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ORIGINAL ARTICLE

Effect of Native and Minimally Modified Low-density Lipoprotein on the Activation of Monocyte Subsets

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Background. In atherosclerosis, monocytes are essential and secrete pro-inflammatory cytokines in response to modified low-density lipoprotein (LDL). Human $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes produce different cytokines. The objective of this research was to determine the number of monocyte subsets positives to cytokines in response to native (nLDL) and minimally modified LDL (mmLDL).

Methods. Human monocytes from healthy individuals were purified by negative selection and were stimulated with nLDL, mmLDL or LPS. Subsequently, human total monocytes were incubated with monoclonal antibodies specific for CD14 or both CD14 and CD16 to characterize total monocytes and monocyte subsets and with antibodies specific to anti-tumor necrosis factor (TNF)-α, anti-interleukin (IL)-6 and anti-IL-10. The number of cells positive for cytokines was determined and cells cultured with nLDL, mmLDL and LPS were compared with cells cultured only with culture medium.

Results. We found that nLDL does not induce in the total monocyte population or in the three monocyte subsets positives to cytokines. MmLDL induced in total monocytes positives to TNF-α and IL-6 as well as in both $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ and in $CD14^{++}CD16^{+}$ monocytes, respectively. Moreover, total monocytes and the three monocyte subsets expressed few amounts of cells positives to IL-10 in response to mmLDL.

Conclusion. Our study demonstrated that nLDL did not induce cells positives to cytokines and that the $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocyte subsets could be the main sources of TNF-α and IL-6, respectively, in response to mmLDL, which promotes the development and progression of atherosclerotic plaque.

Key Words: nLDL, mmLDL, Monocyte subsets, TNF-α, IL-6.

Introduction

Atherosclerosis is a chronic inflammatory disease in which various components of the innate immune system, such as neutrophils, play a role in inflammation due to their effector functions (1). Cells of the monocyte lineage are also important components of the immune defense system and are recruited to lesions during the atherosclerosis inflammatory response. Monocytes are of great interest in atherosclerotic disease due to their phenotypic and functional heterogeneity (1,2). In humans, monocytes are divided into $CD14^{++}CD16^{-}$ (classical), $CD14^{++}CD16^{+}$ (intermediate) and $CD14^{+}CD16^{++}$ (non-classical) subsets (3), which perform different immunological functions. In this context, $CD14^{++}CD16^{-}$ monocytes produce high levels of interleukin (IL)-10 and low levels of tumor necrosis factor (TNF), whereas $CD14^{++}CD16^{+}$ monocytes produce high

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levels of pro-inflammatory cytokines, such as TNF, and low levels of anti-inflammatory cytokines, such as IL-10, in response to lipopolysaccharide (LPS). $CD14^{+}CD16^{+}$ monocytes exhibit a cytokine secretion pattern similar to that of $CD14^{++}CD16^{+}$ monocytes (2,4).

These functional distinctions suggest that monocyte subsets may serve different functions in response to several molecules. In atherosclerosis, low-density lipoprotein (LDL) activates different cell types (5,6). Previous studies have reported that native LDL (nLDL) stimulates C-C chemokine receptor (CCR) 2 expression by monocytes (7) and induces vascular cell adhesion molecule (VCAM)-1 and E-selectin expression in human vascular endothelial cells (8), suggesting that nLDL is capable of stimulating monocytes.

Atherosclerosis also involves the presence of various forms of oxidized LDL (oxLDL) and minimally modified LDL (mmLDL), which act as key pro-inflammatory molecules in this context (5,6). Previous studies have evaluated the effect of mmLDL on mouse macrophages and have found that mmLDL induces the activation of mitogen-activated protein kinases and nuclear factor-κB, which trigger macrophage inflammatory protein-2, monocyte chemoattractant protein-1, TNF-α and IL-6 messenger RNA expression (9). In addition, incubating mmLDL with human monocytes results TNF-α, IL-1β and IL-6 production along with a substantial increase in CD11b/CD18 expression (10−12). This evidence shows that monocytes play a central role in the response to mmLDL. Further, monocyte subsets could respond to endogenous ligands that trigger inflammatory responses. For example, $CD14^{++}CD16^{+}$ monocytes of hypercholesterolemic patients exhibit increased uptake of oxidized LDL via CD36, whereas $CD14^{++}CD16^{-}$ monocytes from the same patients preferentially take up nLDL. In addition, $CD14^{++}CD16^{+}$ monocytes of hypercholesterolemic patients exhibit increased expression of surface proteins such as CD68, stabilin-1, and CD11c in response to oxLDL (13). This evidence shows that monocyte and macrophage populations are capable of secreting pro-atherogenic cytokines in response to mmLDL and that $CD14^{++}CD16^{+}$ monocytes increase CD11c expression in response to oxLDL. Nevertheless, pro-inflammatory cytokine expression by $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{+}CD16^{++}$ human monocyte subsets in response to early forms of modified LDL such as mmLDL remains uncharacterized. In this study, we analyzed the number of human total monocytes and monocyte subsets positive to cytokine in response to nLDL and mmLDL. We found that nLDL does not induce the total monocyte population or monocyte subsets positives to pro-inflammatory cytokine; however, total monocytes were positive to TNF-α and IL-6. In addition, $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes were positives to pro-inflammatory cytokines in response to mmLDL. Furthermore, the three monocyte subsets were very few positives to IL-10 in response to mmLDL stimulation. These results provide a new perspective on the role of mmLDL in the activation of monocyte subsets in association with atherosclerotic inflammation.

Material and Methods

Experimental Protocol

Informed consent was obtained from ten healthy 20−30 year old males and females. All patients were nonsmoking and of normal weight and exhibited normal levels of total cholesterol (<200 mg/dL), triglycerides (<150 mg/dL), LDL (<100 mg/dL) and HDL (>40 mg/dL). The study was approved by the Human Ethics and Medical Research Committees of the Instituto Mexicano del Seguro Social and was developed at the Unidad de Investigación Médica en Inmunología. The study was conducted according to the guidelines of the Declaration of Helsinki.

LDL Isolation and Modification

Human nLDL was isolated from normolipidemic plasma by density ultracentrifugation (10). The nLDL preparation was dialyzed against phosphate-buffered saline (PBS) with 0.5 mmol EDTA, and the protein concentration was determined. Before oxidation, EDTA was removed by extensive dialysis. mmLDL was prepared by incubating nLDL (300 μg/mL) with 10 μmol/L $CuSO_4$ for 1 h at 37°C. Then, mmLDL was dialyzed against PBS supplemented with 0.5 mmol EDTA (10). Oxidative modification of LDL was assessed by measuring thiobarbituric acid-reactive substances, as previously described (10). All LDL preparations used in these experiments were tested for bacterial LPS contamination using a Limulus Amoebocyte Lysate kit (BioWhittaker, Walkersville, MD) according to the manufacturer's instructions.

Monocyte Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from samples obtained from healthy volunteers by density centrifugation using lymphoprep (Axis-Shield, Oslo, Norway). Blood samples were mixed with an equal volume of PBS (pH 7.4), layered over 3 mL of lymphoprep, and centrifuged at 700 *g* for 30 min. PBMCs were washed three times with PBS (pH 7.4). Monocytes were then isolated from PBMCs by negative selection (Pan Monocyte Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). Magnetic microbeads were coupled to an anti−hapten monoclonal antibody and depleted using a magnetic column. Purified cells were stained for CD14 and the purity of the monocytes was determined to be >87%. Flow cytometry was performed on a MACSQuant analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany).