NO AND IRAP MODULATE CYTOKINES PRODUCED BY WEAR PARTICLE ACTIVATED MACROPHAGES

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INTRODUCTION
Nitric oxide (NO) is produced in copious amounts by activated macrophages and is responsible for microbistatic and microbicidal actions during inflammatory responses (1,2). It has also been implicated in the development of aseptic loosening of hip prostheses, a significant clinical concern (3). We reported that NO decreases the anti-inflammatory cytokines and IL-10 while increasing PGE2 and IL-1β production by macrophages activated with LPS (4). The current studies evaluate the role of NO in modulating the cytokine profile of particulate wear debris activated macrophages, and assess the combined effects of IL-1 receptor antagonist (Irap) and inhibition of NO synthesis to modify the cytokine profile of macrophages activated by LPS, polymethylmethacrylate particle (PMMA), and particulate wear debris.

METHODS
Cell Culture. The murine macrophage cell line RAW 264.7 was obtained from the ATCC and used between passages 10-20. The cells were maintained in DMEM, 10% FBS, with antibiotics. They were passaged to 24 well plates for 24 hr to allow the serum to reduce to 1% before addition of agonists and antagonists. LPS, PMMA particles, and endotoxin free[Sigma E-toxate (limulus Amebocyte Lysate) kit] wear particles isolated during revision hip arthroplasty (kindly provided by Dr. P. Wooley, Cleveland, OH) were used to activate the macrophages. Stimulation in the presence and absence of 1 mM L-NMA was used to evaluate the role of NO in activated RAW cell cytokine production. Macrophages producing Irap were trypsinized and seeded onto 24 well plates for experiments to characterize the effects of Irap (± NO) on cytokine production. Briefly, RAW cells were transduced with the retroviral vectors MFG-IacZ or MFG-Irap using a modification of our published procedure (5). Macrophages were seeded onto 24 well plates at the confluence in 25 cm2 flasks. Cells were exposed to 1 ml of the virus (approximately 10^6 pfu) overnight. The viral suspension was removed, the monolayer rinsed, and complete medium added. Culture was continued for 24-96 hr and conditioned media (CM) assayed for Irap to identify successfully transduced cultures, which were then used in these experiments. RAW cell CM to be assayed for cytokine and NO production were removed 24 or 48 hr after activation, and the cells trypsinized for determination of cell numbers. NO production was determined as the nitrite concentration in CM using a spectrophotometric assay based on the Griess reaction. Cytokines were assayed using commercially available ELISA (TGF-β, TNF-α, IL-1β, IL-10, Irap) or EIA (PGE2) kits.

Data analysis and presentation. Experiments were repeated at least 3 times and data is given as mean ± SE, normalized to 10^6 cells. The significance of differences between mean values was determined by t-test.

RESULTS
RAW cells were activated with 10^3-5 micrometer diameter particles/ml or 1 ug/ml LPS and conditioned media collected for assay of cytokines 48 hr later. LPS increased CM NO from 3±0.1 uM to 42±2 uM while debris activation caused an increase to 27±1 uM. NO minimally affected TNF-α while being responsible for a substantial part of PGE2 synthesis in both LPS and wear particle debris activated RAW cells. For example, PGE2 decreased from 440±18 to 140±10 in CM from debris activated cells when NO synthesis was inhibited. Debris activation did not diminish basal TGF-β synthesis/secretion, however the concentrations in CM were increased from 348±76 to 676±93 pg/ml when NO synthesis was inhibited. Total IL-1 was modestly increased by L-NMA inhibition of NO in cells activated with debris, in contrast to the decrease seen in LPS activated cells where CM concentrations decreased from 196±15 to 86±16 pg/ml in the absence of NO. RAW cells were transduced with MFG-IacZ or MFG-IL-1rap as described above. Transduced cells were cultured in D-MEM, 5% FBS for 48 hr, LPS, wear debris or PMMA particles, with or without L-NMA added, and culture continued for a further 48 hr. Conditioned media Irap was 24 ± 3 pg/ml (LacZ) or 2507 ± 50 pg/ml (Irap). CM NO were 10-12 uM (without L-NMA) or 3-4 uM (with L-NMA).

These cells were cultured for 96 hr after transduction to allow substantial accumulation of Irap in the medium, thus they were maintained in D-MEM with 5% FBS, which may account for the lower rate of NO synthesis seen in this series. However, in cells with NO synthesis inhibited, CM IL-10 was increased by an average of 249% (pooling the data from all activation paradigms). Irap enhanced CM IL-10 secretion by macrophages activated by LPS, PMMA particles, or wear debris (92±3%), while the combination of inhibition of NO and presence of Irap increased IL-10 in CM over 5 fold (from 43±2 to 251±16 pg/ml). Similar changes were seen in cytokine production by macrophages activated by LPS or PMMA.

TNF-α was assayed in the CM from the same series of experiments. In this case, inhibition of NO synthesis modestly (but not significantly) increased CM TNF-α production by activated macrophages; Irap alone had no effect on TNF-α concentrations. However, when NO was inhibited in the presence of Irap, TNF-α was reduced from 36±4 to 18±2 ng/ml in the CM of macrophages activated by wear particle debris. These NO/Irap effects on TNF-α were also seen if the RAW cells were activated by LPS or PMMA.

DISCUSSION
These studies show that NO modulates macrophage cytokine production in response to wear particles; it enhances the pro-inflammatory cytokine PGE2, while decreasing the anti-inflammatory cytokines TGF-β and IL-10. Whether similar actions occur in vivo and, if so, whether inhibition of NO synthesis by activated reticuloendothelial cells could significantly attenuate inflammatory responses remains to be explored.

iNOS has been identified in the pseudosynovial membrane which forms subsequent to hip arthroscopy (6) and detection of nitrotyrosine in these membranes provides evidence for the formation and activity of peroxynitrite, a strong oxidant species (7). These findings, coupled with observed actions of NO and PGE2 on bone metabolism, have led to the hypothesis that NO may be a major factor in the development of aseptic loosening (3). The current studies with RAW cells suggest that NO, in addition to direct effects on bone per se, could have a detrimental effect on prosthetic stability, by decreasing synthesis of TGF-β and IL-10. Diminishing TGF-β could diminish anabolic processes in general, while lowering IL-10 may thwart an important anti-inflammatory regulatory pathway in these tissues (8). Further studies are necessary to determine if maneuvers to minimize iNOS induction/NO production in the joint, could be beneficial in minimizing the occurrence of prosthetic loosening.

REFERENCES

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