FIBROBLASTS EXPRESS RANKL AND SUPPORT OSTEOCLASTOGENESIS IN A COX-2 DEPENDENT MANNER FOLLOWING STIMULATION WITH TITANIUM PARTICLES

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Materials and Methods:
Embryonic fibroblasts in culture stimulated with titanium particles were examined by FACS, real time RT-PCR, Northern blot, and Western blot for expressions of VCAM1, RANKL, COX-1 and COX-2, and the four PGE2 receptor isoforms. Experiments were performed in the presence and absence of COX, PKA, and PKC inhibitors, and various EP receptor agonists. Osteoclast formation was examined in co-cultures of PGE2 or Titanium particles pre-treated glutaraldehyde-fixed fibroblasts and primary murine spleen cells in the presence of M-CSF for 7-days.

Results:
Titanium particles stimulated RANKL gene and protein expression in fibroblasts. TNF-alpha markedly elevated the expression of VCAM-1 in the embryonic fibroblasts cell line, similar to effects on synovial fibroblasts. Since VCAM-1 is a synovial fibroblast marker, these cells were used as model for subsequent experiments. A small level of stimulation was observed at 5 x 10^5 particles/ml, progressively increased up to a concentration of 1 x 10^6 particles/ml, where maximal stimulation was observed (5-10 fold). Stimulation occurred by 1 hour, was maximal by 4 hours and remained elevated for at least 24 hours. When cell extracts from fibroblast cultures treated with titanium particles for 0, 8, 24, and 48 hours were similarly harvested and subjected to IP-western for RANKL expression. Similar to ST-2 cells, RANKL protein was undetected under basal conditions, but was induced by 8 hours with maximal levels occurring after 48 hours.

Titanium particles induced COX2 protein expression in fibroblasts. Under basal conditions, COX-2 was undetectable, but expression was observed within 1 hour of exposure to titanium particles. Maximal levels were present by 4 hours and similar at 4 hours following treatment. In contrast, COX-1 expression was constitutive and was not altered following treatment with titanium particles.

RANKL induction in fibroblasts is mediated by PGE2. Fibroblasts were treated with titanium particles for 4 hours in the presence and absence of combinations of indomethacin and PGE2 in order to determine if PGE2 could be blocked by both the non-selective NSAID, indomethacin, as well as by the COX-2 specific inhibitor celecoxib, and also be compensated for by the addition of PGE2 to the cultures. Analysis of EP receptor expression and the use of EP receptor agonists implicated the EP4 receptor as the major mediator of the prostaglandin induced RANKL induction. RT-PCR (30 cycles) showed the absence of EP3 and low levels of EP-1 and EP-2 expression. Substantially higher levels of EP4 expression were observed, suggesting that this is the major receptor subtype in these cells. Using relative specific receptor agonists, the role of four EP receptor subtypes in RANKL induction was assessed by Northern blot analyses. As previously observed, PGE2, which binds to all of the receptors, results in a large induction of RANKL. Iloprost, which binds to the EP1 receptor, and also has affinity for EP3 and IP receptors, caused only a small increase in RANKL expression. Similarly, the EP-2 receptor agonist, butaprost, led to minimal RANKL expression. However, misoprostol, an activator of both EP2 and EP4 receptors, stimulated RANKL to a magnitude similar to that observed with PGE2. To further examine the intracellular signaling that leads to RANKL, PKA (H89) and PKC (Go6976) inhibitors were added to PGE2-stimulated cultures. While the induction of RANKL by PGE2 strongly was inhibited by the H89, only a relatively weak repression was observed with the PKC inhibition, consistent with EP4/PKA mediated signaling.

Discussion:
Following stimulation with titanium particles, fibroblasts synthesize COX-2, secrete prostanoids, and express RANKL. The induction of RANKL was shown to be functionally important, since glutaraldehyde-fixed fibroblasts stimulated with titanium particles directly induced osteoclast formation. The induction of RANKL by particles could be blocked by both the non-selective NSAID, indomethacin, and also by the COX-2 specific inhibitor celecoxib, and could be compensated for by the addition of PGE2 to the cultures. Evidence is accumulating that fibroblastic cells can regulate bone remodeling, particularly in the setting of inflammatory joint disease. In prosthetic loosening, synovial fibroblasts are the most abundant cell type found in the inflammatory membrane and have extensive contact with wear debris particles. Our previous published experiments have shown the importance of COX-2 in wear debris-induced osteolysis since bone loss is ablated in COX-2 -/- mice. Our current studies support a role for COX-2 mediated by PGE2/EP4/PKA signaling events that ultimately regulate the induction of RANKL in synovial fibroblasts. The findings define this pathway as potentially important to regulate particle mediated inflammatory bone loss and have relevance for inflammatory bone loss in general.

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