Modifications of messenger ribonucleic acid level of Toll-like receptors and the adaptor molecules in macrophages after phagocytosis of lipopolysaccharide-coated titanium particles.

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Poster No. 2195  •  ORS 2011 Annual Meeting

Introduction
Aseptic loosening even occurs in the technically well-inserted total joint prostheses: This is the most clinical problem in total hip arthroplasty (THA) [1]. Histopathological analyses have indicated that aseptic loosening of THA is caused by foreign body associated-granulomatous reaction to wear particles, in which abundant monocytes/macrophages were found [2]. Macrophages can phagocytose wear particles released from prosthetic implants and produce various cytokines by themselves in aseptic loosening. In addition, bacterial endotoxins are known to adhere to the wear particles and may increase inflammatory stimulus [3-5]. It has been recognized that adherent lipopolysaccharide (LPS) on particles can induce macrophage activation via Toll-like receptor (TLR) 4 [3]. Recently, abundant expression of TLRs in monocytes/macrophages was found in periprosthetic tissues [6]. However, the precise role of TLRs and their adaptor molecules and the interaction with LPS in the process of aseptic loose connective tissues is still unclear.

We analyzed expression of TLRs, their adaptor molecules and cytokines in macrophages stimulated by titanium particles (Ti) with or without LPS coating to clarify the relationship between role of TLR-mediated signal pathway and adherent endotoxins.

Materials and Methods
1. Culture of macrophages derived from bone marrow:
Bone marrow cells were collected from Wistar rats. The bone marrow cell suspension was filtered by synthetic fiber membrane and cultured under the condition of humidified air admixed 5% CO₂ for 96 hours at 37°C in the media of consisting 90% DMEM, 10% fetal bovine serum (FBS) and with 10 ng/ml macrophage colony stimulating factor (M-CSF). Adherent cells were collected by exposure to tripisin-EDTA solution. The adherent cell fraction (5×10⁶ cells/ml) was harvested. After 24 hours cultivation, supernate containing non-adherent cells was removed, and DMEM was added [7].

2. Titanium particles (Ti) preparation:
Endotoxin-free Ti were prepared by five cycles of alternating treatment with 25% nitric acid and alkali/ethanol [3]. LPS was added back to endotoxin-free Ti by incubating them with 10 ng/ml LPS for 12 hours at 37°C with rotation and then washing the particles 10 times with PBS to remove free LPS [3].

3. Immunocytochemical staining:
Macrophages derived from bone marrow were fixed in acetone and conventional immunocytochemical staining was performed for CD68, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, TLR2, TLR4, TLR5, TLR9, myeloid differentiation primary response gene 88 (MyD88), IL-1 receptor-associated kinase (IRAK)1, IRAK2, IRAK4 and TNF receptor-associated factor (TRAF)6.

4. Quantitative real-time polymerase chain reaction (PCR) analysis on mRNA level after exposure of titanium particle to macrophages:
Soluble LPS (10 ng/ml, sLPS), particles coated by LPS (LPS+ Ti) and endotoxin-free control Ti particles (LPS- Ti) were added to cultured plates. Unstimulated macrophages were cultured as negative control. Total RNA was isolated after 0.5, 1, 3, 6, 12 hours of exposure and reverse transcription was performed. Total RNA was converted into cDNA and enzymatic amplification of the specific cDNA sequences was performed of TLR4, TLR5 and TLR9, MyD88, IRAK1, IRAK2, IRAK4 and TRAF6 was also detected in these adherent cells.

Results
1. Immunocytochemical staining
The cultured, adherent cells from bone marrow were large, had abundant cytoplasm and were more than 99% CD68 immunoreactive. Immunoactive TNF-α, IL-1β, IL-6, TLR2, TLR4, TLR5, TLR9, MyD88, IRAK1, IRAK2, IRAK4 and TRAF6 was also detected in these adherent cells.

2. Quantitative real-time PCR

1) Accuracy of PCR analysis
TNF-α, IL-1β, IL-6, TLR2, TLR4, TLR5, TLR9, MyD88, IRAK1, IRAK2, IRAK4, TRAF6 and GAPDH were detected in all samples. Each PCR performance was estimated by two types of analysis, which were melting curve and quantification analysis. Melting curve analysis showed the fluorescence data that were acquired continuously from a melting experiment and the peaks on curve represented the melting temperatures. Each melting curve analysis showed only single peak, which revealed the accurate PCR performance without nonspecific products.

2) Response of macrophages to LPS dissolved in the medium
mRNAs of proinflammatory cytokines, TLRs, and their adaptor molecules of TLRs were detected in bone marrow derived macrophages stimulated with sLPS. sLPS increased mRNA levels of proinflammatory cytokines standardized by GAPDH at 1, 3, 6 and 12 hours, which indicated macrophages derived from bone marrow had potential to respond to LPS via TLR4-mediated inflammatory signal pathway.

3) Response of macrophages to contact with and phagocytosis of Ti particles
mRNA levels of TNF-α, IL-1β and IL-6 standardized by GAPDH, were significantly higher in LPS+ Ti than LPS- Ti, particularly at 1 and 3 hours. In contrast, mRNA levels of TLR4, TLR5 and TLR9 decreased significantly in LPS+ Ti compared with LPS- Ti, particularly at 3, 6, 12 hours . mRNA levels of MyD88, IRAK1 and IRAK4 also decreased in LPS+ Ti, particularly at 3, 6, 12 hours, and that of TRAF6 was suppressed at 12 hour after transient increase in LPS+ Ti. However, mRNA levels of TLR 2 and IRAK 2 were significantly higher in LPS+ Ti than LPS- Ti, particularly at 3 and 6 hours.

Discussion
Proinflammatory cytokines were much increased by contact with and/or phagocytosis of LPS-coated foreign metallic materials via TLR4-mediated inflammatory signal pathways. Subsequent down-regulation of TLR4, TLR5 and TLR9 at the receptor level, and of MyD88, IRAK1 and IRAK4 at the post-receptor level, suggests that self-protective anti-inflammatory mechanisms are activated in macrophages and injury to innocent bystander cells/tissues after initiation of the host responses. To the contrary, mechanism responsible for a simultaneous marked increase of TLR2 and IRAK2 and a transient increase of TRAF6 in LPS+ Ti stimulations is compared to stimulations with sLPS or control LPS- Ti particles must be precisely examined in future, because it seems that a singular LPS-TLR4 reaction is coupled to a broad-spectrum alteration in TLR/adaptor machinery of the whole cell. This down- and/or up-regulation of the potential TLR-mediated responses to LPS-coated particles reflects the proactive behavior of effector cells in contact with danger-associated molecular patterns to optimize the cellular responsiveness and to inhibit the collateral damage.

References

Figure: Quantitative analysis, mRNA expression levels of TLR4 (A), TLR2 (B) were shown. Graphs showed ratio to LPS-free particles (LPS- Ti) and LPS-coated particles (LPS+ Ti) versus control.