2 may be involved in multiple steps that lead to an increased bone formation, including recruitment of mesenchymal stem cells to the injury site, stimulation of angiogenesis and enhancement of extracellular matrix synthesis. Potential mechanism(s) by which inhibition of COX-2 leads to inefficient bone healing are currently under investigation.