Modulation of IL-1β, IL-6, TNF-α and PGE₂ by pharmacological agents in explants of membranes from failed total hip replacement

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Summary

Introduction and goal: Proinflammatory cytokines and prostaglandin E₂ (PGE₂) play an important role in the pathophysiology of osteolysis and implant loosening. The aim of this study was to evaluate the role of pharmacological agents in the inhibition of Interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) and PGE₂ in explants of interface membranes from failed total hip replacements (fTHR).

Material and methods: Membranes from fTHR were retrieved (N=20) and explants were incubated for 72 h in the absence or presence of tenidap at three different concentrations (5, 20 or 50 µg/ml) or diclofenac (125 µg/l). IL-1β, IL-6, TNF-α, and PGE₂ levels were measured in the culture medium using ELISA Capture or EIA kits. Statistical analysis was done using the Mann–Whitney U-test.

Results: A statistically significant inhibition in IL-1β synthesis was found at tenidap concentrations of 20 µg/ml (71.3%, P<0.05) and 50 µg/ml (79.3%, P<0.02). Tenidap reduced IL-6 levels by 90.4% at 20 µg/ml (P<0.005) and 96.0% (P<0.05) at 50 µg/ml. Tenidap also reduced the synthesis of TNF-α by 66.9% (P<0.05) and 77.4% at concentrations of 20 µg/ml and 50 µg/ml. Tenidap had a marked suppressive effect of over 90% (P<0.001) on PGE₂ synthesis in all three concentrations. Diclofenac (125 µg/l) decreased PGE₂ production by 95% (P<0.0001), but had no significant effect in IL-1β, IL-6, and TNF-α levels in the culture medium.

Conclusion: The ability to simultaneously suppress the release of proinflammatory cytokines and PGE₂ may help control osteolysis and prevent aseptic loosening of THR. This effect could increase implant longevity and lead the way to the pharmacological treatment of this pathology. © 2002 Published by Elsevier Science Ltd on behalf of OsteoArthritis Research Society International.

Key words: Osteolysis, Total hip replacement, Cytokines, Tenidap.
patients\textsuperscript{20,21}, and on a dog model of OA\textsuperscript{12}. Tenidap has been demonstrated to be an effective drug in the treatment of rheumatoid arthritis\textsuperscript{22–24}. \textit{In vivo}, this drug was shown to be capable of reducing, in arthritic patients, several biological markers of inflammation and IL-6\textsuperscript{19,21,22}, the effect of tenidap on CRP and SAA proteins is likely related to the suppression of cytokine synthesis\textsuperscript{19,21,25}. The relevance of tenidap’s action as a cytokine modulating agent vis-à-vis osteolysis is most interesting as several reports have indicated that, at the clinical stage of the disease, variable degrees of inflammation are present\textsuperscript{26,27} and that interface membranes in failed THR synthesize an increased amount of IL-1β, IL-6, TNF-α and PGE\textsubscript{2}\textsuperscript{28}.

The aim of this study was to evaluate the efficacy of simultaneous inhibition of cytokines and PGE\textsubscript{2} in response to tenidap, a potent cytokine modulator, in explants of interface membranes from failed total hip arthroplasties, and to compare their effect with diclofenac, a currently used non-steroidal antiinflammatory drug (NSAID).

### Material and methods

**PATIENT CHARACTERISTICS**

Interface membrane specimens were obtained from the proximal femur at Gruen zones I and VII\textsuperscript{29} at the time of surgery from 20 patients undergoing revision procedures for clinical failure of total hip arthroplasty (Table I). All patients had a failed hybrid THR with a metal on ultra high molecular weight polyethylene (UHMWPE) bearing in order to minimize individual variability due to different wear debris.

The indication for a revision included pain and evidence of loosening. The presence of radiolucent lines at the bone interface and the migration of a component according to radiographic data confirmed loosening. The radiographs from the loose implants with osteolysis contained one or more areas showing ballooning and radiolucent zones with scalloped edges adjacent to the cement mantle or bone.

Twelve patients were men and eight were women. They all had a diagnosis of OA at the time of their primary THR. The mean age (61.7±5.6 years, 65.4±5.6 years old, mean±S.E.M., respectively) and the time to revision (57.0±12.9 months, 49.2±11.5 months, mean±S.E.M., respectively) for both men and women were similar. In all patients, both clinical and radiographic appearances were of aseptic prosthetic failure. Infection cases were excluded by clinical history and microbiology confirmation.

This study was approved by the Institutional Research Committee/Institutional Ethics Committee of the Centre hospitalier de l’Université de Montréal.

### EXPLANT CULTURE

Each interface membrane was aseptically dissected from underlying bone and rinsed in cold saline solution. Tissue samples were sectioned into small fragments of 4–5 mm in diameter. Explants weighing roughly 250 mg were processed, randomly divided, and cultured in duplicate in 12-well plates (Costar, Cambridge, MA, U.S.A.) with 4 ml Dulbecco modified Eagle’s medium (DMEM, GIBCO-Life Technologies, Burlington, ON, Canada), containing penicillin and streptomycin sulfate (100 U/ml and 100 µg/ml, respectively; GIBCO). No serum was present in the culture medium\textsuperscript{20}. Pilot studies were performed at 24 h, 48 h, and 72 h incubation to determine the ideal experimental conditions. Cultures were incubated at 37°C for 72 h in a humidified 5% CO\textsubscript{2}/95% air mixture in the absence or presence of tenidap ([Z]-5-chloro-2, 3 dihydro-3-[hydroxy-2-thienyl methylene]–oxo-1H-indole-1-carboxamide, sodium salt) (Pfizer Central Research, Groton, CT, U.S.A.) with 4 ml Dulbecco modified Eagle’s medium (DMEM, GIBCO-Life Technologies, Burlington, ON, Canada), containing penicillin and streptomycin sulfate (100 U/ml and 100 µg/ml, respectively; GIBCO). No serum was present in the culture medium\textsuperscript{20}. Pilot studies were performed at 24 h, 48 h, and 72 h incubation to determine the ideal experimental conditions. Cultures were incubated at 37°C for 72 h in a humidified 5% CO\textsubscript{2}/95% air mixture in the absence or presence of tenidap ([Z]-5-chloro-2, 3 dihydro-3-[hydroxy-2-thienyl methylene]–oxo-1H-indole-1-carboxamide, sodium salt) (Pfizer Central Research, Groton, CT, U.S.A.) with 4 ml Dulbecco modified Eagle’s medium (DMEM, GIBCO-Life Technologies, Burlington, ON, Canada), containing penicillin and streptomycin sulfate (100 U/ml and 100 µg/ml, respectively; GIBCO).

### Table I

<table>
<thead>
<tr>
<th>Age/sex</th>
<th>Months to revision</th>
<th>Type of failure</th>
<th>Age/sex</th>
<th>Months to revision</th>
<th>Type of failure</th>
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<tr>
<td>MP 75/F 34</td>
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<td>TM 71/F 34</td>
<td>THR dislocation/linear osteolysis</td>
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<tr>
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<td>MB 78/F 118</td>
<td>Linear osteolysis</td>
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<td>Massive linear/cavitary osteolysis</td>
<td>TK 68/M 62</td>
<td>Linear osteolysis</td>
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<tr>
<td>AL 68/F 40</td>
<td>Massive cavitary osteolysis/femoral fracture</td>
<td>GR 42/F 38</td>
<td>Linear osteolysis/peri prosthetic fracture</td>
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<tr>
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<td>Linear/cavitary osteolysis</td>
<td>AP 62/M 49</td>
<td>Linear osteolysis/peri prosthetic fracture</td>
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<td>JP 59/M 41</td>
<td>Massive linear wear/linear osteolysis</td>
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<td>AO 79/M 119</td>
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<td>Linear osteolysis</td>
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<tr>
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<td>Massive cavitary osteolysis</td>
<td>SO 69/M 41</td>
<td>Linear osteolysis</td>
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</table>

All patients had a diagnosis of hip osteoarthritis at the time of the primary total hip replacement. Only failed hybrid THR with metal on UHMWPE bearings were included in the study to standardize patient selection.

*THR: Failed total hip replacement.
CYTOKINE (IL-1β, IL-6 AND TNF-α) DETERMINATION

The levels of IL-1β, IL-6 and TNF-α were determined in the culture medium of membrane explants by a capture antibody in a sandwich enzyme-linked immunosorbent assay (ELISA Capture)33. The IL-1β, IL-6 Kit and TNF-α (high sensitivity) were obtained from R&D Systems, (Minneapolis, MN, U.S.A.). Each ELISA was performed according to the manufacturer’s specification. Sensitivity to the culture medium was 0.3 pg/ml for IL-1β according to the manufacturer’s specification. Sensitivity to (Minneapolis, MN, U.S.A.). Each ELISA was performed (high sensitivity) were obtained from R&D Systems, R&D Systems.

PGE2 DETERMINATION

PGE2 was measured in the explant culture supernatants using the PGE2 Immunoassay kit (Cayman Chemical, Ann Arbor, Michigan, U.S.A.). This assay is based on the competition between PGE2 and a PGE2-acetylcholinesterase conjugate (PGE2 tracer) for a limited amount of PGE2 monoclonal antibody33.

All reactions were measured on a micro-ELISA Vmax photometer (Molecular Devices Corp., Menlo Park, CA, USA). Data were expressed in ng/g tissue wet weight (ng/gww, IL-6 or PGE2) or pg/g tissue wet weight (pg/gww, IL-1β or TNF-α)32.

STATISTICAL ANALYSIS

Twenty patients were included in this study (N=20). Each membrane explanted from a given patient was tested for every experimental condition. To avoid discrepancies in cytokine and PGE2 levels when working with explants, two samples of the same membrane were cultured for every experimental condition and the mean result was included in the statistical analysis as the only corresponding data for a given patient. Therefore, our statistical analysis was based on 20 separate results for each variable (N=20). Mean values and standard errors of the mean were calculated, and statistical analysis was done using the Mann–Whitney U-test. P values equal or less than 0.05 were considered significant.

Results

CYTOKINE AND PGE2 SYNTHESIS IN EXPLANTS

Preliminary experiments were carried out using revision interface membranes explants, and the levels of inflammatory mediators were measured. Cytokine levels over various lengths of incubation time were also measured, and the optimal incubation time was found to be 72 h.

At first, we examined the level of cytokine and PGE2 synthesis in membrane specimens of loosened implant with osteolysis. The membrane explants produced IL-1β, IL-6, TNF-α and PGE2. Cytokine and PGE2 production varied considerably between patient samples. The secretion of IL-1β (194.72±52.47 pg/gww, mean±S.E.M.) and TNF-α (4.04±0.70 pg/gww, mean±S.E.M.) were in the pico-gram range, while the levels of IL-6 (183.34±110.04 ng/gww, mean±S.E.M.) and PGE2 (791.53±310.99 ng/gww, mean±S.E.M.) were in the nanogram range.

EFFECTS OF DRUGS ON CYTOKINES AND PGE2

IL-1β was detected on all the specimens used. As shown in Fig. 1, a statistically significant decrease in the IL-1β synthesis was found at two tenidap concentrations tested; with inhibition of 71.3% (P<0.05) at a therapeutic (20 μg/ml) concentration and 79.3% (P<0.02) at a supra-therapeutic concentration (50 μg/ml). Although the level of IL-1β in the tenidap-treated specimens was reduced by 51.0% at a concentration of 5 μg/ml, these results are not statistically different from the untreated (control) specimens.

Diclofenac (125 μg/l) had no significant effect on the inhibition of IL-1β synthesis. The interface membrane explants produced a very high level of IL-6 compared with the two other cytokines studied (IL-1β and TNF-α). Our results showed that tenidap, at a sub-therapeutic concentration (5 μg/ml), reduced IL-6 production by 77.5%, but there was no significant difference with the control group (Fig. 2). The reduction was statistically significant at 90.4% (P<0.005) and 96.0% (P<0.05) at a supra-therapeutic concentration (50 μg/ml). Although the level of IL-6 in the tenidap-treated specimens was reduced by 51.0% at a concentration of 5 μg/ml, these results are not statistically different from the untreated (control) specimens.

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Inhibition higher than 90% (P<0.0001) was observed for all tissues examined. Similarly, diclofenac significantly inhibited (P<0.0001) PGE$_2$ production at the interface membranes by 95% using therapeutic concentrations of the drug (125 $\mu$g/l) (Fig. 4).

**Discussion**

This study shows that tissue retrieved from fTHR synthesized significant levels of proinflammatory cytokines and PG. Indeed, high levels of PGE$_2$, IL-1$\beta$, IL-6 and TNF-$\alpha$ were synthesized by the tissue surrounding loose implant with osteolysis. This phenomenon suggests that numerous cytokines and other proinflammatory mediators are produced by bone and interface membranes of loosened prosthesis and that specific cytokines, namely IL-1$\beta$, IL-6 and TNF-$\alpha$ are associated with osteolysis$^{34}$. We have run preliminary assays at 24 h, 48 h and 72 h in a pilot study to address the question whether tissue extracts should have been used instead of culture medium after 72 h incubation in order to detect fine differences in our in vitro system. TNF-$\alpha$ was not detectable in significant levels prior to 72 h incubation in the culture medium and, yet, a TNF-$\alpha$ high-sensitivity kit from R & D Systems (Minneapolis, U.S.A.) had to be used. Moreover, IL-1$\beta$ levels were also at the picogram range at 72 h. Since TNF-$\alpha$ is considered to be the single most important cytokine implicated in wear-debris induced osteolysis$^{35-37}$, it seems logical that the other cytokines and PGE$_2$ should also be measured at 72 h. Our group has already successfully measured cytokine levels in the culture medium of RA synovial membranes explants at 72 h incubation with consistent results$^{39}$.

This corroborates the numerous studies that suggest the excess synthesis and secretion of proinflammatory cytokines from macrophages stimulated with wear particles play a major role in the pathophysiology of osteolysis and loosening process$^{38,39}$. Cytokines exert their action by binding to specific cell membrane receptors and transducing intracellular signals. IL-1 and TNF-$\alpha$ are very potent inducers of proteolytic enzymes, such as MMP, which can destroy the interface tissues and remove osteoid, thereby promoting osteolysis and providing a surface for osteoclastic bone resorption$^{34,40}$. IL-6 is also implicated in the modulation of bone resorption by the osteoclasts$^{40}$. The level of IL-6 was found in synovial fluid to be 10 times higher in revision total joint arthroplasties with osteolysis$^{2}$. There is higher cytokine expression in tissues surrounding cemented prostheses when compared to prostheses without cement$^{28}$. The excess synthesis of PGE$_2$ by interface membranes at the site of aseptic loosening is also one of the factors contributing to osteolysis and the loosening process$^{34,41-43}$.
Our results demonstrate that tenidap significantly inhibited the expression of several cytokines from 67% up to 90% in therapeutic concentrations. The inhibition of TNF-α by tenidap was between 66.9% and 77.4% (P<0.05) depending on the concentration used and that of IL-1β was between 71% (P<0.05) and 79% (P<0.02). Moreover, the level of IL-6 production was reduced by 90.4% (P<0.005) at the lower tenidap concentration and by 96.0% (P<0.05) at the higher concentrations. Tenidap also had a marked suppressive effect of over 90% (P<0.0001) on PGE2 synthesis at all three concentrations used.

Tenidap, a drug from the chemical class of the oxindoles, has been shown to modulate cytokine (IL-1β), IL-6 and TNF-α production in synovial membrane of RA patients. Tenidap also reduced in vivo IL-1β activity in synovial fluid as well as the activity and/or expression of MMP in cartilage in a canine experimental model. Tenidap was chosen for this study because of its very potent cytokine synthesis inhibition both in vitro and in vivo. On the other hand, diclofenac, a NSAID belonging to the arylacetic group of phenylacetic acid, significantly inhibited the synthesis of PGE2, a major factor associated with the pathophysiology and symptoms of osteolysis and implant loosening. However, no effects were observed on the synthesis of cytokines, which makes it known to be very important modulators of osteolysis in periimplant tissues.

Much of the work to find a pharmacological treatment for aseptic loosening has focused on bisphosphonates and TNF-α antagonists. The potential of TNF-α antagonists has already been demonstrated in an animal model in osteolysis. Thus, the reduction in TNF-α production by tenidap should have a similar effect on osteolysis prevention. Also, the production of IL-6 and PGE2 by particle-activated osteoblasts is thought to negatively influence the gene expression of procollagen and favor recruitment of osteoclastic cells. The inhibition of PGE2 observed in this study was not associated to a concomitant decrease in cytokine levels in the diclofenac group. PGE2 is directly related to IL-6 expression. Significantly suppressing PGE2 by 95% from 0 h to 72 h using either drug has most likely downplayed the increased levels of IL-6 found in the culture medium. Nevertheless, a significant inhibition of PGE2 should have also theoretically decreased IL-6 levels in the diclofenac group, which was not the case. Contrary to diclofenac, tenidap also seemed to have a direct inhibitory effect on IL-1, IL-6 and TNF-α, which explains its efficacy even at 72 h of incubation compared to PGE2 inhibition alone. The reduction of IL-6 and PGE2 secretion by tenidap could help restore the normal balance between bone formation and bone resorption in implant failure.

The interaction between osteoblasts, macrophages and osteoclasts in aseptic loosening of orthopedic implants is underestimated in the literature. However, accumulating evidence suggests that the osteoblasts play a central role in recruiting and activating cells responsible for bone destruction. Modulation not only of proinflammatory cytokines but also PGE2 synthesis by pharmacological agents is of prime importance in avoiding orthopedic implant loosening and osteolysis. Our findings suggest a possible role for multiple cytokine inhibition in the treatment/prevention of patients suffering from aseptic loosening. To our knowledge, this is the first time that simultaneous inhibition of multiple cytokines and PG in the interface membrane by a pharmacological agent has been reported. Further studies in vivo are necessary to evaluate the simultaneous inhibition of these proinflammatory cytokines in the pathophysiology of implant loosening, as well as its correlation to osteoblastic activity. These findings pinpoint specific targets for potential pharmacological prevention of failed total joint arthroplasties and provide alternatives to surgical treatment.

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