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Inhibition of cyclooxygenase-2 down-regulates osteoclast and osteoblast differentiation and favours adipocyte formation *in vitro*☆

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenases (COX) and are widely used for post-trauma musculoskeletal analgesia. In animal models, NSAIDs have been reported to delay fracture healing and cause non-union, possibly due to the drug-induced inhibition of osteoblast recruitment and differentiation. To further investigate the cellular effects of these drugs in the context of bone healing, we examined the effects of COX-1 inhibitor indomethacin and COX-2 inhibitors, parecoxib and NS398 on osteoclast and osteoblast differentiation and activity *in vitro*. We discovered that all tested COX-inhibitors significantly inhibited osteoclast differentiation, by 93%, 94% and 74% of control for 100 μM indomethacin, 100 μM parecoxib and 3 μM NS398, respectively. Furthermore, inhibition of COX-2 reduced also the resorption activity of mature osteoclasts. All tested COX-inhibitors also significantly inhibited osteoblast differentiation from human mesenchymal stem cells. Simultaneously, the number of adipocytes was significantly increased. The adipocyte covered areas in the cultures with 1 μM indomethacin, 1 μM parecoxib and 3 μM NS398 were 9%, 29% and 24%, respectively, as compared with 6% in the control group. This data suggests that COX-2 inhibition disturbs bone remodelling by inhibiting osteoclast differentiation and diverting stem cell differentiation towards adipocyte lineage instead of osteoblast lineage. In conclusion, our results further suggest cautious use of COX-2 inhibitors after osseous trauma.

Keywords: Cyclooxygenase; Human mesenchymal stem cell; Adipocyte; Parecoxib

1. Introduction

Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins. Two forms of COX enzyme have been characterized extensively thus far: cyclooxygenase-1 (COX-1) and COX-2. COX-1 is involved in the maintenance of physiologic functions like haemostasis and gastric protection. The cyclooxygenase-2 enzyme (COX-2) is produced by a variety of cells *in vivo* in response to trauma and inflammation (Noor and Gajraj, 2003; Okada et al., 2003; Zhang et al., 2002). COX-3 has only recently been discovered in dogs and rats and its function may be related to paracetamol-induced hypothermia and analgesia (Botting and Ayoub, 2005; Kis et al., 2005).

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Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the synthesis of COX-enzymes and hence the production of prostaglandins, which mediate pain. The various NSAIDs exhibit differences in COX isoenzyme inhibition profiles (Gierse et al., 1999). Due to their analgesic and anti-inflammatory effects, these drugs are also widely used in the treatment of post-fracture pain and swelling. In addition to their role as mediators of pain, prostaglandins are also potent regulators of bone cell functions (Okada et al., 2003; Rawlinson et al., 2000). Especially prostaglandin E_2 (PGE_2) can stimulate the differentiation of both osteoblasts and osteoclasts (Liu et al., 2005). The effects of prostaglandins on osteoclast formation are mediated via enhancing the action of receptor activator of nuclear factor-kappa B ligand (RANKL) on osteoclast precursors. This induction is mediated by osteoblasts. The enhanced action of RANKL in osteoclast precursors also stimulates the differentiation of osteoblasts. (Okada et al., 2003; Liu et al., 2005; Choi et al., 2005; Wei et al., 2005). NSAIDs have different capabilities to inhibit different COX isoenzymes (Gierse et al., 1999).

Bone repair is a tightly regulated process which involves a highly orchestrated participation of several cell types. During the initial step of fracture healing, a haematoma covers the fracture area and inflammatory cells, such as macrophages and leukocytes, are attracted to the site of trauma (Ozaki et al., 2000; Simon et al., 2002). These cells provoke an inflammatory response, which involves various cytokines, growth factors and arachidonic acid metabolites and activated osteoclasts, all of which contribute to the osteoblastic proliferation and differentiation of stem cells. The final osteosynthesis occurs 3–6 weeks after the fracture. First, the organic extracellular matrix is produced and mineralized after which bone is continuously remodelled by resorption and reformation (Noor and Gajraj, 2003; Barnes et al., 1999; Einhorn 1998).

Data from previous animal studies suggests that inhibitors of COX-2 may interfere with the bone healing process (for review see Li et al., 2006). More specifically, Zhang et al. have demonstrated that healing of stabilized tibia fractures was significantly delayed in COX-$2^{-/-}$ mice (Zhang et al., 2002). Goodman et al. (2002) have showed that a COX-2 selective NSAID decreased bone growth *in vivo*. Giordano et al. reported a significant delay in fracture healing in tenoxicam group and the osseous union was more incomplete the sooner the NSAID-treatment was initiated (Giordano et al., 2003) So far, there are no prospective clinical studies on COX-2 and fracture healing. The only clinical studies thus far have been retrospective and focused on spinal fusions (Glassman et al., 1998; Reuben and Ekman, 2005).

The aim of this study was to further clarify the effects of the COX-inhibitors, with differences in COX-inhibition profiles, on osteoblast and osteoclast differentiation and activity, using various *in vitro* assays. Our results further suggest that COX-2 inhibition significantly decreases osteoclast differentiation (Sato et al., 1997; Kotake et al., 1999; Okada et al., 2000). We also show that COX-2 inhibitors decrease osteoblast differentiation and promote stem cell commitment towards the adipocyte lineage in human mesenchymal stem cell cultures.

2. Materials and methods

2.1. Reagents

Indomethacin was purchased from Alpharma OY (Helsinki, Finland), parecoxib from Pfizer OY and NS398 (*N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) from Sigma (St. Louis, MO, USA). α-Modified Essential Medium (α-MEM), phosphate buffered saline, pH 7.2 (PBS), 1 M HEPES Buffer, Trypsin–EDTA solution and penicillin/streptomycin solution (10 000 units/ml penicillin G sodium and 10 000 μg/ml streptomycin sulphate in 0.85% saline) were purchased from Gibco BRL (Paisley, the UK). Peroxidase-conjugated WGA-lectin, dexamethasone, L-ascorbic acid 2-phosphate, β-glyserolphosphate, bovine serum albumin, 4-*p*-nitrophenylphosphate and TRACP histochemical kit no 386 were purchased from Sigma (St. Louis, MO, USA). *O*-cresolphthalein-complexone was purchased from Roche Diagnostics (Mannheim, Germany). Oil Red O was obtained from Sigma.

Trizol reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA), Prime RNAse inhibitor from Eppendorf (Hamburg, Germany), M-MLV reverse transcriptase from Invitrogen Life Technologies and all primers used were from Oligomer (Helsinki, Finland), and Dynazyme II from Finnzymes (Helsinki, Finland).

2.2. Osteoclast resorption assay

The bone slices were prepared from frozen bovine cortical bone shafts and they were cut with a diamond saw to a 200 μm thickness, sterilized with sonication, rinsed briefly in 70% ethanol and then transferred to fresh 37 °C medium into 24-well plates. Osteoclasts were isolated from the long bones (femur, tibia, humerus) of 1- to 2-day old newborn male Sprague–Dawley rats, as previously described in detail. (Boyde et al., 1984; Chambers et al., 1984). Briefly, osteoclasts were scraped from the endosteal surface of split long bones into α-MEM and centrifuged at 200 ×g for 10 min. The cells were then resuspended in the same medium and an aliquot of 50 μl was added to bone slices which were placed on a piece of parafilm. The cells were allowed to attach onto the bone slices for 30 min at 37 °C 5%CO_2/95% air. After that, the non-attached cells were rinsed off with sterile PBS. The bone slices with the attached cells were then placed into 24-well plates and cultured in α-MEM buffered with 20 mM HEPES, 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% heat-inactivated fetal calf serum, in the presence or absence of 1 μM, 10 μM and 100 μM indomethacin or parecoxib or 0,03 μM, 0,3 μM and 3 μM NS398.

The cultures were stopped after 48 h by fixing the cells with 3% paraformaldehyde and 2% sucrose for 5 min. To detect apoptotic osteoclasts, the cells on the bone slices were stained for tartrate resistant acid phosphatase (TRACP) using a histochemical kit according to the manufacturer's instructions (Sigma) and with Hoechst, to detect the nuclei, as described in detail previously (Selander et al., 1996). In some experiments