NF-KB SIGNALING BLOCKADE ABOLISHES IMPLANT PARTICLE-INDUCED OSTEOCLASTOGENESIS.

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Introduction: Periprosthetic osteolysis with resultant bone loss and implant loosening is the major problem that limits survivorship of total hip and knee replacements. The biologic response to implant wear debris is manifested as chronic inflammation mediated by various cytokines (IL-1, IL-6, RANKL, TNF-α) that stimulate osteoclast differentiation and activation. The osteoclastogenic effects of these cytokines are mediated through induction of the nuclear transcription factor NF-kB which is known to be essential for developmental osteoclastogenesis. NF-kB is most commonly activated by phosphorylation of its inhibitory protein IkB with subsequent dissociation and NF-kB translocation to the nucleus where it regulates target gene transcription. We and others have recently demonstrated that this signaling pathway is activated in osteoclast precursor cells after exposure to implant debris (1) suggesting that NF-kB signaling may play a role in particle-directed osteoclastogenesis (2). Nevertheless, the specific function of the NF-kB signaling pathway in particle-directed osteoclastogenesis has not been determined. Therefore, we have investigated the effect of NF-kB signaling blockade on polymethylmethacrylate (PMMA) particle-induced osteoclastogenesis in vitro.

Methods: Commercially available PMMA microspheres (Polysciences, Inc.) 1-10 um diameter (mean, 4.5um) were used for all experiments and particles tested negative for endotoxin (BioWhittaker, Inc.). Human phenylalanine chloromethyl ketone (TPCK) and Calpain Inhibitor I (CPI) (inhibitors of the NF-kB pathway) were obtained from Calbiochem, Inc. The IkB mutant:TAT peptide fusion inhibitor was prepared as previously described (3). NF-kB Electrophoretic Mobility Shift Assay (EMSA): Osteoclast precursor cells in the form of bone marrow macrophages were isolated from C3H/HeN mice, treated at confluence and analyzed by EMSA (1). Nuclear extracts were prepared and incubated with an oligonucleotide probe from the KB3 site of the TNF promoter. Samples were fractionated by 4% TBE gel electrophoresis and analyzed by autoradiography. Immunoblot Analysis: Equal amounts of nuclear and cytosolic protein extract were electrophoresed, transferred to nitrocellulose membrane and analyzed by immunoblot with anti-p50 (NF-kB) antibodies (1). Immunoblot and EMSA experiments were performed three times with similar results. Osteoclastogenesis Assays: Murine (C3H/HeN) osteoclast precursor cells were maintained in the presence of RANKL (20mg/ml) for 3 days and then treated with control media or experimental conditions for an additional 24 hours (4). Alternatively, murine whole bone marrow cultures were maintained in the presence of 10nM 1,25-(OH)2D3 for eight days and then treated with control or experimental conditions for an additional 2 days (4). Cultures were then fixed, Tartrate Resistant Acid Phosphatase (TRAP) stained and average osteoclast (multinucleated, TRAP positive cells) counts determined. Each condition was run in triplicate and experiments performed three times with similar results. Results were compared with an unpaired t-test.

Results: We have previously demonstrated that PMMA (0.6mg/ml) particles markedly activate NF-kB in osteoclast precursor cells within 30 minutes of particle exposure. Thus, we first wanted to determine if particle-induced NF-kB activation as measured by EMSA could be inhibited by blocking phosphorylation and/or degradation of the inhibitory protein IkB. Protein-DNA binding experiments demonstrate that the inhibitors TPCK (1µM, inhibits IkB phosphorylation) and CPI (10µM, inhibits IkB proteolysis) completely block particle-induced NF-kB activation in osteoclast precursor cells. Additionally, immunoblot confirmed that particle-stimulated nuclear translocation of the p50 NF-kB subunit was abrogated by these inhibitors indicating cytosolic retention of the transcription factor. After establishing effective NF-kB signaling blockade with these agents, we next tested the effects on osteoclast formation. RANKL-primed osteoclast precursor cells were treated with particles in the presence or absence of TPCK and CPI. These inhibitors did not effect basal osteoclastogenesis, yet particle stimulation of osteoclastogenesis (8 fold) was inhibited (95%) in the presence of TPCK and CPI (p<0.001).

Most importantly, we utilized a specific inhibitor in the form of an IkB mutant:TAT peptide fusion protein (TAT-IkBα46-317) to selectively block NF-kB activation. This IkB mutant that lacks the functional phosphorylation sites binds to and prevents NF-kB dissociation and translocation to the nucleus. The TAT-IkB mutant inhibited particle-stimulated NF-kB activation in osteoclast precursor cells and completely blocked osteoclastogenesis (99%, p<0.001) in RANKL-primed osteoclast precursor cultures. To test the role of alternative osteoclastogenic signaling pathways mediated by supporting stromal cells, we turned to whole bone marrow cultures. In the whole bone marrow microenvironment, particle-induced osteoclast formation (5.5 fold) was completely (99%) inhibited by addition of the fusion protein to cultures on days 0, 2 and 4 (and maintenance to day 10) (Figure). Addition of the inhibitor on days 6 and 8 resulted in a 90% and 80% reduction of osteoclasts, respectively (p<0.005).

Significance: These data demonstrate that: 1) the NF-kB inhibitory agents TPCK and CPI effectively block NF-kB activation in osteoclast precursor cells, 2) these inhibitors abolish particle-induced osteoclastogenesis in RANKL-primed osteoclast precursor cells and 3) the IkB mutant:TAT peptide fusion protein selectively blocks particle-induced NF-kB activation and osteoclast formation in both the osteoclast precursor cell and whole bone marrow osteoclastogenic assays. These findings suggest that the NF-kB signaling pathway plays an essential role in mediating implant particle-enhanced osteoclast formation. Thus, the nuclear transcription factor NF-kB and its regulatory molecules should be considered excellent targets for therapeutic modalities designed to inhibit particle-induced osteoclastogenesis and the resultant periprosthetic osteolysis.

References:

Figure Eight day Osteoclastogenic whole bone marrow cultures were treated with control media (a), PMMA (0.6mg/ml) (b), the IkBα46-317:TAT peptide inhibitor (50nM, days 2-10) (c), or PMMA and IkBα46-317:TAT peptide (d) as indicated. Cultures were fixed and TRAP stained on day ten. Arrow indicates TRAP positive multinuclear osteoclast.