

NSAIDs inhibit *in vitro* MSC chondrogenesis but not osteogenesis: implications for mechanism of bone formation inhibition in man

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Abstract

The non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for analgesia but may inhibit bone formation. We investigated whether the reported NSAID effect on bone is related to inhibition of bone marrow mesenchymal stem cell (MSC) proliferation and osteogenic and chondrogenic differentiation and evaluated both cyclooxygenase (COX)-1 and COX-2 specific drugs. The effects of seven COX-1 and COX-2 inhibitors on MSC proliferation and osteogenic and chondrogenic differentiation were tested using Vybrant, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT), functional and quantitative assays of MSC differentiation. The MSC expression of COX-1 and COX-2 and prostaglandin E2 (PGE-2) levels were evaluated serially during lineage differentiation by quantitative PCR and ELISA. None of the NSAIDs at broad range of concentration (range 10^{-3} to 100 $\mu\text{g/ml}$) significantly affected MSC proliferation. Surprisingly, MSC osteogenic differentiation inhibition was not evident. However, NSAIDs affected chondrogenic potential with a reduction in sulphated glycosaminoglycans (sGAG) content by 45% and 55% with diclofenac and ketorolac, respectively ($P < 0.05$ compared to controls). Parecoxib and meloxicam, more COX-2 specific reagents inhibited sGAG to a lesser degree, 22% and 27% respectively ($P < 0.05$ compared to controls). Cartilage pellet immunohistochemistry confirmed the above results. Pellet chondrogenesis was associated with increased COX-1 expression levels but not COX-2, and COX-1 specific drugs suppressed MSC PGE-2 more than COX-2 specific inhibitors. These findings suggest that NSAIDs may inhibit bone formation *via* blockage of MSC chondrogenic differentiation which is an important intermediate phase in normal endochondral bone formation.

Keywords: bone healing • MSCs • NSAIDs • COX-1 • COX-2

Introduction

The suppression of new bone formation may be either advantageous or disadvantageous depending on the clinical scenario. The stimulation of bone repair during fracture healing or in spinal fusion procedures is highly desirable [1, 2]. Conversely, the inhibition of bone repair in settings such as heterotopic ossification or spinal fusion associated with ankylosing spondylitis is equally

sought [3–6]. Although the molecular basis for bone repair is very complex, a wealth of experimental evidence supports the importance of prostaglandins in bone biology, and perhaps of the utmost clinical relevance is the involvement of prostaglandins in bone formation, given that their antagonists or cyclooxygenase (COX) inhibitors are widely used for the treatment of pain associated with fractures and in many orthopaedic and rheumatologic settings [7–9].

Numerous experimental evidence supports the importance of prostaglandins in bone biology. For example, impaired production of prostaglandin E2 (PGE-2) in COX knockout animals is associated with fracture non union and conversely PGE-2 agonists accelerate bone formation [10–12]. PGE-2 may be the most abundant prostaglandin involved in bone biology [13]. It can stimulate new bone formation, increase bone mass and also regulate both bone morphogenetic protein 2 (BMP-2) and BMP-7 expression [11, 12,

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14, 15]. In animal models where PGE-2 synthesis was blocked, bone healing was compromised but bone homeostasis was otherwise not affected [16]. Impaired production of PGE-2 in COX knockout animals is associated with fracture non-union and conversely PGE-2 agonists accelerate bone formation [10–12]. Although the molecular basis for bone repair remains unclear, prostaglandins antagonists or COX inhibitors are widely used for the treatment of pain associated with fractures and in many orthopaedic and rheumatologic conditions. Given that prostaglandins may be involved in bone formation, this raises clinically relevant concerns [7–9].

The traditional non-steroidal anti-inflammatory drugs (NSAIDs) inhibit both COX-1 and COX-2 thus reducing the levels of PGE-2. The introduction of more potent COX-2 inhibitors has also been associated with the diminution of PGE-2 expression in bone tissues [17, 18]. Given the aforementioned importance of PGE-2 in bone metabolism, it is not surprising that strong experimental evidence indicates that both traditional NSAIDs and the more specific COX-2 inhibitors may prevent bone formation in different settings including fracture healing and postoperative bone repair in spinal fusion [5, 6]. However, randomized controlled trials on the effects of NSAIDs on bone repair in man are lacking and some conflicting results show a negative effect or no effect at all [3–5, 19–22].

The cellular basis for bone repair *in vivo* is poorly understood but highly proliferative multipotential mesenchymal stem cells (MSCs) are thought to be the key cellular orchestrators of this process [2]. With respect to bone formation and fracture repair, two principal mechanisms are involved: intramembranous ossification with the direct formation of bone from MSC or endochondral ossification where a hypertrophic cartilaginous intermediate stage exists [23]. Although the effects of NSAIDs on bone formation are thought to be mediated by their interference with the PGE-2 pathway, data regarding their effect on MSC proliferation and differentiation are lacking. This study therefore investigated the effects of NSAIDs on the MSCs potential for proliferation and differentiation towards the osteogenic and chondrogenic lineages.

Materials and methods

Patients and cells

Between January and August 2006 patients were invited to participate in this study. Ethics committee approval was obtained (Ref number Q1206/127). Inclusion criteria included healthy adult patients admitted in our institution with lower limb or pelvic fractures requiring operative treatment. The exclusion criteria included pathological fractures, local and systemic inflammatory conditions and use of steroids or previous radiotherapy. In total 10 consecutive patients met the inclusion and participated in this study. There were five males and five females with a mean age of 43 years (range 18 to 81 years). MSCs were isolated from trabecular bone (TB, normal bone adjacent to fracture site, $n = 5$) or bone marrow (BM) aspirated from superior iliac crest, $n = 5$) [24]. BM aspirates were immediately transferred to 5 ml EDTA containing Vacutainer tubes, filtered

through a cell strainer and the mononuclear cell (MNC) fraction of the BM was isolated after density centrifugation using Lymphoprep (Axis-Shield, Dundee, UK). TB-derived MSCs were isolated using enzymatic digestion with collagenase 0.25% (Stem Cell Technologies, Vancouver, Canada) for 4 hrs (37°C, 5% CO₂). Of note both sources of MSCs were used for experiments since studies have suggested that both populations are able to proliferate and differentiate to osteogenic and chondrogenic lineages but also MSCs from both of those sites take part in the process of bone healing [25–27]. A total of 20×10^6 cells collagenase-released TB cells or BM MNCs were placed into 25 cm² flasks and grown till passage (p) 3. At p3 they were frozen in liquid nitrogen prior further use.

Cell phenotyping

Cultured MSCs were used for flow cytometry at 10^5 cells/test. Test antibodies were: phycoerythrin (PE)-conjugated nerve growth factor receptor (NGFR)/p75, CD106/vascular cell adhesion molecule-1 (VCAM-1), CD146/Muc18, CD166/activated leukocyte cell adhesion molecule (ALCAM), CD73/SH3 (all from Pharmingen, Cowley, UK) and CD105/SH2 (Serotec, Kidlington, UK), and fluorescein isothiocyanate (FITC)-conjugated CD45 (DAKO, High Wycombe, UK) and CD13 (Serotec). D7-FIB-PE was labelled in-house from purified D7-FIB (Serotec). Isotype-specific negative control antibodies were purchased from Serotec. Dead cells were gated out based on propidium iodide exclusion (Sigma, Dorset, UK). All flow cytometry data were analysed with WinMDI v8 (Scripps Research Institute, La Jolla, CA, USA).

NSAIDs preparations

The NSAIDs used in this study were selected on the basis of clinical use and soluble preparations being available. These included diclofenac sodium (Novartis, Athens, Greece), piroxicam (Pfizer, Athens, Greece), parecoxib (Pfizer, Athens, Greece), lornoxicam (Nycomed, Athens, Greece), meloxicam (Boehringer-Ingelheim, Athens, Greece), ketoprofen (Rhône-Poulenc Rorer AEBE, Athens, Greece) and ketorolac (Sigma). Peak plasma concentrations were 1 µg/ml for diclofenac sodium, 1.5 µg/ml for piroxicam, 1 µg/ml for parecoxib, 2 µg/ml for lornoxicam, 2 µg/ml for meloxicam, 4 µg/ml for ketoprofen and 1.1 µg/ml for ketorolac [28–33]. All seven NSAIDs were used for the proliferation and osteogenic differentiation but four were selected for chondrogenic differentiation. These included ketorolac and diclofenac as being more COX-1 specific, and celecoxib and ketoprofen as COX-2-selective NSAIDs.

Analysis of proliferation

The effect of NSAIDs on MSC cell proliferation was assessed by three different methods according to the number of viable cells, passage number and size of the colonies formed. Cellular adherence was allowed without the influence of the drugs. In the standard assay (3 days of culture), cells were seeded in triplicate wells of flat-bottomed 96-well tissue culture plates at seeding densities of 4000, 2000, 1000 and 500 cells per well. In the long-term assay (7 days of culture), the format was similar, but lower cell densities were used to avoid confluence (2000, 1000, 500 and 250 cells per well). The XTT assay was used to measure number of viable cells. (Roche, Burgess Hill, UK) At the end of the assay, 50 µl of XTT dye was added according to manufacturers' instruction. After 4 hrs (37°C, 5% CO₂)

Table 1 Primers used for real time PCR

Gene target	Forward (5'-3')	Reverse (5'-3')
GAPDH	AAC AGC GAC ACC CAC TCC TC	CAT ACC AGG AAA TGA GCT TGA CAA
COX-1	TCT GGG AGT TTG TCA ATG CCA	GCT GAT GTA GTC ATG TGC TGA GTT G
COX-2	CAA ATC ATC AAC ACT GCC TCA ATT	TTA ATG AGC TCT GGA TCT GGA ACA C
Runx2	GCA GCA CGC TAT TAA ATC CAA ATT	ATT CTG CAA CTA GAA AAA ACA GTT
Sox 9	GAC ACC TCT GGG GTC CCT TC	TCC TCA AGG TCG AGT GAG CTG T
Col2a	TTT CCC AGG TCA AGA TGG TC	TGC AGC ACC TGT CTC CAC CA

Primer concentration was 0.5 pmol/μl except for Cox-1 and Cox-2 which were 0.3 pmol/μl.

the optical densities (ODs) were read on spectrophotometer (OpsysMR, Dynex technologies, Chantilly, VA, USA) using 450 nm optical filter.

Secondly, cells were labelled with the fluorescent dye CFDA and their divisions were tracked using the Vybrant CFDA SE assay. (Invitrogen, Paisley, UK) In this assay cells were labelled with 1 μl/ml of Vybrant dye for 15 min. in 37°C, 5% CO₂. After incubation, the dye was removed, adherent cells were washed thoroughly (four times) with Dulbecco's phosphate buffered saline (PBS) and fresh growth media, with or without the studied NSAIDs. Cells were harvested on the first, third and fifth days after labelling. On the day of the harvest, the cells were trypsinized and analysed on flow cytometer.

Thirdly, 1000 cells per well were seeded in 6-well tissue culture plates in triplicate. On the seventh day of culture, adherent cells were washed with PBS, fixed with 1% paraformaldehyde (Sigma) for 15 min. and stained with Crystal Violet (1% in water, Becton Dickinson, Oxford, UK) for 2 min. Colony formation (CFU-F) was then measured by manually counting colonies.

Analysis of osteogenic differentiation

Osteogenic induction was performed with p3 cells in all experiments as previously described [34]. The two assays used to define the osteogenic potential of MSCs were the quantitative measurement of alkaline phosphatase (ALP) activity in the cellular protein fraction at day 7 and the calcium deposition at day 21 of osteogenic differentiation. The ALP was measured using p-nitrophenyl phosphatase (Sigma). The protein content was measured on the same cell lysates as ALP using the Bio-rad Protein kit (Bio-Rad, Hemel Hempstead, UK) following manufacturer's instructions and finally the quantification of calcium deposition was performed with the cresolphthalein complexone method (Thermo, Cramlington, UK).

Analysis of chondrogenic differentiation

For chondrogenic differentiation, a serum-free chondrogenic medium was used as previously described [34, 35]. Cells were allowed to differentiate for 21 days and form pellets. The drugs diclofenac sodium and ketorolac were included in the medium in their stated plasma concentration. Cells were fed three times per week with half-medium changes. At day 21, pellets after digestion with Papain (Sigma, how much how long) were assayed for their sGAG content using a commercially available assay (Wieslab AB, Boldon, UK). Additional immunohistochemical analysis was performed on day 21 pellets for as previously described [36].

Prostaglandin E2 assay

Total PGE-2 production in culture supernatant was also measured during the proliferation and chondrogenic assays. At the time of feeding, the discarded medium was assayed for the amount of PGE-2 present (R&D Systems, Minneapolis, MN, USA). The culture medium PGE-2 content was taken into consideration and deducted in the calculation of cell supernatant PGE-2 content.

Quantitative real-time PCR

RNA was isolated from cultured MSCs by guanidium-based cell lysis followed by a phenol/isopropanol extraction as previously described [36]. Removal of contaminating DNA (by DNase treatment) was confirmed by an intron-spanning PCR. First strand cDNA was synthesized using superscriptII (Invitrogen) and oligodT (Promega, Southampton, UK). Real time PCR was performed with an ABI7900 sequence detection system (Applied Biosystems, Warrington, UK) in the presence of SYBR-green. Primers for each gene were designed using Primer Express 2 (Applied Biosystems). These primer sequences are presented in Table 1. Glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) was used as a house-keeping gene.

Statistical analysis

Assumption of normality was tested with a one-sample Kolmogorov–Smirnov test. Data are expressed as mean (standard deviation) or median (range) as appropriate. Parametric and nonparametric data were compared using the unpaired Student's t-test and nonparametric paired test, respectively. The cut-off value for significance was $P = 0.05$. All calculations were done using the SPSS software standard version 13.0 for Windows.

Results

MSCs phenotype

As previously stated, studies have suggested that both BM and TB MSC populations are able to proliferate and differentiate towards osteogenic and chondrogenic lineages. Importantly, MSCs from both of sites can take part in the process of bone healing [25–27]. We therefore used both sources of MSC to perform this work. A

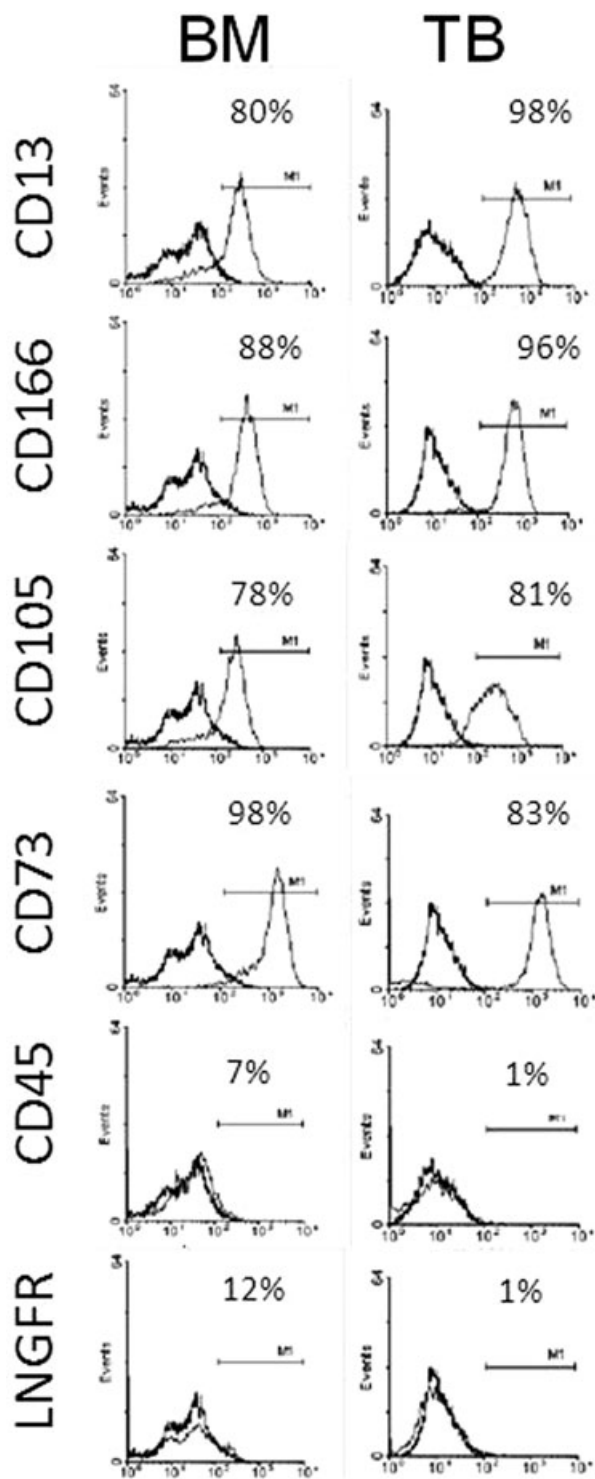


Fig. 1 MSCs from TB and BM are uniformly positive for CD13, CD166, CD105, CD73 and negative for CD45 and LNGFR. The bold line represents the isotype control antibody while the thin line represents the actual marker antibody.

panel of MSC markers was used to investigate surface phenotype of p0 MSCs derived from BM or TB. MSCs from both sources were uniformly positive for CD13, CD166, CD105, CD73 and negative for CD45 and low-affinity nerve growth factor receptor (LNGFR) confirming the phenotype for culture expanded MSCs (Fig. 1).

NSAIDs do not affect MSCs proliferation *in vitro*

Treating growing MSC cultures with all NSAIDs at their peak plasma concentration levels resulted in no statistically significant inhibition of the proliferation of MSCs *in vitro* as assessed by modified tetrazolium salt XTT and CFU-F assays. Of note, diclofenac sodium meloxicam and piroxicam but not the others (ketorolac, lornoxicam, parecoxib and ketoprofen) in concentration of 100 $\mu\text{g/ml}$ induced a decrease in MSC proliferation ($P < 0.05$) (Fig. 2A and B). These inhibitory concentrations are more than 100 times higher than the expected therapeutic plasma concentrations. The effect of Diclofenac sodium and ketorolac (at their peak plasma concentrations) were also evaluated using the Vybrant assay. No differences were found between treated and control cells at any time-points studied, which confirmed the XTT assay findings (Fig. 2D). Using the CFU-F assay, no difference in the number and size of colonies was found suggesting that both diclofenac sodium and ketorolac in an extended range of concentrations analysed (0.01 to 100 $\mu\text{g/ml}$) had any effect on MSC proliferation (data not shown). However, the highest concentration of diclofenac sodium showed again a negative effect confirming the findings obtained by XTT. These findings indicated that NSAIDs in physiological concentrations did not impair MSC proliferation.

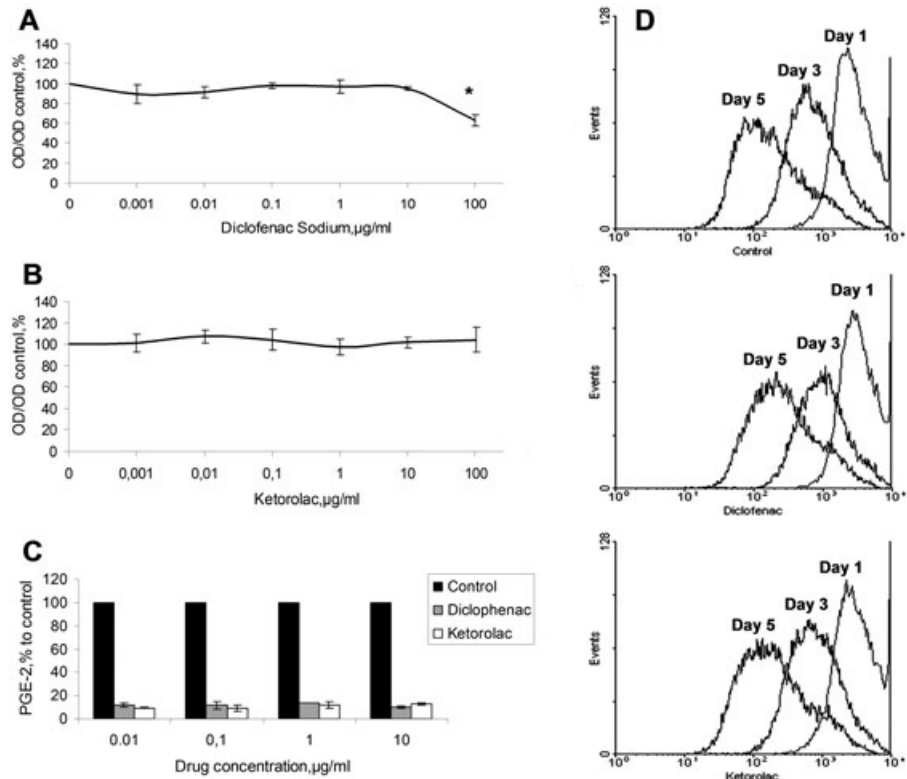
PGE-2 measurements in cell supernatants during proliferation

PGE-2 measurements were performed in MSC culture supernatants to verify whether or not endogenous COX-1/2 activity was blocked by NSAIDs in growing MSCs. In representative cultures, PGE-2 was measured in supernatants supplemented with either diclofenac or ketorolac. Basal production of PGE-2 was evident in all control cultures with a range of 342 to 562 pg/ml. A strong inhibition of PGE-2 production (average 90%) was observed for both drugs at all studied concentrations ($P < 0.05$) (Fig. 2C). These results demonstrate that endogenous production of PGE-2 by MSCs was severely decreased by both diclofenac sodium and ketorolac confirming that these drugs were active in this culture system but had no adverse effect on MSC proliferation at pharmacologically utilized doses.

The effect of NSAIDs on osteogenic differentiation of MSCs *in vitro*

Diclofenac sodium and ketorolac were used to treat MSC during differentiation assays towards osteogenesis. There was no

Fig. 2 (A) No statistically significant difference was found in the expected plasma concentrations of diclofenac sodium. A 38% decrease in proliferation was observed at 100 µg/ml of diclofenac sodium for all studied seeding densities suggesting a toxic concentration affecting the viability of the cells. (B) Ketorolac had no effect on MSC proliferation in the whole range of studied concentrations. (C) The culture medium supplementation with NSAIDs diminishes the PGE-2 production by the proliferating MSCs. (D) Flow cytometric analysis of growing cultures on days 1, 3 and 5 of culture. Vybrant fluorescence was read in FL1 (green) channel. Identical rates of proliferation for both treated and control cells are seen. * $P < 0.05$



significant difference of ALP activity/protein at day 7 for any of the studied concentrations of both diclofenac sodium and ketorolac (Fig. 3A, B and C). Similarly, no statistically significant difference was observed in calcium deposition at day 21 when medium was supplemented with both diclofenac sodium and ketorolac. Calcium deposition was also not affected by any of the other studied NSAIDs (data not shown) and no difference was noted between the COX-1 and COX-2 NSAIDs.

The effect of NSAIDs on chondrogenic differentiation of MSCs *in vitro*

Diclofenac sodium, ketorolac, parecoxib and meloxicam were used in differentiation towards chondrogenesis (Fig. 4). Combined data on sGAG content at the end of the chondrogenic differentiation assay (day 21) showed a significant decrease in the sGAG content per pellet in the presence of all four studied NSAIDs. Both diclofenac and ketorolac significantly decreased sGAG production (45% and 55%, respectively, $P < 0.05$). A lesser decrease was noted with parecoxib and meloxicam (by 22% and 27%, respectively, $P < 0.05$). The higher level of inhibition by ketorolac suggested a potentially predominant involvement of COX-1 in the chondrogenic differentiation of MSCs. It is also obvious that the less COX-1 selectivity, the less sGAG production by the growing chondrocytes. Chondrogenic differentiation data were confirmed

by Toluidine Blue staining (Fig. 4C). In some donors disturbances in the architecture of the pellets were also noted.

PGE-2 measurements in cell supernatants during differentiation

In order to demonstrate the inhibition of endogenous COX-1 and COX-2 activity by the added drugs, PGE-2 was measured in the pellet culture supernatants. Control data from both experiments showed a large increase in PGE-2 production at the earlier stages of chondrogenesis (above 5 ng/ml) (Fig. 4B). From the 10th day of differentiation, however, PGE-2 production rapidly decreased and remained constantly low until the end of the experiment. In the treated pellets, PGE-2 production was inhibited by both ketorolac and diclofenac sodium at all time-points. Furthermore, the peak levels of PGE-2 produced by control MSCs undergoing chondrogenic differentiation were approximately 15-fold higher than those produced by the treated cells.

Molecular basis for the inhibition of MSC chondrogenesis by NSAIDs

To explore the molecular mechanism by which NSAIDs may inhibit chondrogenesis, we measured COX-1 and COX-2 expression

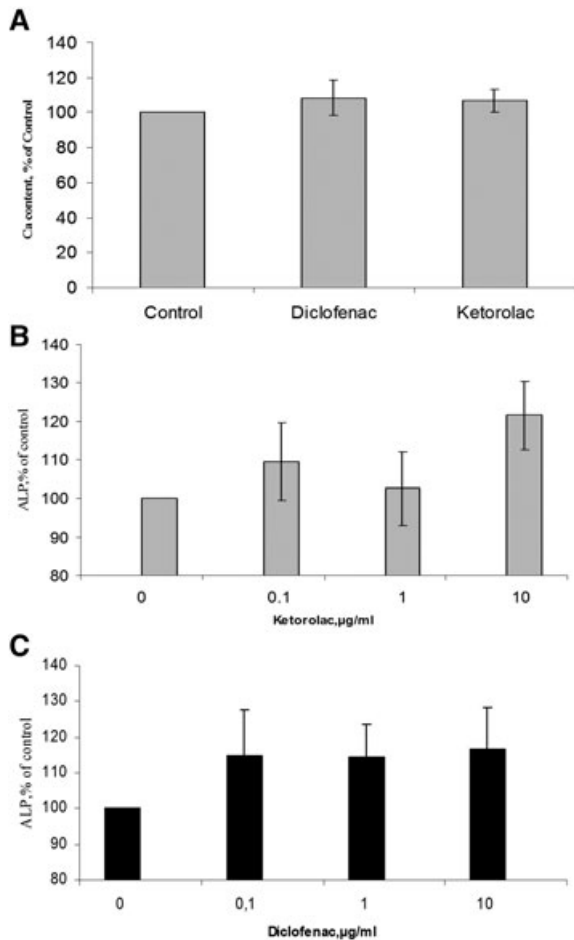


Fig. 3 (A) No difference of calcium content is observed in cells supplemented with diclofenac sodium and ketorolac at their expected peak plasma concentration. (B, C) The effect of diclofenac sodium and ketorolac on the ALP activity of MSCs on day 7. Both diclofenac sodium and ketorolac had no effect on the ALP activity of MSCs undergoing osteogenic differentiation.

during MSC osteogenic and chondrogenic differentiation using quantitative real time PCR ($n = 4$ donors). Firstly, progression towards osteogenic and chondrogenic differentiation was confirmed by a gradual increase in the expression of the transcription factor Runx2 and sox-9 (triggers of osteogenesis and chondrogenesis, respectively) followed by an accumulation of the message for mature bone-related protein osteocalcin and of Collagen II in cartilage formation (Fig. 5).

A gradual increase in COX-1 expression, paralleled by a slow decrease in COX-2 expression (approximately 7- and 5-fold, respectively) was observed during osteogenesis. This could lead to an overall 'no effect' result, explaining our findings of no inhibition of osteogenesis by NSAIDs. In contrast to osteogenesis, a substantial increase in COX-1 message (over 10,000-fold) was

observed, with only minor changes detected for COX-2 message during chondrogenic progression (Fig. 5). This could explain our results showing an increase in PGE-2 production during chondrogenesis and more pronounced inhibition of chondrogenesis by the selective COX-1 inhibitor ketorolac.

Discussion

There is an emerging consensus that NSAIDs may have a negative effect on bone formation but the cellular basis for this is incompletely understood. The present study used well established *in vitro* MSC assays to investigate the effect of NSAIDs on the proliferation and differentiation of MSCs.

Our findings suggested that the detrimental effect of MSCs on bone is more likely to be relevant when bone repair uses a cartilage intermediate stage, namely endochondral ossification.

Our first finding was that different types of NSAIDs only inhibited MSC proliferation at concentrations 50- to 90-fold higher than the peak plasma concentration. Similar findings were observed on canine osteosarcoma cells where anti-proliferative effects were only noted at very high concentrations of meloxicam [38]. On the other hand, our data are in disagreement with reports showing that physiological range of diclofenac, ketorolac or indomethacin inhibited both MSCs and osteoblast proliferation [39, 40]. However, the methodology and assays applied as well as the source of cells could account for this discrepancy. Finally, it is well established that NSAIDs have an anti-proliferative effect on abnormal, neoplastic cells of myeloid leukaemia, colon cancer, abdominal and hepatocellular carcinoma lineages [41].

We were surprised to find that NSAIDs had no direct effect on MSCs osteogenic differentiation. However, the literature supports the idea that NSAIDs are not inhibitory with respect to mature osteoblast function. Increased ALP activity during osteogenic differentiation was reported by Sell *et al.* who noted small increases in treated osteoblasts [39]. Differing effects on ALP activity and type I collagen, both of which increased, and osteoblast proliferation which decreased were noted by Evans and Butcher, following indomethacin treatment [42]. Ho *et al.* found that both ALP and type I collagen increased with various doses of ketorolac [40]. Our results, showing the lack of NSAID effects on bone, are in accordance with these studies.

Endochondral ossification is an important part of fracture healing when bone fixation permits a small degree of movement. In this study the results showed that both diclofenac sodium and ketorolac inhibited sGAG production of MSC chondrogenic pellets by 45% and 55%, respectively. Similarly, parecoxib and meloxicam decreased sGAG production but to a lesser degree and these findings were confirmed by immunohistochemistry.

This finding is open to a number of potential speculations explaining NSAIDs effect on growing cartilage. NSAIDs are organic acids, acting as reversible and competitive inhibitors of COX activity, decreasing the conversion of arachidonic acid to prostaglandins

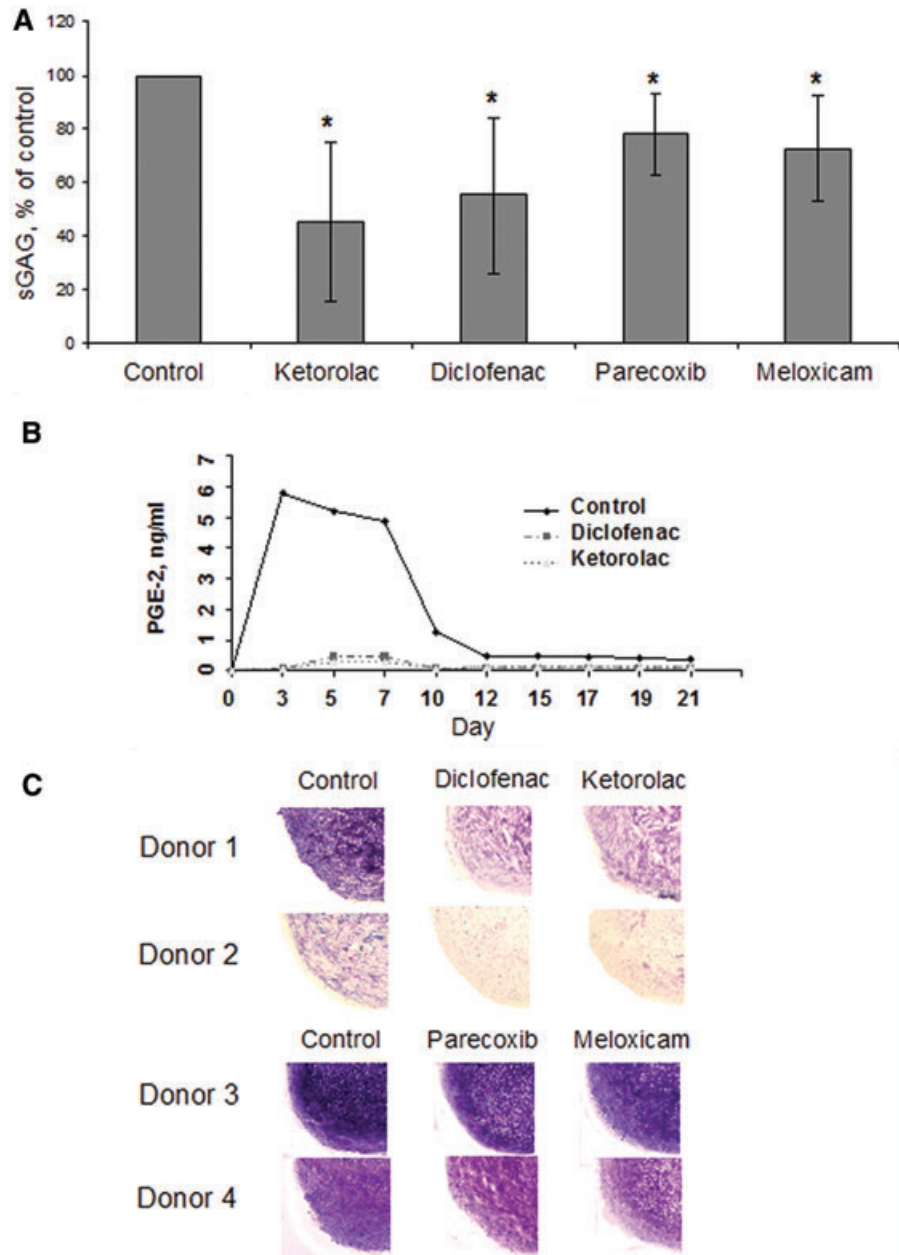


Fig. 4 (A) All studies NSAIDs down regulated sGAG production by the growing chondrocytes. Diclofenac sodium down regulated the sGAG production by 45% ketorolac by 55%, parecoxib by 22% and meloxicam by 27% ($P < 0.05$). **(B)** An increase in PGE-2 production takes place up to the 7th day of chondrogenic differentiation followed by a rapid decline and stabilization till the end of differentiation. Both drugs substantially decrease PGE-2 production in both experiments. $*P < 0.05$. **(C)** Immunohistochemistry of pellets confirming quantitative sGAG assay.

[43]. Therefore the first hypothesis consists of our finding that during the first week of chondrogenic differentiation a high endogenous production of PGE-2 takes place. If this production of PGE-2 is important for chondrogenesis, NSAIDs with their detrimental effect on this stage could result in an overall negative effect. This theory is strengthened by studies on animal models supporting the idea that early administration of NSAIDs inhibits bone healing while late does not have any significant effect [44]. In addition our data show this effect might relate to a greater physiological rise in COX-1 enzyme compared to COX-2 during the effector phase of MSC

chondrogenic differentiation. Another hypothesis is based on the COX-independent effect of NSAIDs mediated through modulation of the activity of various intracellular kinases that include the JNK, ERK, Akt, p38 MARK [45–50]. These can lead to a change in expression and activity of certain transcription factors like the NF- κ B, AP-1, Egr-1, p53, PPARgamma [45–50]. These signalling pathways could drive cell proliferation, motility, survival and regulate metabolic processes as glycogen and collagen production [45–50]. Unfortunately there are no available studies specifically on chondrocytes however these pathways are well studied in other cell

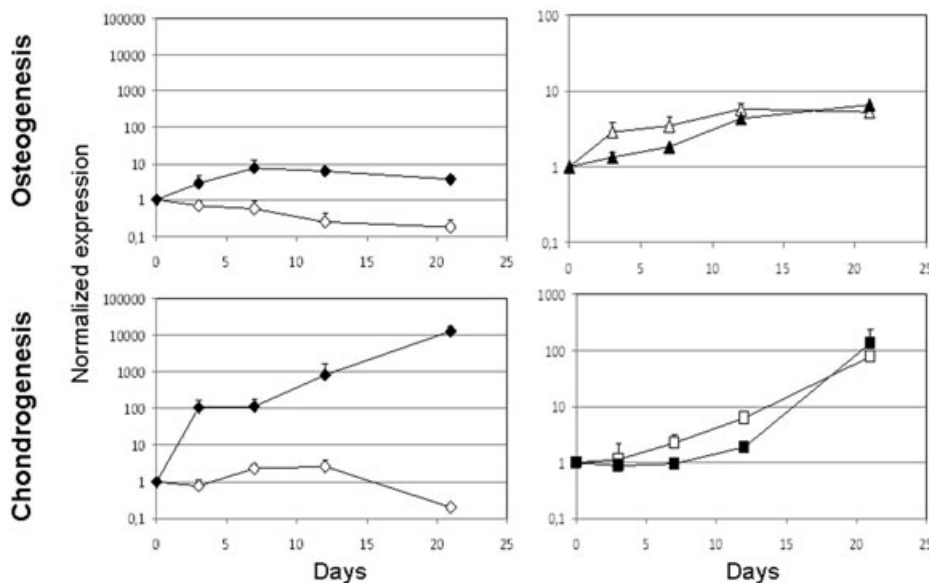


Fig. 5 Expression of COX-1 (black diamonds), COX-2 (open diamonds), Sox9 (black squares), Runx2 (black triangles), Osteocalcin (open triangles) and Collagen II (open squares) was measured by real time PCR and normalized to GAPDH. During both osteo- and chondrogenesis, COX-1 increased and COX-2 decreased however to a greater extent in chondrogenesis. Both transcription factors and end product increased steadily in their respective differentiation assay.

types in order to elucidate the anti-proliferative, pro-apoptotic and anticancer effect of NSAIDs.

Our results showed that the drugs diclofenac sodium and ketorolac decreased the PGE-2 production of MSCs in culture by 90% in the whole range of studied concentrations and that this was associated with reduced chondrogenesis. Similar results were reported by Ho *et al.* [40] PGE-2 amongst other prostaglandins (PGD-2 and PGF-2a) was found to increase hyaline cartilage matrix deposition and enhance chondrogenic differentiation [51]. This is in agreement with the present finding showing the inhibition of sGAG production and impaired chondrogenesis in the pellet system.

Not only are our findings relevant to bone biology but they could be equally relevant for articular cartilage biology. In cases of growing cartilage, such as during skeletal system development or endochondral ossification, the use of NSAIDs remains in question. In an *in vitro* model studying the effects of meloxicam on growing chondrocytes, Bassleer *et al.* found no effect upon DNA, collagen II and proteoglycans synthesis [52]. Other drugs like sodium naproxen shared similar effects in contrast to aspirin which was found to impair the formation of chondrocytes [53, 54]. In a COX knockout model Zhang *et al.* reported that although the fractures of COX-2 knockout mice failed to calcify, they produced substantial amounts of un-mineralized cartilage [54]. This suggests pure COX-2 enzyme inhibition or the complex mitogenic milieu *in vivo* could account for these changes.

There is evidence that the overall action of NSAIDs could be dose and time dependant. Karachalios *et al.* indicated that the low therapeutic doses of highly COX-2 had a minor effect on bone healing of rabbits [55]. Similar finding were reported by a number of authors who also suggested minor reversible negative effect [3, 44, 55]. Late administration of NSAIDs was reported to have no significant effect of bone healing as NSAIDs seem to interfere with

the early stages of bone healing, *i.e.* inflammatory and haematoma stage, and when NSAIDs administration is discontinued healing is restored and the negative effects are reversed [55].

In conclusion, the findings of the herein study suggest that NSAIDs do not interfere with MSCs potential to proliferate and differentiate into osteogenic lineage. However, they do inhibit the chondrogenic potential of MSCs *in vitro*. If this also occurs *in vivo*, it suggests that NSAIDs could interfere with fractures where endochondral ossification occurs and bridging callus is required in order to minimize motion permitting the ossification of the fracture. In this context, their use should be limited. On the other hand, this study does not support the discontinuation of NSAIDs on simple well-stabilized fractures where primary bone healing is anticipated. It could also provide evidence that administration of NSAIDs during the first week after fracture, when the pain is more intense, is safe since endochondral bone formation begins later. On another positive note, these data would support a mechanistic role for NSAID use in ankylosing spondylitis where both traditional and COX-2 specific drugs may retard new bone formation at enthuses which is thought to be mediated through an endochondral bone formation like pathway.

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Conflict of interest

All authors have no conflicts of interest.

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