Minimally Modified LDL Induces Actin Polymerization in Macrophages via CD14 Signaling Pathway.

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Introduction

The LDL particle is composed of an outer layer of phospholipids and proteins surrounding a lipid core and is used to transport cholesterol throughout the body. An excess of plasma LDL leads to an excessive deposition in the artery walls where LDL-phospholipids will undergo oxidation. Mild oxidation of these LDL particles creates minimally modified LDL also known as mmLDL. MmLDL is a pro-inflammatory and pro-atherogenic lipoprotein that participates in the early stages of oxidation, and is as pertinent to atherosclerosis as fully oxidized particles of LDL. Oxidized LDL is recognized by macrophage scavenger receptors and its uptake is unregulated and leads to cholesterol accumulation and “foam-cell” formation, the first step in atherogenesis. MmLDL, unlike fully oxidized LDL, is still recognized by the LDL receptor, but is not recognized by macrophage scavenger receptors. MmLDL is capable of inducing endothelial cells of the blood vessel wall to display adhesion molecules, leading to attraction of monocytes. In turn, the monocytes enter the artery wall, convert to macrophages and then continue to take up OxLDL. In addition, monocytes serve the important function of taking up apoptotic cells (cells undergoing “programmed cell death”) thus removing potentially toxic material from the artery wall. We found that mmLDL also acts to stimulate actin polymerization and spreading of macrophages, which decreases the uptake of apoptotic cells but enhances the uptake of OxLDL. We proposed that this F-actin response is due to mmLDL binding to macrophage CD14 receptor and activation of TLR4 signaling cascade. These data will allow us to begin to decipher the mechanism of macrophage reception to mmLDL, which seems to manipulate aspects of atherogenesis.

Materials and Methods

F-actin assay. Filamentous actin response is when actin molecules begin to polymerize into long chains, which is essential for many cellular processes, one of which is cell spreading. We used the J774 macrophage cell line and a mutant of J774 called LR-9 that is resistant to LPS, which is due to CD14 deficiency. J774 cells were plated at 0.3 x 10^6 cells per well in 10%FBS/RPMI 1640 and incubated at 37° for 2 hours. J774 cells were treated with sense or antisense DNA oligonucleotides for CD14 receptors. Antisense is a Post-Transcriptional gene silencing factor that blocks mRNA translation by binding to the sense strand of endogenous CD14 mRNA and prevents CD14 protein synthesis. We worked specifically with CD14 antisense (5'AAGCACACGCTCCATGGTCGGTAG-3') and CD14 sense (5'-CTACCGACCATGGAGCGTGTGCTT-'3), the latter as a negative control. Each treatment was applied twice and cells were incubated for 48 hours. In half of the wells media was removed and replaced with 0.9ml of mmLDL and incubated for 1 hour. 0.3 ml of F-actin stain composed of 15% Formaldehyde, 0.8% Saponin, 0.4μM FITC – phalloidin, and 1.2μM phalloidin was added to the wells. The cells were incubated for 30 minutes then scraped into FACS tubes for analysis of F-actin content.
**CD14 Expression.** J774 and LR-9 cells were plated at 0.5 x 10^6 cells per well for 24 hours, then washed and harvested into cold BSA/PBS. The cells were sequentially incubated on ice for 30 minutes with a blocking agent, primary and secondary antibodies, and analyzed by flow cytometry. Optimization of the staining procedure is outlined in Results. Cells were then subject to FACS assay.

**Results and Discussion**

To test for F-actin response in correlation to CD14 expression on J774 cells we used a Post Transcriptional Gene Silencing Factor antisense DNA, as well as its control, sense DNA. Non-treated cells were used as a control for F-actin response. Our experiment showed no significant change in F-actin response in cells treated with sense, but the expected decrease in F-actin response in cells treated with antisense. In our control cells there was a 80% increase of fluorescence indicating increased F-actin formation between cells in media only and cells in mmLDL (fig1). In cells treated with antisense there was only a 30% increase in F-actin between cells in media only and cells in mmLDL (fig1). In contrast, in cells treated with sense there was a 125% increase in F-actin between cells in media only compared to cells in mmLDL (fig1). The amount of F-actin found in these cells is believed to have a linear relationship with fluorescence readings given in FACS assay.

We believe that mmLDL induces the F-actin response through its activation of the CD14 receptor. The antisense applied to cells was supposed to block the CD14 mRNA and prevent CD14 protein synthesis. In congruence with our hypothesis, we found that the cells with less CD14 receptors would be less affected by mmLDL and therefore have less of an F-actin response. This experiment was repeated two times and Figure 1 is an average of the two experiments. Our experiment shows that the cells treated with antisense to CD14 show a blunted response to mmLDL, with an induction of only 37%.
increase in F-actin formation compared to a 80% increase in the absence of added mRNA. As a control, the sense treatment did not decrease the F-actin response. These results place much emphasis and significance that mmLDL does in fact interact with CD14 receptors to activate filamentous actin polymerization.

To validate that our sense/antisense DNA treatment has indeed changed CD14 expression, we estimated CD14 expression on J774 and LR-9 cells (mouse cell lines), using a rat anti-mouse CD14 monoclonal antibody (Pharmingen) in two formats, non-labeled and FITC-conjugated. We tested whether FITC-antiCD14 is sensitive enough compared to indirect fluorescent labeling using antiCD14 antibody followed by a secondary FITC-anti-ratIgG antibody. Non-specific ratIgG, either FITC-conjugated or non-labeled, respectively, were used as reference baselines of non-specific binding. We also tested whether blocking with non-specific ratIgG prior to addition of a specific antibody resulted in reducing non-specific background staining. We found that our readings of specificity of the CD14 receptor staining with FITC-antiCD14 controlled by FITC-ratIgG, without ratIgG blocking, provided us with enough difference to believe these antibodies are optimal in testing for CD14 expression. It is thought that using a primary and secondary antibody, in testing for CD14 expression, is a more sensitive process that will give more precise results, although the process is more complicated and time consuming. Indeed, using anti-CD14 as a primary antibody and FITC-antiratIgG as a secondary antibody we found 98% more signal in LR-9 cells and a 188% higher signal in J774 cells (fig 2) compared to using a direct labeling method. These findings do support the increased sensitivity of using primary plus secondary antibody, yet we have shown it is more feasible to use only a labeled primary antibody.

Surprisingly, LR-9 cells in fact showed more CD14 expression than J774 cells. When using indirect fluorescence labeling, LR-9 showed 52% more CD14 expression and in the direct fluorescence labeling experiment LR-9 expression for CD14 was 121% greater than J774. Contrary to assumption, based on literature, belief that LR-9 is deficient of CD14 expression, these cells expressed more CD14 compared to J774. This unexpected result raises the question of the specificity of the antibody to CD14, or the
validity of the LR-9 cells as being deficient in CD14. A preliminary Western blot analysis suggests that the anti-CD14 antibody binds to multiple bands, suggesting that it is non-specific. Tests are currently underway to utilize other anti-CD14 antibodies to study this issue.

CD14 expression tests were simultaneously performed on J774 cells treated with antisense and sense DNA. To determine CD14 expression we used direct fluorescence labeling with FITC-αCD14 and the antibody FITC-ratIgG was used as a baseline for non-specific binding. J774 cell line was treated with antisense and sense twice over a period of 48 hours. The CD14 antisense treated cells were believed to become deficient in CD14 receptors, similar to the condition of LR-9 cells. However, antisense treated cells showed a greater amount of CD14 expression, at 4 times the amount of by cells treated with CD14 sense(fig 3). These results on CD14 expression using the Pharmingen’s α-CD14 do not conform with published data on LR-9 and with functional F-actin test. However the observations noted above call into question the specificity of the anti-CD14 antibody. Further experiments using alternative specific antibodies together with Western Blot analysis will be needed to asses CD14 expression in antisense and sense treated J774 and LR-9 cells.

We hypothesized that mmLDL induces an F-actin response through CD14 signaling which has a linear relationship to the amount of CD14 receptors. The two experiments testing this hypothesis appear to support our expectations. However, in subsequent testing to verify CD14 expression it was found that LR-9 and antisense treated J774 cells expressed higher levels of CD14. This raises the question of validity of LR-9 cells as well as specificity of the antibody to CD14. This issue is under current investigation through Western blot analysis and use of substitute antibodies.
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