Inhibition of polymethylmethacrylate particle-induced monocyte activation and IL-1β and TNF-α expression by the antioxidant agent N-acetylcysteine

Kevin J Mulhall[1], William A Curtin[1] and H Frederick Given[2]

Departments of [1]Orthopaedics, Merlin Park Hospital, Galway and [2]Surgery, University College Hospital, Galway, Ireland. E-mail: kjm@indigo.ie
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ABSTRACT – We investigated the effectiveness of an antioxidant agent, N-acetylcysteine (NAC), in suppressing macrophage activation and mediator release in response to particulate debris.

Polymethylmethacrylate (PMMA) particle-stimulated monocyte-macrophages were cultured alone and with varying concentrations of NAC. Tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) expression in the resultant cultures were measured using enzyme-linked immunosorbant assays. The ultrastructural effect of treatment was also assessed by electron microscopy. Cell viability in the various cultures was measured to rule out an effect of cytotoxicity.

NAC treatment reduced TNFα and IL-1β expression by the monocyte-macrophages. Culturing with NAC was also associated with less ultrastructural activation of the monocytes. Furthermore, NAC was not associated with any adverse effect on cell viability in the concentrations used.

Our findings demonstrate the effectiveness of the antioxidant N-acetylcysteine in suppressing the cell activation and TNFα release seen on exposure to wear debris. This represents a novel potential therapeutic method in the prevention or treatment of periprosthetic osteolysis.

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The importance of wear and loosening secondary to periprosthetic osteolysis as limiting factors in the long-term outcome of arthroplasty are well recognized (Murray and Rushton 1990, Schmalzried et al. 1992, Wright and Goodman 1995). The exact pattern of osteolysis is further related to the effective joint space, and to joint pressures in the effective joint space, allowing access of wear debris to the bone cement/implant interface.

The use of pharmacological agents in the prevention of this osteolytic response to particulate debris has attracted much interest, with particular emphasis on anti-inflammatory agents and bisphosphonates (Haynes et al. 1996, Horowitz et al. 1996, Pandey et al. 1996, Shanbhag et al. 1997, Sabokbar et al. 1998, Hiroi-Furuya et al. 1999). N-acetylcysteine (NAC) is an antioxidant agent that has been investigated for possible use in sepsis and that has been shown to suppress TNF-α expression (Mendez et al. 1995, Fox et al. 1997). The antioxidant activity of NAC is thought to occur via inhibition of Nuclear Factor-κB (NF-κB) activation through an effect on cellular thiol status and not by the more typical antioxidant mechanism of passive free radical resorption (Bellezzo et al. 1998). It has been further shown that NAC actively induces synthesis of a nuclear protein before NF-κB activation. This modulates cellular signal transduction cascades (Fox et al. 1997). Macrophage activation and expression of cytokine mRNA are thus suppressed by NAC at the nuclear level. However, the use of NAC or related agents has not been assessed as a potential method of suppressing the responses of cell activation, phagocytosis and cytokine expression to orthopedic wear debris.

We investigated the effect of the antioxidant agent NAC in reducing the activation and cytokine response of mononuclear cells to particles of polymethylmethacrylate.

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Methods and materials

Production of particles for use in cultures

Particulate polymethylmethacrylate 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 then resuspended in plain RPMI at a concentration of 1000 μg/mL. Particle size, as determined by electron microscopy, was, on the average, 1 μm.

Electron microscopy of particles

Particles were prepared in dry state for scanning electron microscopy (SEM) analysis. The particles were initially mounted on an aluminum stub using adhesive tabs. Following this, they were sputter-coated with gold and then viewed with a Hitachi S-4300 FES electron microscope with KV.

Preparation of mononuclear cell suspension

A previously established protocol for obtaining an isolated population of mononuclear cells for culture with orthopedic particles was employed (Herman et al. 1989, Schmalzried et al. 1992, Wright and Goodman 1995). In brief, healthy volunteers donated blood, which was then immediately processed. The fresh peripheral anticoagulated blood was first diluted with an equal volume of Hank's Balanced Salt Solution (HBSS). Each aliquotof 20 mL of the diluted blood was then layered onto 10 mL of Lymphoprep in 50 mL centrifuge tubes. These tubes were centrifuged at 1400 rpm at room temperature for 30 min. The 'buffy' coat of mononuclear cells formed at the interface between the serum and mainly red blood cells with this centrifugation was then transferred into clean tubes and diluted with an equal volume of phosphate-buffered saline/fetal bovine serum 10% (PBS/FBS 10%). The resultant tubes were then centrifuged at 1400 rpm at 4 ºC for 10 min (wash procedure). After discarding the supernatants, the mononuclear cells were resuspended in 10 mL of PBS/FBS 10% and the 'wash' procedure was repeated. All specimens were then centrifuged at 1400 rpm at 4 ºC for 10 min. Resuspension of the resultant pellets was then done using 20 mL of PBS/FBS 2% (with 0.6% Na citrate) on a Vortex shaker. The cells were characterized and counted using flow cytometry on a FACScan (Becton Dickinson) flow cytometer with Lysis II version 1.1 software on a Hewlett Packard 98785A computer. Standard Trypan Blue dye techniques with a Kovaslide counting chamber were also used in all samples. Polypropylene tubes were employed for all cell suspensions to prevent cell adherence while performing the cell counts and flow cytometry.

Methods of cell cultures and treatment

To obtain cultures of monocyte-macrophages alone, the monocytic cell suspensions obtained above were plated out in cell culture dishes for 3 hours to allow adherence of the monocyte-macrophages. Following this period of adherence, nonadherent cells (i.e., lymphocytes) were gently washed off using culture medium and the remaining cells were then lifted off and resuspended in RPMI supplemented with 10% fetal bovine serum, 5 μg/mL penicillin and 50 U/mL streptomycin. The mononuclear cell lineage of the cells in the final cultures was further verified at this point using the specific cytochemical stain α-naphthyl acetate esterase.

The definitive cell cultures were then established using 2.5×10^6 cells per well of a 24-well culture plate. These were treated with N-acetylcysteine, in concentrations of 15 mmol/L, 30 mmol/L and 60 mmol/L. Cells were treated in 2 ways, either being preconditioned with NAC added 1 hour before the addition of particles (which will be referred to as the 'pretreated' group) or having the NAC added 1 hour after adding the particles (which will be referred to as the 'posttreated' group). The cells were then incubated at 37 °C in 5% CO_2 for 24 hours.

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

Cytokine assays

When the various cell cultures had been done, the supernatants were carefully eluted, and centrifuged

once at 2000 rpm for 3 minutes (Sorvall RMC 14, Du Pont) to eliminate the possibility of any residual cell material or particles interfering with the assays. Solid phase enzyme-linked immunosorbant assays (ELISA) were then performed in duplicate, specifically according to the detailed manufacturer s instructions, to determine the levels of both TNF-α (TNF-α immunoassay, R&D Systems Europe, Quantikine) and IL-1β (IL-1β Immunoassay, R & D systems Europe).

Macrophage electron microscopy

Obtaining appropriate cells for EM followed the cell culture protocols outlined above. Adherent cells were lifted out of culture, using a gentle cell scraper technique without trypsinization. These cells were immediately fixed in 2% cacodylate-buffered glutaraldehyde, then postfixed in 1% phosphate-buffered osmium tetroxide, and suspended in Agar. The Agar blocks were processed as conventional tissue blocks and embedded in epoxy resin mixture (Agar 100/Araldite CY212/DDSA). Cells for examination were randomly located by a blinded observer on semi-thin (0.5 micron) survey section stained with 1% toluidine blue. Ultra-thin sections (90–100 nm) were cut on a Reichert-Jung Ultracut ultramicrotome using glass knives, mounted on copper grids, stained with uranyl acetate and Reynolds' lead citrate and examined in a JEOL 1200-EX transmission electron microscope. The cells were blindly allocated to 2 groups as either showing a monocytic or a macrophagic phenotype, including the amount of phagocytosis seem.

Cell viability in culture

The cell viabilities following 24-hour cultures with varying concentrations of PMMA particles and NAC were analyzed and compared to control cultures. The cultures were established by the methods outlined above and 3 concentrations of PMMA particles, 100, 500 and 1000 μg/mL, were used, as also were the 3 concentrations of NAC used in the cultures described above. All cultures were performed in triplicate and the viabilities assessed at 3 points in time over the 24-hour culture period. Cell viability was analyzed with the Trypan blue exclusion technique.

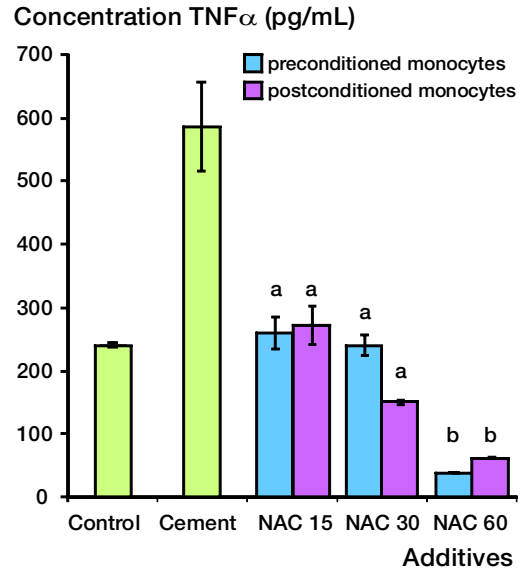

Figure 1. TNFα expression in monocyte cultures.
[a] $p < 0.05$ versus cement particle control group
[b] $p < 0.05$ versus plain control group

Statistics

Statistical analysis of the data from the controls and the treatment groups was done with a one-way analysis of variance (ANOVA) employing SigmaStat software, version 2.0, a Tukey test for multiple comparisons being used to determine specifically which treatment groups differed from others significantly. A Mann-Whitney rank sum test was used to compare levels of cell activation and phagocytosis. Cell viability analyses were performed using the Student's t-test.

Results

Cytokine assay results

Control cultures showed that the plain control cultures expressed both TNF-α and IL-1β, but the addition of particles of PMMA produced a rise in the expression of both TNF-α ($p = 0.005$) and IL-1β ($p = 0.00003$).

Pretreatment of the monocytes with NAC was shown to be associated with significant reductions in TNF-α expression versus the PMMA-stimulated controls at all concentrations of NAC (Figure 1). The reduction in TNF-α expression was dose-

IL-1β expression in monocyte cultures

	Mean	95% CI [a]	P-value vs. CTC [b]
Preconditioned monocyte values			
Control	269.7	26.0	0.00003
Cement	1043.7	0.6	–
NAC 15	272.4	24.6	0.00002
NAC 30	256.9	4.8	0.000001
NAC 60	77.6	15.0	0.000006
Postconditioned monocyte values			
Control	269.7	26.0	0.00003
Cement	1043.7	0.6	–
NAC 15	1077.0	40.6	0.07
NAC 30	270.8	30.1	0.00004
NAC 60	269.0	28.4	0.00003

[a] 95% CI = 95% confidence interval of the mean
[b] CTC = cement-treated control group

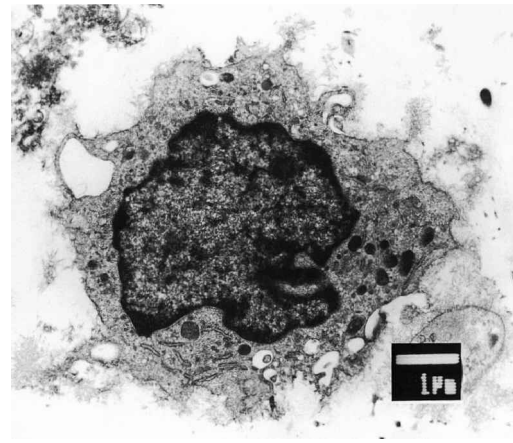

Figure 2. Electron micrograph of monocyte-macrophage after 24-hour culture with PMMA particles, showing ultrastructural signs of activation and macrophage morphology (60kV × 6000; scale as shown).

dependent, however, with a significantly greater effect recorded at the higher concentration. The response to the highest dose of NAC was such that even the baseline TNF-α expression by plain control cells was suppressed ($p = 0.00005$), a result not achieved with the lower doses.

Similar results were achieved regarding IL-1β expression at all doses of NAC, with again a reduction of IL-1β expression ($p = 0.0007$) versus the baseline levels of the control cultures (Table).

As for the posttreatment group, the lowest concentration of NAC had no significant effect on IL-1β expression, but the other doses of NAC significantly suppressed IL-1β versus the PMMA challenged control ($p = 0.00004$). All doses of NAC, however, effectively reduced TNF-α expression in about the same way as the pretreated group. Again, the highest dose of NAC was associated with a lower TNF-α than the plain control cells ($p = 0.0001$).

Direct comparison of pretreatment with posttreatment groups showed better suppression of both TNF-α and IL-1β with pretreatment, particularly at the highest dose of NAC ($p = 0.0004$ for TNF-α and $p = 0.0008$ for IL-1β), which suggested that at this dose level NAC is more effective if available before particulate debris acts on the cells.

Macrophage electron microscopy

The ultra-structural appearances of the cells were found to correlate well with the treatment. Following the random selection process there were 10 cells from each type of culture examined, with 8 of the NAC-treated monocytes cultured with particulate PMMA at 1000 μg/mL retaining their original monocytic morphology over 24 hours. The same proportion of untreated cells exposed to particulate PMMA differentiated into active cells, with the morphological appearance of macrophages. The untreated cells (Figure 2) showed activation and differentiation into 'mature'-looking macrophages, with more phagocytosis and phagocytic inclusions, multiple surface cytoplasmic outgrowths and lysosomes in the cytoplasm than treated cells ($p = 0.001$). More general signs of cell activity include an enlarged size of the cells, prominent nucleoli and enlarged nuclei with more diffuse chromatin than was observed in the original monocytes. It is also readily apparent that the cytoplasm in these cells was much more active with abundant rough endoplasmic reticulum (RER) and mitochondria. A well-developed RER reflects protein synthesis, and in this case shows the greatly increased expression of cytokines measured in response to the particles. NAC-treated cells, on the contrary, were found to have a typical monocytic morphology, with a large central nucleus and somewhat condensed chromatin, a relatively small surrounding cytoplasm and a relatively inactive cytoplasm with little evidence of mitochondrial activity and formation of RER (Figure 3).

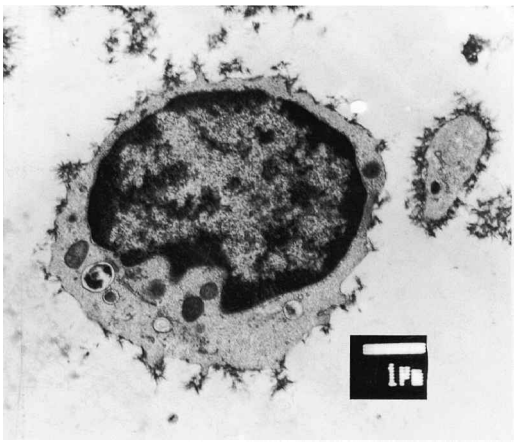

Figure 3. N-acetylcysteine-treated monocyte after 24-hour culture with particles of PMMA showing absence of activation and persistent monocytic morphology (60kV × 6000; scale as shown).

Results of cell viability in culture

The results of our analysis of cell viabilities, using the data obtained from the Trypan blue exclusion at 3 times during the 24 hours of incubation for plain, control cells and also for cultures with varying concentrations of PMMA particles and NAC, showed reductions in cell viability over time in all culture conditions.

Comparison of the results between the various cell cultures revealed that neither the PMMA particles in the concentrations used (i.e., 1000 μg/mL; $p = 0.53$) nor the NAC at any concentration ($p = 0.7$) was associated with a reduction in cell viability when compared to control cultures.

Discussion

Our findings show that NAC leads to a predictable and dose-dependent suppression of monocyte/macrophage activation. We have further shown that NAC has no adverse effect on cell viability in vitro, which is of relevance in proposing it as a possible therapeutic agent. 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 (Trindade et al. 1999).

As the mechanisms of osteolysis and the response to wear debris have been further defined, methods of modulating these responses have been investigated (Schwarz et al. 2000). Bisphosphonates have been of particular interest, etidronate and disodium pamidronate being shown to be effective in reducing bone resorption in vitro following exposure of a macrophage/bone co-culture model to PMMA particles (Pandey et al. 1996, Hiroi-Furuya et al. 1999). Note that it has recently been demonstrated that anti-inflammatory agents can inhibit the osteolysis associated with titanium particles (Blaine et al. 1997).

It has been shown that pretreatment with the antioxidant agent N-acetylcysteine suppresses LPS-mediated production of TNF-α and PGE2 by resident lung macrophages and that NAC suppresses NF-κB activation associated with reactive oxygen species (Mendez et al. 1995, Fox et al. 1997, Isaacs et al. 1999, Li et al. 2000). NF-κB activation is a critical convergence point of the inflammatory, cytotoxic and cytokine/TNF-α expression modulating pathways (Schreck et al. 1992). We have now demonstrated that NAC represents an effective method of suppressing the monocyte/macrophage response to particles of PMMA. The finding that NAC significantly prevented phagocytosis of particles also indicates that this therapy may be of use for other types of particles.

Although we found the effect of NAC to be significant and dose-related whether cells were preincubated with NAC or had NAC added after the particles, there was a significantly greater suppression of activation when cells were pretreated. The timing of therapy is therefore an issue and there is evidence that the mechanisms of aseptic loosening begin directly after implantation, whether or not clinical or radiographic evidence of loosening is present—e.g., roentgen stereophotogrammetric analysis studies show that implants subsiding more than 2 mm initially are more likely to loosen and fail early and histological studies reveal that the pseudomembrane can appear as early as 2.5 months after cemented arthroplasty (Korovessis and Repanti 1994, Kiss et al. 1996). Therefore, early therapy may reasonably be expected to help prevent loosening through prevention of this initial inflammatory membrane formation. A relevant observation in this regard was that macrophages in

the joint capsule increase the production of TNF-α at an early phase and in the absence of clinical loosening (Nivbrant et al. 1999). These investigators established that TNF-α release precedes and leads to osteolysis, rather than being a tissue response to already established loosening; thus emphasizing the possible importance of early prevention of macrophage activation and TNF-α expression. Using agents as additives in cement in a similar fashion to the antibiotics currently employed or in other local delivery systems at the time of implantation may be possible applications of the findings of this work (Corry and Moran 1998). A significant advantage of NAC in this regard is that its use is well established in humans and it has had a good safety profile.

Although there are several goals in reducing the incidence of aseptic loosening and failure of implants, such as optimization of the bearing materials and surgical techniques, we believe modulating the immune response using pharmacological treatments, such as described here, may have significant future potential as regards reducing periprosthetic osteolysis. We have introduced the concept of using antioxidant therapy to suppress monocyte activation, but further studies, particularly in vivo assessments of these interventions, are needed to define the precise role of such treatment in promoting the longevity of implants.

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