ANALYSIS OF THE NFKB-BINDING SITES IN THE TNF-ALPHA PROMOTER IN MACROPHAGES STIMULATED BY TITANIUM WEAR DEBRIS

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Introduction: Osteolysis following joint arthroplasty is believed to be mediated by implant-derived wear debris. Once phagocytosed by macrophages, the particles stimulate cytokine production. The molecular mechanisms regulating this cytokine synthesis are not clear at this time. TNFα is a central mediator of aseptic loosening, thus understanding the pathways leading to TNFα induction are very important for clinical translation.

One of the well known mediators of TNFα induction is the transcription factor NFκB. The role of NFκB in Ti stimulated TNFα synthesis has not been completely determined, thus we analyzed different NFκB-binding sites in the TNFα promoter in macrophages following Ti stimulation. We also determined the kinetics of cellular TNFα mRNA levels, because of the existence of a negative regulatory loop in TNFα induction. This loop includes tristetraprolin (TTP) – a Zn-finger protein that binds to AU-rich mRNA domains and induces their rapid degradation. TNFα mRNA appears to be a target for TTP, thus analysis of Ti regulation of TTP could help clarify the mechanism of TNFα synthesis and autoregulation.

Methods: The ANA-1 murine macrophage cell line was kindly provided by Dr. G. Cox. Cells were grown on DMEM+10% FBS (GIBCO). Ti particles (Alpha AESAR, MA) were used at a concentration 5x10⁶ particles/ml. Conditioned medium was collected after 18h of treatment (if not specified) and analyzed by ELISA for cytokine concentration. mRNA was isolated after specified times with QIAGEN kit and 10⁻¹⁰ M α TNF was added. The TTP cDNA probe was provided by Dr. P. Blackshear.

Results: Ti particles stimulated the dose-dependent release of TNFα from ANA-1 cell cultures. Significant effects were observed as early as 2 hours following stimulation and a maximal 20-fold effect was observed at a concentration of 10⁶ particles/ml, 18 hours following addition of Ti. TNFα mRNA levels were markedly elevated within 30 minutes, and were maximal at 4 hours, remaining elevated for 12 hours following stimulation with Ti. In contrast, the stimulation of IL-6 was delayed, with significant effects on mRNA and protein synthesis initially observed only at four hours following stimulation. To examine how NFκB may regulate TNFα synthesis, ANA-1 cells were transfected with the pNFκB-luc construct. Ti caused a 2-fold increase (p<0.05) in luciferase activity (Fig. 1), demonstrating upregulation of this signaling pathway by particulates. To determine whether activation of the TNFα gene is associated with NFκB signaling, the TNFα-luciferase-reporter constructs were transfected into ANA-1 cells. Ti (5x10⁶ particles/ml) stimulated a 60% increase (p<0.005) in luciferase activity with the wild type construct. Ti particles stimulated a similar increase in luciferase activity with constructs containing mutations in the NFκB binding at the kB1 site (-510) resulted in slightly decreased luciferase activity (Fig. 2). Mutation in the kB2 site (-630) resulted in a 30% decrease (p<0.01) in luciferase activity (Fig. 2). Mutation in the kB1 site (-510) resulted in slightly enhanced transcriptional activity, which was not statistically significant. Experiments with LPS stimulated ANA-1 cells demonstrated a similar effect of the kB2 site on luciferase stimulation.

To assess the kinetics of NFκB activation, ANA-1 cells were stimulated with Ti particles and nuclear extracts obtained for gel shift assays. NFκB binding was markedly elevated by 30 minutes, peaked at one hour, then gradually declined by four hours following stimulation with Ti particles. Since IkBα is an inhibitor of NFκB activation, we assayed for its presence in cytoplasmic extract from ANA-1 cells stimulated with Ti. We observed minor changes in IkBα levels 15 minutes after Ti stimulation, which returned to control levels at long time points following stimulation.

NFκB is known to regulate Tristetraprolin (TTP) expression. TTP mRNA levels were undetectable in control but markedly elevated 30 min following Ti stimulation.

Discussion: As more research is being done toward understanding wear debris mediated inflammation, it is important to know the pathways leading to cytokine expression. TNFα is one of the most general pro-inflammatory proteins, whose synthesis can be regulated as at the transcriptional and post-transcriptional levels. Transcriptional regulation of NFκB by NFκB appears to be the central part of the Ti induced inflammatory response. Using reporter gene assays we demonstrated a direct role of NFκB in Ti induced TNFα mRNA and protein synthesis. We showed that only one NFκB binding site in TNFα promoter significantly changes the transcription of this cytokine. This is the evolutionary conserved kB2a site, which was previously demonstrated to be necessary for LPS signaling.

NFκB is autoregulated to prevent the toxic effects of this cytokine on all cells. One proposed mechanism by which TNFα could be autoregulated is by inducing an enzyme (TTP) which destabilizes its mRNA. Here we show that this may be how TNFα is down regulated following Ti stimulation. The activation of NFκB by Ti stimulation in macrophages leads to the transcription of both TNFα and TTP. The subsequent de novo protein synthesis of TTP can then act to down-regulate TNFα production in the cell.

Future experiments utilizing dominant-negative forms of IkB and TTP will further clarify the role of this pathway in pro-inflammatory cytokine responses to particulate wear debris, and will further validate these molecules as drug targets for the prevention of aseptic loosening.