Comparison of Different Anti-Inflammatory Agents in Suppressing the Monocyte Response to Orthopedic Particles

Kevin J. Mulhall, MCh, FRCSI(Tr & Orth)*
William A. Curtin, MCh, FRCSI(Tr & Orth)*
H. Frederick Given, MCh, FRCSI†

Abstract

Three different anti-inflammatory agents—diclofenac, dexamethasone, and N-acetylcysteine—were compared to evaluate their effectiveness in suppressing monocyte-macrophage cell culture activation and mediator release (tumor necrosis factor-α [TNF-α] and interleukin-1β [IL-1β]) in response to polymethylmethacrylate particulate debris. N-acetylcysteine and diclofenac were most effective in suppressing TNF-α and IL-1β expression by the monocyte-macrophages. Dexamethasone reduced TNF-α expression but was not as effective in suppressing IL-1β expression. N-acetylcysteine and dexamethasone had no effect on cell viability whereas diclofenac at the highest concentrations decreased cell viabilities. N-acetylcysteine and diclofenac, but less so dexamethasone, are effective in suppressing wear debris-related cell activation and mediator release and thus potentially represent therapeutic or preventive modalities for periprosthetic osteolysis.

The importance of wear and loosening secondary to periprosthetic osteolysis as a limiting factor in the long-term outcome of arthroplasty is well recognized.1-3 Wear-related loosening of joint replacements occurs as a result of a combined mechanical and biological process; mechanically generated wear particles are associated with the biological host response of inflammation, osteolysis, and eventual loosening.

It has been demonstrated in vitro that particulate debris, particularly that of micron and submicron dimensions, stimulates macrophages, which then produce bone resorption directly and indirectly, via mediators, through osteoclasts.1,4

Macrophages are central to this proposed process in that they are the foremost cells of phagocytosis, although osteoclasts and other cells have also been shown to have phagocytic capability.2 The osteolysis observed in any individual case has been related to the effective joint space and joint pressures in the effective joint space, allowing access of wear debris to the bone cement/implant interface.

Although many pro-inflammatory cytokines and growth factors contribute to this process, interleukin-1β and, in particular, tumor necrosis factor-α are pivotal.4,5 For example, it has been demonstrated that TNF-α depleted mice fail to produce osteolysis in response to polymethylmethacrylate particulate debris, that orthopedic particle-induced osteolysis can be blocked with anti-TNF-α tumor neutralizing antibody and that mice failing to express any TNF-α receptor are protected from polymethylmethacrylate-induced bone resorption compared with controls, verifying that TNF-α is essential for periprosthetic osteolysis.10

The use of pharmacological agents in preventing the osteolytic response to particulate debris has attracted interest, with particular emphasis on anti-inflammatory agents and bisphosphonates.11-16 N-acetylcysteine is an antioxidant agent that has been shown to suppress TNF-α expression.17-20 N-acetylcysteine is atypical in its antioxidant activity, acting not through passive free radical resorption, but by actively inducing synthesis of a protein prior to nuclear factor-κB activation, which modulates cellular signal transduction cascades. Diclofenac is a nonsteroidal agent widely used in orthopedics and rheumatology. Dexamethasone is a steroid agent well known for its anti-inflammatory properties. However, the use of these agents has not been

From the *Department of Orthopedics, Merlin Park Hospital, and the †Department of Surgery, University College Hospital, Galway, Ireland.
Reprint requests: Kevin J. Mulhall, MCh, FRCSI(Tr & Orth) 74 Chapeltown, Drumcondra, Dublin 9, Ireland.

www.orthobluem journal.com
assessed and compared as potential methods of suppressing the immune response to orthopedic wear debris.

This study compared the effectiveness of N-acetylcysteine, diclofenac, and dexamethasone in reducing the activation and cytokine response of mononuclear cells to polymethylmethacrylate particles.

**MATERIALS AND METHODS**

**Particle Production for Culture Use**

Particulate polymethylmethacrylate (Howmedica International Inc, Shannon, Ireland) was used in all cultures. The cement was supplied sterile as spherical particles, and prior to use in cultures was washed with phosphate buffered saline and resuspended in plain Roswell Park Memorial Institute (RPMI) medium at a concentration of 1000 µg/mL. Particle size, as determined by electron microscopy, averaged 1 µm.

**Electron Microscopy of Particles**

Particles were prepared in dry state for scanning electron microscopy analysis. The particles were initially mounted on an aluminum stub using adhesive tabs. They were sputter-coated with gold and viewed with a Hitachi S-4300 FBS electron microscope (Hitachi Technology Corp, Tokyo, Japan).

**Mononuclear Cell Suspension Preparation**

A previously established protocol for obtaining isolated mononuclear cells for culture with orthopedic particles was used. Healthy volunteers donated blood, which was immediately processed. The fresh peripheral anticoagulated blood was first diluted with an equal volume of Hanks Balanced Salt Solution.

Aliquots of 20 mL of the diluted blood were each layered on 10 mL of Lymphoprep (Progen Diagnostics, Heidelberg, Germany) in 50 mL centrifuge tubes. These tubes were centrifuged at 1400 rpm at room temperature for 30 minutes. The “uffy” coat of mononuclear cells that formed at the interface between the serum and red blood cells with this centrifugation was removed and placed in clean tubes and diluted with an equal volume of phosphate buffered saline/fetal bovine serum 10% (PBS/FBS 10%)

The resultant tubes were centrifuged at 1400 rpm at 4°C for 10 minutes (wash procedure). After discarding the supernatants, the mononuclear cells were resuspended in 10 mL of PBS/FBS 10% and the “wash” procedure repeated.

All specimens were centrifuged at 1400 rpm at 4°C for 10 minutes. Resuspension of the resultant pellets was performed using 20 mL of PBS/FBS 2% (with 0.6% Na citrate) on a Vortex shaker (G. Kisker Glok, Steinfurt, Germany). The cells were characterized and counted using flow cytometry on a FACSscan flow cytometer with Lysis II version 1.1 software (Becton Dickinson & Co, Franklin Lakes, NJ) on a Hewlett Packard 98785A computer (Palo Alto, Calif). Standard Trypan Blue dye techniques with a KovaSlides counting chamber (Hycar Biomedical Ltd, Edinburg, UK) were used for all samples. Polypropylene tubes were used for all cell suspensions to prevent cell adherence while performing the cell counts and flow cytometry.

**Cell Culture and Treatment Methods**

To obtain cultures of only monocyte-macrophages, the mononuclear cell suspensions were plated in cell culture dishes for 3 hours to allow adherence of the monocyte-macrophages. Following adherence, nonadherent cells (ie, lymphocytes) were gently washed off using culture medium and the remaining cells were lifted off and resuspended in RPMI supplemented with 10% FBS, 5 µg/mL penicillin, and 50 U/mL streptomycin. The mononuclear cell lineage for final cultures was further verified at this point using the specific cytochemical stain α-naphthyl acetate esterase (Sigma Diagnostics, Dorset, United Kingdom).

The definitive cell cultures were established using 2.5×10⁶ cells per well of a 24-well culture plate. Two different doses of each agent were used to treat cells. N-acetylcysteine (Evans Medical Ltd, Leatherhead, England) was administered in concentrations of 30 mM/L and 60 mM/L, diclofenac sodium (Diclac; ROWA Pharmaceuticals, Cork, Ireland) in 10⁻⁶ M and 10⁻⁵ M per well, or dexamethasone Na Ph (David Bull Laboratories, Warwick, United Kingdom) in 10 µg and 100 µg per well. Cells were treated with these agents in two ways: 1) pre-conditioned with the agent added 1 hour prior to addition of particles (“pretreated” group), or 2) the agent added 1 hour after addition of the particles (“post-treated” group). The cells were then incubated at 37°C in 5% CO₂ for 24 hours.

To ensure the absence of a soluble mediator of monocyte activation in the polymethylmethacrylate (eg, lipopolysaccharide) that could be responsible for any activity seen, polymethylmethacrylate was incubated for 3 hours and particles were removed using sterile microfilters. These filtered media were cultured for 24 hours with monocytes as the main experiments but demonstrated no difference in cytokine expression (TNF-α and IL-1β) versus control cultures using plain culture medium.

**Cytokine Assays**

After the various cell cultures had been performed, the supernatants were carefully eluted and centrifuged once at 2000 rpm for 3 minutes (Sorvall RMC 14; Du Pont) to eliminate any residual cell material or particles interfering with the assays. Solid phase enzyme linked immunosorbant assays were then performed in duplicate, according to the detailed manufacturers instructions, to determine the levels of TNF-α (TNF-α Immunoassay, Quantikine; R&D Systems Europe, Oxon, United Kingdom) and IL-1β (IL-1β Immunoassay, R&D Systems Europe).

**Cell Viability in Culture**

The cell viabilities following 24-hour cultures with varying concentrations of polymethylmethacrylate parti-
icles, N-acetylcysteine, diclofenac, and dexamethasone were analyzed and compared with control cultures. The cultures were established by the methods outlined above and three concentrations of polymethylmethacrylate particles (100, 500, and 1000 μg/mL) were used, as were the two concentrations of the various agents used in the cultures described above. All cultures were performed in triplicate and the viabilities assessed at three time points over the 24-hour culture period. Cell viability was analyzed using the Trypan blue exclusion technique.

**Statistical Analysis**

Statistical analysis of the data was performed using a one way analysis of variance (ANOVA) using SigmaStat software (version 2.0; SPSS, Chicago, Ill) with a Tukey test for multiple comparisons to determine which treatment groups had significant differences. Cell viability analyses were performed using Student t test.

**RESULTS**

**Cytokine Assay**

Control cultures demonstrated that the plain control cultures expressed both TNF-α and IL-1β, but addition of polymethylmethacrylate particles produced a significant rise in TNF-α (P<.010) and IL-1β (P<.001) expression.

Pretreatment of the monocytes with all of the agents was associated with significant reductions in TNF-α expression versus the polymethylmethacrylate stimulated controls (Figure 1). The reduction in TNF-α expression was dose-dependent for N-acetylcysteine and diclofenac, but not dexamethasone, with significantly greater effect recorded at the higher concentrations. Although all agents used also suppressed baseline TNF-α expression by plain control cells, this effect was most significant for the higher dose of N-acetylcysteine and all doses of diclofenac (P<.001).

All of the agents significantly suppressed IL-1β expression in the pretreatment groups compared with particle stimulated cultures. However, only N-acetylcysteine and diclofenac at the higher doses significantly reduced IL-1β expression compared with plain control cultures (P<.005) (Figure 2).

In the post-treatment group, IL-1β expression was suppressed by N-acetylcysteine and diclofenac at all concentrations (P<.001) whereas dexamethasone failed to produce a significant reduction. All agents used as post-treatments were, however, significantly effective in reducing TNF-α expression comparable to the pretreated group, with N-acetylcysteine and diclofenac the most effective in this regard (P<.000).

The highest dose of N-acetylcysteine and both doses of diclofenac in the post-treatment group were associated with significantly reduced TNF-α compared to plain control cells (P<.01). None of the agents in post-treatment produced this response for IL-1β.

Direct comparison of pretreatment with post-treatment groups showed significantly better suppression of TNF-α and IL-1β (P<.05) with pretreatment with the highest doses of N-acetyl-
cysteine and diclofenac, indicating that at this dose level these agents are more effective when available before particulate debris acts on the cells.

Cell Viability in Culture

The results of our analysis of cell viabilities using the data obtained from the Trypan blue exclusion results at three time points over 24-hour incubation for plain, control cells and also for cultures with varying concentrations of polymethylmethacrylate particles and the other agents demonstrated decreases in cell viability over time in all culture conditions.

Comparison of the results between the various cell cultures revealed that the polymethylmethacrylate particles in the concentrations used (ie, 1000 µg/mL) and N-acetylcysteine or dexamethasone at any concentration were not associated with a statistically significant reduction in cell viability when compared to control cultures (P=.53 and P=.7, respectively). However, although diclofenac had no adverse effect on cell viability at 6 hours, it had effects at later times. At 12 and 24 hours, a significant effect was noted on mononuclear cells in the higher dose group (P=.003).

DISCUSSION

Given the extent of aseptic loosening around orthopedic implants, a pharmacological therapy preventing or treating periprosthetic osteolysis may be beneficial. Several anti-inflammatory agents have been compared to demonstrate their relative effectiveness in suppressing monocyte responses to polymethylmethacrylate particles. The circulating monocytes used in this study represent the precursors of the tissue macrophages and osteoclast-like cells implicated in aseptic loosening, and their use in analyzing responses to orthopedic particles is well established. Two types of cell culture were performed, pre- and post-treatment. Post-treatment cultures were performed to examine whether the proposed treatments would have any effect on cells that are already activated, which mimics to some extent the clinical scenario.

Our results demonstrated that pre- and post-treatment with diclofenac leads to significant decreases in TNF-α and IL-1β expression versus baseline and polymethylmethacrylate stimulated controls. Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID), a class of agents that act through inhibition of prostaglandin synthesis. Nonsteroidal anti-inflammatory drugs, as well as N-acetylcysteine, also inhibit TNF-α gene expression by macrophages, and may therefore theoretically prevent TNF-α induced osteoclastogenesis and osteolysis.

The common use of diclofenac as an anti-inflammatory agent in orthopedics made analysis of its effects of interest, but other NSAIDs, including indomethacin and mefanamic acid, have been evaluated in related research. These agents have been added to polymethylmethacrylate and cultured with human periodontal ligament fibroblasts, producing significant reductions in Prostaglandin E2 expression by lipopoly saccharide challenged cells. Although diclofenac was significantly effective in preventing mediator release in all of the experiments in this study, it also had significantly deleterious effects on cell viability in the higher dose. This lends doubt to the reliability of the findings on mediator release with the higher dose used, as a cytotoxic effect would also decrease mediator synthesis and release. This may undermine its potential usefulness as a reasonable local treatment for immune-mediated osteolysis.

Dexamethasone is an immune-modulating glucocorticoid steroid not frequently used in orthopedics. Although it is not a practical agent for osteolysis treatment, we analyzed it to assess its relative anti-inflammatory effects. Regarding the action mode of dexamethasone, Auphan et al demonstrated that it induces expression of IkB-α, which was previously shown to be a mechanism for modulation of NF-κB activation. Remick et al, using dexamethasone 3 hours prior to endotoxia challenge, significantly reduced macrophage TNF-α and IL-1β expression by 80% and 60%, respectively. It produced significant reductions of TNF-α release when compared with baseline and particle stimulated cells in the current study. However, it did not lead to significant reductions for IL-1β in post-treatment or versus baseline expression but reduced IL-1β in pretreatment compared to polymethylmethacrylate stimulated cultures. Overall, it was less effective than the other two agents across the range of experiments and specifically led to less significant reductions in mediator release than the other agents. Despite its well-documented anti-inflammatory effects, dexamethasone does not lead to consistent suppression of the specific particle associated activation of macrophages and does not appear to be an effective treatment choice for periprosthetic osteolysis.

Recent evidence indicates that pretreatment with the antioxidant agent N-acetylcysteine suppresses Lipopolysacchandi-mediated production of TNF-α and Prostaglandin E2 by resident lung macrophages and that N-acetylcysteine suppresses NFκB activation associated with reactive oxygen species (a critical convergence point of the inflammatory, cytotoxic, and cytokine/TNF-α expression modulating pathways). We therefore assessed whether N-acetylcysteine would be effective in suppressing the monocyte/macrophage response to polymethylmethacrylate particles. Our findings demonstrated that pre- and post-treatment with N-acetylcysteine significantly suppressed TNF-α and IL-1β expression. This was dose-dependent and was not associated with cytotoxicity. N-acetylcysteine thus appears to be a potentially useful agent in preventing monocytes activation, with significant effectiveness demonstrated in all experiments.

As mentioned earlier, it has become apparent that TNF-α is one of the foremost mediators of immune-based osteoclastogenesis and osteolysis, and is therefore the object of current analysis. Regarding the specificity of TNF-α in
mediating the osteolytic response, Merkel et al.\(^{16}\) demonstrated that TNF-\(\alpha\) is the primary soluble mediator of particle-induced osteolysis by showing that polyethylene particles induce macrophage expression of TNF-\(\alpha\) in a dose-dependent fashion, and that macrophages from TNF-\(\alpha\) receptor deleted mice failed to express an osteoclast phenotype in response to polyethylene particles. This also showed that mice failing to express any TNF-\(\alpha\) receptor are protected from polyethylene-induced bone resorption compared with controls.

As the effectiveness of the agents was greater when cells were pretreated, the timing of therapy also is an issue. Nivbrant et al.\(^{27}\) observed that macrophages in the joint capsule increase the production of TNF-\(\alpha\) at an early phase and in the absence of clinical loosening. This established that TNF-\(\alpha\) release precedes and leads to osteolysis, rather than being a tissue response to ongoing loosening and emphasizes the importance of early prevention of macrophage activation and TNF-\(\alpha\) expression. Using agents, such as those in this study, as additives in cement in a similar fashion to the antibiotics currently used or in some other local delivery system at implantation therefore suggests itself as a possible direct clinical application of the findings of this work.\(^{23}\)

**CONCLUSION**

The use of immune modulating agents, combined with improvements in techniques and bearing surfaces, may represent an important treatment modality for periprosthetic osteolysis. Of the agents assessed in this study, diclofenac and N-acetylcysteine were effective in suppressing monocyte activation in response to polyethylene particles. The higher concentration of diclofenac, however, was associated with some cytotoxicity therefore caution is required in extrapolating from the results for that agent. Further investigation, such as in vivo assessments of these interventions, is required to define the precise role of such treatment in reducing the burden of aseptic loosening of orthopedic implants.

**REFERENCES**


