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Lipoproteins induce tyrosine phosphorylation in human natural killer cells

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Abstract: Lipoproteins, chylomicrons (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL) and acetyl modified LDL (AcLDL) can stimulate natural killer (NK) cells upon binding to specific receptors. Using flow cytometry and a specific monoclonal antibody against phosphotyrosine, we assessed the effect of lipoproteins on total intracellular expression of immunoreactive phosphotyrosine. Tyrosine phosphorylation increased when lipoproteins were added to purified NK cells, previously cultured overnight in RMPI–0.5% fatty acid free bovine serum albumin. The optimal effect occurred at 15 minutes for all the lipoproteins used. The concentrations of lipoprotein were 10 μg of protein/ml of CM, and 20 μg of protein/ml for the other lipoproteins. The effect of lipoproteins on phosphotyrosine positivity was as follows: LDL > VLDL > CM > HDL > AcLDL. Thus lipoproteins may activate NK cells through tyrosine phosphorylation and this signal transduction pathway seems to be enhanced when these cells are activated with LDL.

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Introduction: Lipoproteins, chylomicrons (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) have always been considered important for cell and tissue physiology [1]. However, deregulation of the metabolic control of lipoprotein may be the cause of vascular pathologies such as atherosclerosis [2,3].

Even though NK cells have not been directly involved in the development of atherosclerosis, diet [4], lipid emulsions [5] and lipoproteins [6] alter NK lytic efficiency against K562 and P815 cells. At optimal concentrations, CM, VLDL and LDL enhanced while HDL and acetyl-modified LDL (AcLDL) inhibited the NK cytotoxic response against K562 [6]. On the contrary, all lipoproteins enhance the NK proliferative response [6]. The mechanism by which this effect is produced seems to be dependent on the stimulatory signals generated upon ligand binding with the specific receptor.

Materials and methods: Lipoproteins were separated as described previously [6]. The levels of oxidized lipoproteins, assessed by the thiobarbituric acid assay (TBARS), were undetectable [6]. LDL was acetylated as described by Basu *et al.* [7]. All the lipoproteins were washed, dialysed and filter-sterilized before the assay.

Immunofluorescent quantification of tyrosine phosphorylation of cellular proteins was performed as described previously by Far *et al.* [8]. The assay was conducted with purified NK cell populations from three different donors (> 85% $CD16^+$, < 1% $CD14^+$, < 1% $CD3^+$ and < 1% $CD19^+$) as described previously [6].

The cells were incubated with RMPI–0.1% BSA fatty acid free for 18 h before the stimulation with lipoprotein and subsequently washed and incubated with 10 μg of protein/ml of CM or 20 μg of protein/ml of one of the lipoproteins (VLDL, LDL, HDL and AcLDL) for different time points.

Afterwards, the cells were fixed with 1% paraformaldehyde permeabilized with saponin, incubated with 0.01 M phosphate buffer solution (PBS)–0.1% bovine serum albumin (BSA) 0.1% Tween-20 to block non-specific binding and finally incubated with anti-phosphotyrosine-FITC (Sigma Chemical Company, St Louis, MO, USA). As controls, the cells were treated with pervanadate (positive) and 1 μM staurosporine (negative) as described previously [8].

Specificity was assessed by adding phosphotyrosine or incubating pervanadate-stimulated cells with unlabelled anti-phosphotyrosine before incubation with the labelled antibody. Finally, the fluorescence intensity was quantified in an EPICS ELITE flow cytometer (Coulter Corporation,

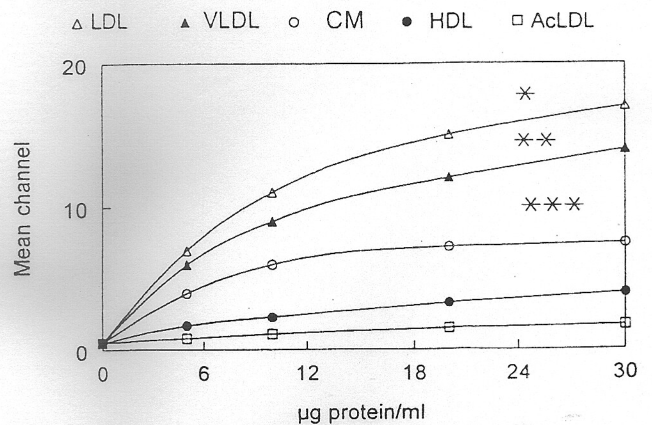

Figure 1. Effect of lipoproteins on tyrosine phosphorylation. The mean channel fluorescence intensity (logarithmic units) represents the amount of immunoreactive phosphotyrosine detected with anti-phosphotyrosine-FITC and assessed by flow cytometry.

check beads.

ANOVA test and Student's t-test were used to analyse the results. Data were considered significant when P values were < 0.05.

ence in the intensity of fluorescence that was dependent on the concentration of the lipoprotein used. Figure 1 illustrates the effect of lipoprotein on phosphotyrosine positivity in three sets of experiments. The cells stimulated with LDL had the

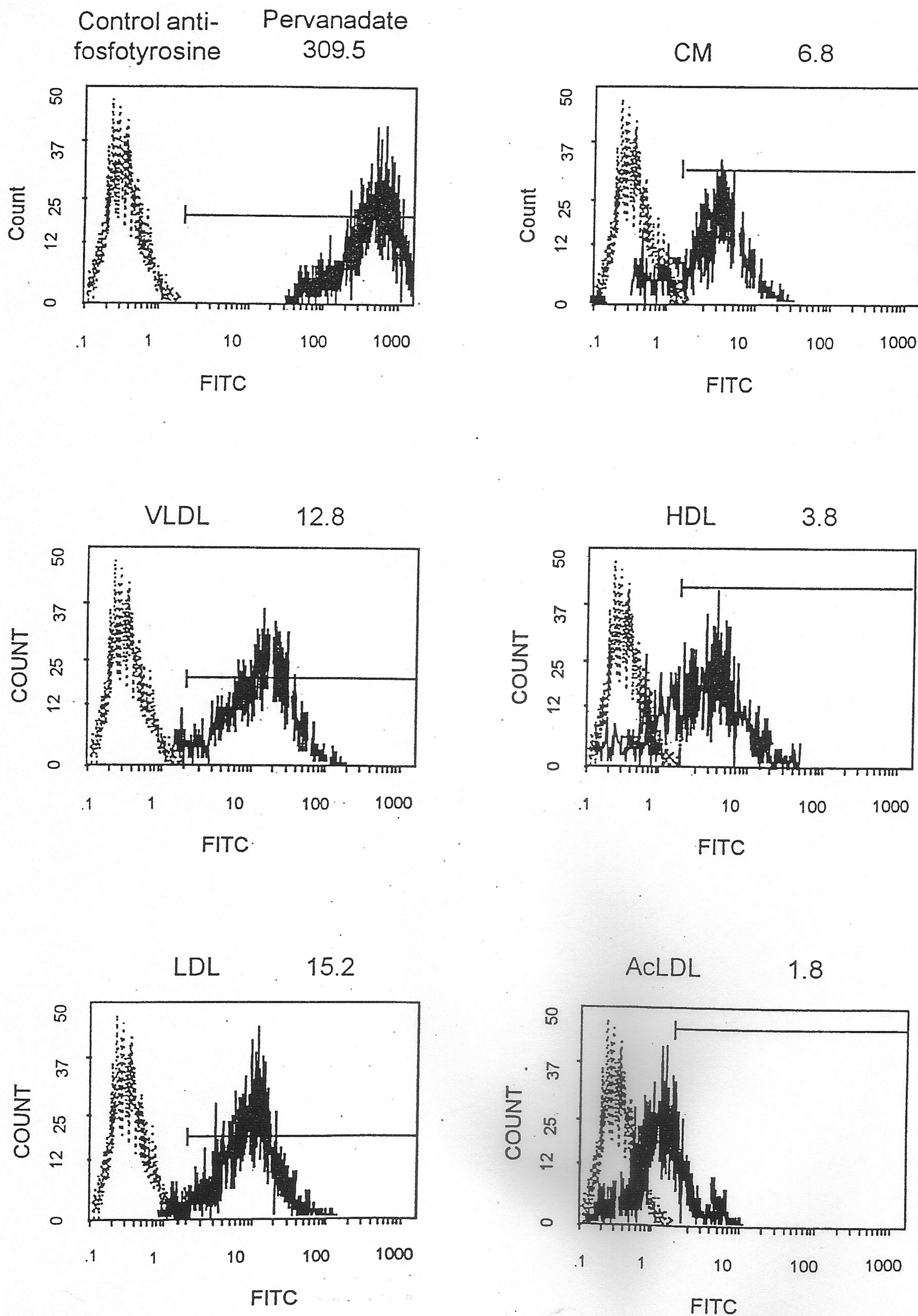

Figure 2. Flow cytometry histograms of stimulated cells. In this typical flow cytometry experiment, the dotted line refers to control (non-stimulated cells) and the dark lines represent the lipoprotein-stimulated cells. The number on the top right of the histograms represents the mean channel fluorescence intensity in logarithmic units.

highest levels of phosphotyrosine. These were significantly higher than those of cells stimulated with HDL or AcLDL ($P < 0.001$) and those of CM stimulated cells ($P < 0.05$). Similarly, the cells stimulated with VLDL expressed higher levels of phosphotyrosine as compared to HDL or AcLDL ($P < 0.005$) and CM ($P < 0.05$) stimulated cells.

Figure 2 represents a typical assay for phosphotyrosine determination, at optimal concentrations of lipoprotein, using flow cytometry. The mean channel fluorescence intensity, reported in logarithmic units, was assessed in the total cell population. The dotted line represents the basal phosphotyrosine expression. The dark lines represents the phosphotyrosine from stimulated cells.

In concordance with the figure, the amount of phosphotyrosine recorded following LDL stimulation was the highest, contrasting to the low levels observed with AcLDL and HDL.

Discussion: Lipoproteins can modulate NK proliferative and cytotoxic responses [6]. Thus the signal transduction generated upon lipoprotein binding to its receptor may be important in the physiological response of NK cells.

The effects of the different types of lipoprotein on tyrosine phosphorylation are clear. High levels of phosphotyrosine occurred upon LDL and VLDL stimulation, contrasting with the low levels seen upon AcLDL, HDL and CM stimulation.

Previous studies [9,10] have shown that LDL activation induces phosphorylation of the mitogen-activated protein kinase (MAP) in the U937 cell line and smooth muscle cells. As a consequence of this activation, the proliferative response of these cells increased upon LDL activation. The effect of LDL was inhibited by HDL.

Similarly, Lara *et al.* [11] have shown that LDL increased the translocation of PKC to the particulate membrane fraction of polymorphonuclear cells, with a corresponding rise in peroxide production. Miki *et al.* [12] suggested a direct activation of the *lyn* tyrosine kinase upon AcLDL binding to the scavenger receptor in the THP-1 cell line, indicating a direct involvement of this kinase with the receptor.

These reports lead us to postulate that lipoprotein-induced signal transduction can be an important event in NK cell activation.

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