The influence on human osteoblasts in vitro of non-steroidal anti-inflammatory drugs which act on different cyclooxygenase enzymes

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There is increasing evidence that non-steroidal anti-inflammatory drugs (NSAIDs) can adversely affect bone repair. We have, therefore, studied the in vitro effects of NSAIDs, which differentially inhibit cyclooxygenases (COX), the prostaglandin/thromboxane synthesising enzymes, on human osteoblasts. Indomethacin and the new nitric oxide (NO)-donating NSAIDs block the activity of both COX-1 and COX-2. Indomethacin and 5,5-dimethyl-3-(3 fluorophenyl)-4-(4 methylsulphonal) phenyl-2 (5H)-furanone (DFU) reduced osteoblast numbers in a dose-dependant manner and increased collagen synthesis and alkaline phosphatase activity. The reduction in osteoblast numbers was not caused by loss of adhesion and was reversible. Neither NSAID influenced DNA synthesis. There was no difference between the effects of indomethacin and DFU. NO-NSAIDs did not affect cell numbers.

These results suggest that care should be taken when administering NSAIDs to patients with existing skeletal problems and that NO-NSAIDs may be safer.

Bone is a dynamic organ, undergoing both resorption and formation in response to changing demands from the external environment. Skeletal homeostasis is disturbed in pathological conditions such as osteoporosis and injury. Homeostasis is maintained by many factors including prostaglandins (PGs). PGs are important in normal and pathological bone turnover. For example, oestrogen deficiency can increase PG synthesis in bone and elevated PG concentrations are implicated in aseptic loosening. PGs modulate osteoblast proliferation and differentiated functions and exogenous PGs have biphasic effects, dependant upon dosage. In addition, exogenous PGs have a biphasic effect on osteoblast proliferation so that the effect of a PG may be stimulatory or inhibitory to bone cells, depending upon its concentration.

PGs are synthesised by the enzyme cyclooxygenase (COX). The activity of COX is modulated by an autocrine feedback loop. COX exists in two different forms, a constitutive isoenzyme, COX-1, and an inducible one, COX-2, which are differentially regulated. Very recently, the identification of an alternative splicing of the COX-1 gene has been demonstrated, encoding a third enzyme, called COX-3. Different molecules of NSAIDs can inhibit either COX-1 or COX-2 activity, although few molecules are completely enzyme specific. Commonly used NSAIDs such as aspirin and indomethacin block the activity of both isoenzymes, although their effect is greatest on COX-1. New types of NSAIDs have been developed which affect COX-2 activity dramatically more than COX-1. Recently, in a totally different approach to the development of NSAIDs, nitric oxide (NO) has been coupled to classical and widely used NSAIDs. The chemical approach uses a spacer arm as a linker of the NO moiety to the molecule, allowing release of NO from the drug in vivo. As NO is an important modulator of bone metabolism these drugs may have a role to play in the treatment of orthopaedic patients. The healing response to bony injury is a complex process involving a cascade of linked events, each of which must occur at the correct place in the sequence for bone to be formed successfully. NSAIDs are often given to these patients. There has been an increase in the use of COX-2 inhibitory NSAIDs, which have fewer gastrointestinal side effects than traditional NSAIDs, but there have been few studies into any potential side effects that COX-2 inhibitors may have on the skeleton. NSAIDs have been shown to delay fracture healing in animals and to affect bone formation adversely, through a mechanism that is unclear.
delay in healing of fractures in patients.\textsuperscript{25,26} Another study showed there was a greater risk of fracture for users of NSAIDs compared with non-users, whilst other studies have shown that NSAIDs decreased the amount of bony ingrowth into an orthopaedic implant.\textsuperscript{27,28}

Studies \textit{in vitro} have shown that NSAIDs inhibit osteoblast proliferation and stimulate protein synthesis, but the great majority of these studies used either animal tissues or cell lines.\textsuperscript{4,6} There is only one report in the literature using human osteoblasts.\textsuperscript{32,34} First passage cells were removed by the explant technique of Beresford et al\textsuperscript{32} from samples of human trabecular bone obtained from the femoral heads of patients undergoing total hip replacement for osteoarthritis. Informed consent was obtained before using the samples for this study. Any patients with infections or with clinical conditions which might compromise the viability of the bone cells were excluded. The cells were grown to confluency in Minimal Essential Medium with 10\% foetal calf serum plus supplements (Life Technologies, Inchinnan, UK), as described previously.\textsuperscript{33} We, and others, have previously shown that these cells have many of the characteristics of osteoblasts.\textsuperscript{32,34} First passage cells were removed by enzyme digestion for use in our experiments.

\textbf{NSAIDs.} The NSAIDs tested in this study were indomethacin, which inhibits COX-1 and COX-2, DFU, a selective inhibitor of COX-2 and the NO-donating NSAIDs, NCX 4016, NCX 701, HCT 1026, which are all non-specific COX inhibitors.

\textbf{Cell numbers.} The osteoblasts were seeded at 1 x 10\(^4\) per well into a 24-well plate (Life Technologies) and cultured for up to four days with the NSAIDs (3 x 10\(^{-7}\) IND or DFU). Cell numbers were assessed every 24 hours. The osteoblasts were removed by trypsinisation and cell numbers counted as before. Enzyme digestion for use in our experiments.

\textbf{Collagen synthesis.} Osteoblasts were cultured in 48-well plates at a cell density of 2.5 x 10\(^4\) cells per well. NSAIDs were added after 24 hours and the osteoblasts incubated for a further 48 hours. The culture media were removed, snap frozen and stored at -20\(^\circ\)C. Total collagen content was assayed using a commercial kit (BioColor, Belfast, Ireland) and cells counted as before.

\textbf{Alkaline phosphatase activity.} Osteoblasts were cultured at 5 x 10\(^4\) cells per well in 24-well plates and NSAID were added after 48 hours in culture. A further 24 hours, the medium was removed and the cells were lysed by freeze-thawing and were assayed for ALP activity using the method of Lowry.\textsuperscript{37}

\textbf{Statistical analysis.} Each experiment used a separate patient sample with internal controls (i.e., cells not treated with NSAID). The results from individual experiments for a specific parameter were pooled for analysis. Statistical analysis was undertaken using the Student's \textit{t}-test when two variables were under analysis and ANOVA for more than one variable. Each set of experiments was carried out using cells from a minimum of four patients.

\textbf{Results}

\textbf{Cell numbers.} The first set of experiments, which examined the effect of chronic exposure of osteoblasts to NSAID,
demonstrated an overall decrease in cell numbers in cultures incubated with NSAID compared to control cultures (Fig. 1). As would be expected, the numbers of cells in control cultures rose steadily over the entire culture period, increasing from $1.0 \times 10^4$ cells per well at the start of the experiment to $1.8 \times 10^4$ at the end. The number of cells in cultures incubated with both NSAID declined with time so that at the end of the experimental period the number of osteoblasts in cultures with indomethacin were only 13% of the control. A similar difference was seen in the cultures with DFU, where numbers were only 22% of the controls. A statistically significant difference between test and control cultures ($p < 0.001$) was seen as early as day two onwards for both drugs and remained statistically significant thereafter. The data show that both drugs reduced osteoblast numbers overall. For DFU this effect was delayed until day two but there was no statistically significant difference in the effect the two drugs had on osteoblast numbers ($p > 0.05$).

The second set of experiments examined whether the NSAID had a dose-dependant effect on osteoblast numbers. Table I shows a dose dependant decrease in cell numbers with increasing concentrations of NSAID. The difference became statistically significant ($p \leq 0.05$) at $0.003 \mu M/l$.

Recovery of osteoblasts after incubation with NSAID. The osteoblasts were able to recover from 24 hours incubation with NSAID, as shown by the increase in cell numbers seen over the rest of the experimental period (Fig. 2). Incubation with NSAID caused a statistically significant decline ($p < 0.001$) in osteoblast numbers to 77% (indomethacin) and 81% (DFU) of the control. Only 24 hours after removal of the drug cell numbers had increased to 92% (indomethacin) and 95% (DFU) of control.

DNA synthesis. Table II shows that after 24 hours incubation, uptake of TCA-insoluble H3-Tdr was similar in osteoblast cultures incubated with or without NSAID ($p > 0.05$). This lack of response to NSAID was seen in cultures seeded at both $2 \times 10^4$ and $5 \times 10^4$ cells per well. Cell counts on
parallel cultures demonstrated the expected decrease in cell numbers, as was seen in the experiments examining the effect of NSAID on cell numbers described above (data not shown). The decrease was statistically significant ($p < 0.001$).

**Collagen synthesis.** An increase in the amount of collagen synthesised and secreted per cell was seen for osteoblasts incubated with NSAID (Fig 3). DFU increased the amount of collagen synthesised by 85% and indomethacin by 48% compared to control cultures. The range of values was, however, wide and the increase in collagen secretion was not statistically significant for either drug ($p > 0.05$). Furthermore, using the normalised data to minimise individual variability no significant difference was found in the effect of the two NSAID on collagen synthesis ($p > 0.05$).

**ALP activity.** Incubation of osteoblasts with NSAID stimulated ALP activity as demonstrated by the increase in specific activity of the enzyme exposed to both indomethacin and DFU for 48 hours. Pooling of normalised data from six separate experiments gave an increase of ALP activity for osteoblasts with indomethacin of 36%; ratio of Test/Control activity, expressed as $\mu$M para-nitrophenol (PNP) converted per cell, = $1.36 \pm 0.36$. For osteoblasts incubated with DFU, ALP activity increased by 41%; ratio of Test/Control activity, expressed as $\mu$M PNP converted per cell, = $1.41 \pm 0.19$. These increases in ALP activity did not reach statistical significance as the range of endogenous activity of ALP was wide (specific activity of controls ranged from 0.53 to 87 $\mu$M PNP). There appears to be a negative correlation ($r = 0.89$) between endogenous ALP activity and the effect of DFU on the level of ALP activity (Fig. 4a), such that, when endogenous ALP activity is high, little change in activity is seen in osteoblasts incubated with DFU and vice

<table>
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<tr>
<th>Drug</th>
<th>Seeding density 2 x 10^6</th>
<th>Seeding 5 x 10^6</th>
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<tr>
<td></td>
<td>$^3$H uptake %</td>
<td>$^3$H uptake %</td>
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<tr>
<td>Control</td>
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<td>2.07 ± 1.17</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>3.47 ± 2.17</td>
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</tr>
<tr>
<td>DFU</td>
<td>3.70 ± 2.22 $p$</td>
<td>1.81 ± 1.34</td>
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**Table II.** The effect of NSAID on DNA synthesis by osteoblasts. Results are expressed as means ± SD from at least three separate experiments with three replicates per treatment. Results are expressed as TCA-insoluble tritiated thymidine taken up by the cells as a percentage of the total radioactivity added.

![Fig. 3](image)

The effect of NSAID on collagen secretion by osteoblasts: the total collagen synthesised was assayed and cell numbers counted. Results are the means of data from four separate experiments and are expressed as $\mu$g collagen per 10^6 cells.

![Fig. 4a](image)

The relationship between basal level ALP activity and the effect of a) DFU and b) indomethacin. The results are from four separate experiments and are expressed as the log of the basal level of ALP activity ($\mu$M PNP per 10^6 cells) plotted against % increase in ALP activity.
versa. This relationship did not occur in osteoblasts exposed to indomethacin (Fig. 4b).

Effect of NO-NSAIDs. Unlike the results seen with conventional NSAIDs, none of the NO-donating NSAIDs tested had any effect on cell numbers (Table III) after 24 hours incubation with the drugs. There was no statistical difference in cell numbers (Wilcoxon signed-ranks test, p > 0.05) in test and control wells.

Discussion

This study compared two NSAIDs, indomethacin and DFU, which differentially affect COX isoenzymes. Indomethacin inhibits the activity of both COX-1 and COX-2, the constitutive and inducible forms of the enzyme, respectively, whilst DFU preferentially blocks the inducible COX-2 with a specificity of >1000 (expressed as a ratio of COX-2: COX-1). The aim was to investigate whether both NSAIDs had similar effects on osteoblasts in vitro or whether preferential inhibition of COX-2 had less effect on osteoblast metabolism than did inhibition of both isoenzymes.

Our results demonstrated that there were fewer osteoblasts in cultures incubated with either indomethacin or DFU, compared to control cultures and that this decline in cell number continued over the experimental period. This effect was seen equally for both drugs tested and was dose-dependent. The only other study in the literature, which recorded cell counts using rat calvaria, also showed a reduction in the number of osteoblasts.4 We also found that osteoblasts could recover and increase in number over time after only a brief exposure to NSAIDs, suggesting that the adverse effect on cell number of the two NSAIDs tested is reversible. This finding has implications for the timing of administration of such drugs to patients. Recent evidence in a rat model of bone remodelling shows different effects of indomethacin at different time points.38 These in vitro findings need further study to clarify the relationship between length of exposure to NSAID and the ability of cells to recover from the insult.

We were unable to demonstrate that either NSAID affected DNA synthesis by osteoblasts. The cell numbers in parallel cultures increased as usual, so this lack of response could not have been due to the lack of proliferation by the particular osteoblasts used. Data from the literature are inconsistent regarding the influence of NSAIDs on DNA synthesis in osteoblast-like cells.4,29

Discrepancies between studies can be attributed, partially, to the use of animal rather than human cells, differences in the timing of drug addition and in cell numbers. In our study, collagen synthesis by osteoblasts was increased by both NSAIDs when expressed as total amount per cell, but the range of the results was too wide to achieve statistical significance. As PGs inhibit collagen synthesis this increase is not unexpected.1,5,20 Similar studies using animal cells found either increases in collagen synthesis or no effect.4,39 In vivo investigations of bone healing showed that NSAIDs impaired this process, possibly because of a change in collagen synthesis.20,23,24,40

Similarly, non-significant increases were found in ALP activity after incubation with NSAID. Again, other studies have found variable effects of NSAIDs on osteoblasts.6

The only study on human osteoblasts showed a decrease in ALP activity with indomethacin, but it used different units of activity and a lower cell number, at which the negative regulatory effect of the genes important in cell proliferation could act on ALP.4,31

The finding that the stimulatory effect of DFU (but not IND) varied depending upon the endogenous level of activity of ALP leads us to speculate that suppression of COX-2 only might indirectly send a signal to the ALP gene to increase transcription, thus increasing a low level of ALP activity. There is evidence of negative regulation of ALP activity by PGE2 by modulation of genes important in osteoblast differentiation.31,42 To our knowledge these results are the first to demonstrate the effect of a COX-2 inhibitor on human osteoblasts. Our data demonstrate that both a COX-2 inhibitor and a non-specific NSAID have similar effects on most of the osteoblast parameters tested (p > 0.05). The exception to this was the effect of the COX-2 inhibitor DFU on ALP activity. A recent in vivo study using knockout mice demonstrated the integral role of COX-2 in bone repair.43

The results of our study demonstrate that both NSAIDs tested reduced osteoblast numbers without a concomitant decrease in DNA synthesis and that the osteoblast population could recover from these inhibitory effects if the NSAID are removed. This suggests that the NSAIDs may either initiate apoptosis or necrosis of osteoblasts, so that there are fewer osteoblasts in the culture. Preliminary results suggest that incubation with NSAID did not initiate apoptosis of osteoblasts, but these findings require confirmation and expansion.

The preliminary study using NO-donating NSAIDs showed that osteoblast numbers were unaffected at the concentration used, but further work is needed to confirm these findings and to examine the effect of these drugs on other parameters of osteoblasts activity, given the pivotal role of NO in bone growth and remodelling.

Clinically, NSAIDs are widely used to control pain and inflammation related to bone damage. In pathological conditions where maximal new bone formation is required and where suppression of osteoblast proliferation is undesirable, these results suggest that NSAIDs should be used with care. It would appear that in fracture healing an alternative analgesic is preferable where an increase in osteoblast numbers is important.38 Probably at one to two weeks post-

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<th>Control</th>
<th>NCX 4016</th>
<th>NCX 701</th>
<th>HCT 1026</th>
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<tr>
<td>1.7 (0.06)</td>
<td>1.8 (0.18)</td>
<td>2.45 (0.051)</td>
<td>2.0 (0.07)</td>
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fracture their use is acceptable and could even be desirable at stages where optimisation of ALP activity is important, e.g. during matrix synthesis and mineralisation of callus. Our results also show that a specific COX-2 inhibitor, DFU, which will have fewer adverse side effects on the gastrointestinal tract, had a similar effect on osteoblasts as a traditional NSAID, indomethacin. In the light of the previous published data on inhibition of bone resorption and the preliminary evidence reported in this paper, particular interest should be addressed to the new class of NO-donating NSAIDs, which are gastrointestinal safe and may possess a sparing profile on the bone compartment, interfering less with the osteoclast/osteoblast balance.15,20,44

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References