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Lipoprotein lipase expression in natural killer cells and its role in their cytotoxic activity

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SUMMARY

Lipoprotein lipase (LPL) is the key enzyme in the metabolism of triglyceride-rich lipoproteins. The patterns of LPL mRNA expression and secretion of the enzyme have not yet been established in natural killer (NK) cells. We show in the present communication that $CD3^-$ $CD16^+$ cells (NK cells) transcribe LPL mRNA, express LPL on the surface and secrete the enzyme. In contrast, there is no LPL expression on the surface of highly purified B and T lymphocytes. Stimulation of NK cells with interleukin-2 (IL-2) reduced the expression of LPL on their surface and augmented the secretion of LPL by the cells. The addition of anti-LPL antibodies to NK cells in culture led to a complete abrogation of cytotoxicity of NK cells against the K562 tumour cell line. Furthermore, IL-2 stimulation of effector cells reversed the anti-LPL antibody-induced inhibition of cytotoxic activity. Overall, these findings suggest that LPL plays a key role in the cytotoxic activity of NK cells.

INTRODUCTION

Lipoprotein lipase (LPL; E.C. 3.1.1.34) is a key enzyme in the catabolism and anabolism of triglyceride-rich lipoproteins, chylomicrons and very low density lipoprotein, as well as other lipoproteins.[1] It has been established that LPL is secreted by adipose tissue (white/brown), heart, mammary gland (lactating), skeletal muscle, adrenal, ovary, thoracic aorta, spleen, small intestine, testis, lung, kidney, brain (hippocampus), neonatal liver[1] and macrophages,[2–4] and is thought to be important in the development of atherosclerosis since it generates a high quantity of remnant lipoproteins that are avidly taken up by macrophages.[5,6] Natural killer (NK) cells have not been shown to be involved in the development of atherosclerosis; however, modifications of NK cell activity due to diet or modified cholesterol have been observed.[7–9]

NK cells ($CD3^-$ $CD16^+$ $CD56^+$) represent a subset of lymphocytes distinguishable from T and B lymphocytes by their morphology, phenotype and functional capacity to spontaneously kill tumour cells or virally infected cells.[7,8] Treatment of NK cells with interleukin-2 (IL-2) results in a significant augmentation of their tumoricidal activity.[7,8]

Different mechanisms have been proposed to explain NK cytotoxicity, including: (1) secretion of cytotoxic molecules such as interferon, etc., and (2) membrane interaction with infected cells or tumour cells. Among the products of NK metabolism, arachidonic acid metabolites are essential in mediating the lysis of target cells.[8,10] Selective inhibition of arachidonic acid metabolites or an increase in cAMP levels by other means is directly associated with the inhibition of cell lysis.[8,10] On the other hand, the supplies of fatty acids needed for NK cytotoxicity may be furnished by the products of LPL in NK cells.

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MATERIALS AND METHODS

Chemicals

Fetal calf serum (FCS), human recombinant IL-2, L-glutamine, penicillin–streptomycin and RPMI-1640 medium and RPMI-1640 select-amine kit were purchased from Gibco BRL (Gaithersburg, MD). Percoll and Ficoll–Paque® were purchased from Pharmacia LKB (Uppsala, Sweden). dCTP, Expre^{35}S^{35}S protein-labelling mix and En^{3}Hance were purchased from New England Nuclear (Mississauga, Ontario, Canada). All other reagents were acquired from Sigma Chemical Co. (St Louis, MO).

Antibodies

Monoclonal antibody anti-Leu-11c–PE (CD16; FcRIII receptor) was purchased from Becton Dickinson (Mountain View, CA); NKH-1 RD_1 (CD56; asialo-GM_1), NKH-1 unlabelled, T3 (CD3) unlabelled, T3 (CD3)–FITC and MO_2 (CD14)–FITC cytostat antibodies were purchased from Coulter Immunology (Hialeah, FL); anti-CD11b/CD18 (anti-MAC-1) was purchased from Boehringer Mannheim (Indianapolis, IN).

Cell purification

Blood samples were taken from normal healthy donors (blood bank of the Central University Hospital, Caracas, Venezuela). Human large granular lymphocytes (LGL) were separated by passage of the non-adherent mixed population of cells through

nylon wool, and subsequent centrifugation on Percoll gradients.[11] The cells isolated from the Percoll gradients were treated with anti-CD3 monoclonal antibody plus complement, to deplete $CD3^+$ cells. Finally, $CD3^-$ $CD16^+$ were isolated by positive sorting of brightly stained $CD16^+$ cells, > than 10^2 in the logarithmic scale, using the Leu-11c–PE monoclonal antibody in an EPICS 753 flow cytometer (Coulter Corporation, Hialeah, FL).[12]

Purified NK ($CD3^-$ $CD16^+$) cells were then cultured for 18 hr in RPMI-1640 medium supplemented with 10% FCS in the absence or presence of 50 IU of IL-2/10^6 cells/ml.

Monocytes were purified by centrifugation on Percoll gradients and subsequent adherence to plastic. T lymphocytes were isolated by passage through nylon wool, centrifugation on Percoll gradients, and treatment with anti-CD56 monoclonal antibody to deplete NK cells. B cells were purified by depleting T and NK cells by rosetting to neuraminidase-treated sheep red blood cells followed by Ficoll–Hypaque separation. All fractions were tested for CD14 expression (monocytes), CD3 expression (T cells) and CD5 (B cells).

Immunoprecipitation of LPL

The anti-LPL antibody (IDI-1) used in the present study has been described previously,[13] and has similar affinities for the human, bovine and murine enzymes due to the high degree of homology among LPL produced by different species.[14] This antibody inhibits the catalytic activity of human, bovine and murine LPL that is mediated by the dimer form of the enzyme.[1]

LPL from NK cells was immunoprecipitated using the standard protocol for immunoprecipitation described elsewhere,[15,16] with a minor modification, namely anti-LPL–Sepharose was used instead of antibody plus protein A–Sepharose. Briefly, purified NK cells were rinsed with methionine cysteine-free medium (RPMI-1640 select-amine) and metabolically labelled with 0·1 mCi/ml of Expre^{35}S^{35}S labelling kit overnight in the presence of 10% FCS. The cells were then centrifuged and lysed in phosphate-buffered saline (PBS) solution containing 1% nonidet P-40 (NP-40), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 0·1 mM leupeptin. After centrifugation of the lysed cells, an aliquot of the supernatant was pretreated with normal rabbit IgG coupled to protein A–Sepharose. The mixture was incubated for 2 hr at room temperature, centrifuged and the supernatant was mixed with 20 μl of anti-LPL–Sepharose, prepared as detailed elsewhere,[13] for 1 hr at room temperature. The mixture was centrifuged, and the pellet was washed twice with 0·05% NP-40 and 1 mM PMSF, and twice with 0·05 M Tris–HCl, pH 6·8. Finally, the pellet was resuspended in SDS buffer and heated at 95° for 10 min. Samples were then subjected to a SDS–polyacrylamide gel electrophoresis using 12% acrylamide as separating gel. After the electrophoresis run, the gels were soaked in En^{3}Hance, dried and autoradiographed with an amplified screen using Kodak Xomat AR film (Rochester, NY).

A murine macrophage cell line, ANA-1, was used as a control. ANA-1 cells were metabolically labelled as before, but were incubated for 3 hr at 37°. The antibody is able to recognize the monomer band of LPL (apparent MW 55 000), and the dimer form (apparent MW 100 000). The dimer form is considered to be the catalytically active form of the enzyme.[1]

Labelling of anti-LPL with FITC

For use in flow cytometry, anti-LPL antibody and a control (irrelevant) IgG antibody were labelled using fluorescein isothiocyanate (FITC) as described in detail elsewhere,[17] and adjusted to 1 mg/ml in PBS 0·02% Na azide. Anti-LPL FITC had both the same ability to inhibit the catalytic activity of LPL, and the same affinity for LPL as the unlabelled antibody, as determined by ELISA (results not shown).

In order to determine the lytic effect of the antibody on NK cells, NK cells were labelled with ^{51}Cr for 1 hr at 37°, washed, and then incubated with 10 μl/10^6 cells of anti-LPL for 30 min at 4°; the cells were then washed with PBS and incubated with complement for 30 min at 37°. Background cytotoxicity was determined by the analysis of cell supernatants following the addition of anti-CD4 plus complement to the controls. Lytic anti-MAC-1 (CD11b/CD18) and anti-CD56 were also used as controls.

Enzyme expression

The expression of membrane-bound LPL was monitored by flow cytometry, using an EPICS 753 flow cytometer (Coulter) equipped with a 5-W argon laser and dye laser. Anti-LPL–FITC and an irrelevant IgG–FITC from untreated rabbits were included as a control. The instrument was calibrated for fluorescence and light scatter using DNA-check calibrating beads (Coulter Immunology). Excitation wavelength was set at 488 nm and all parameters except forward angle light scatter (FALS) were assessed using a logarithmic scale. For two-colour analysis, a 530 nm short-pass filter for FITC and 590 nm long-pass filter for phycoerythrin (PE) were employed.

For single and double labelling studies, performed using whole blood, 100 μl of sample (containing between 6000 and 12 000 leucocytes/mm^3) was used and incubated with 5 μl of different monoclonal antibodies coupled to FITC, PE or anti-LPL–FITC. After 30 min of incubation at room temperature, the erythrocytes were lysed with Immunoprep solution (EPICS; Coulter Diagnostics) and the samples were analysed as described above. Results were expressed as the percentage of total lymphocytes.

For single labelling of purified cell populations, 1×10^6 cells were incubated with 5 μl of the antibody for 30 min at 4°. The cells were then washed twice with PBS containing 1% bovine serum albumin (BSA) and 0·02% Na azide, and once with PBS alone. The cells were sorted based on high expression of CD16 (bright fluorescence > 10^2 in the logarithmic scale). In order to determine the level of expression more precisely, sorted $CD16^+$ cells were washed extensively, cultured overnight at 37° and re-analysed for CD16 expression. Then, anti-LPL–FITC was added, and the level of LPL expression on the cells was determined as described previously.

Enzymatic activity assessment

LPL activity was assayed according to the method of Nilsson-Ehle & Schotz.[18] Briefly, 100 μl of a sample medium was incubated with 100 μl of substrate mixture containing glycerol tri[9,10(n)-^{3}H] oleate (New England Nuclear, Boston, MA), 1 mg triolein, 2 mg bovine-free fatty acid-free albumin, 0·12 mg lecithin, 16–20% of heat-inactivated human or bovine serum and 16% (vol/vol) glycerol in 0·2 M Tris–HCl (pH 8·0). The substrate mixture was incubated for 60 min at 37°. The specific activity of the substrate was 100 d.p.m./nmol fatty acid. One unit of enzyme activity was defined as the release of 1 nmole of fatty acid/min/ml of media. Samples were tested in quadruplicate in each of the separate experiments.

Cell-surface LPL activity was also assayed using 2×10^6 cells/ml of 0·2 M Tris–HCl in the presence of 4·5 mM of substrate, and 30 mg/ml of fatty acid-free BSA. After 20 min of incubation at 37°, the cells were centrifuged, and the supernatant was assayed for enzyme activity. Control supernatant containing substrate in the absence of serum (lacking the activator of LPL), was used to determine background levels of enzymatic activity. Varying concentrations of the antibody were used to monitor anti-LPL-induced inhibition of the catalytic activity.

Determination of LPL immunoreactive mass

The LPL immunoreactive mass was measured by ELISA using affinity-purified antibodies specific for LPL, according to the technique previously described[13] with some modifications. Briefly, 1 μg/well of purified antibody against bovine LPL was adsorbed in 96-well plates (Immulon II; Dynatech, Alexandria, VA) and incubated overnight with different dilutions of the sample (in PBS or 0·154 M NaCl) or a human LPL standard. The wells were then washed with PBS 0·05% Tween 20, and affinity-purified antibody to LPL was added. Anti-rabbit IgG peroxidase was added to the wells and incubated for 3 hr. The peroxide reaction was developed by adding peroxide substrate (0·3 mg/ml o-phenylenediamine, 0·012% hydrogen peroxide in PBS, pH 7·2). The reaction was stopped with 25 μl of 2 M sulphuric acid and the results were read in an ELISA plate reader (Microwell System; Organon Teknica, Austria). The samples were analysed in quadruplicate.

LPL mRNA expression assessment by RT–PCR

The polymerase chain reaction (PCR) was performed using total RNA isolated from unstimulated or stimulated (50 IU/ml of IL-2 for 18 hr) NK and T cells. The RNA was purified according to the method of Chomczynski & Sacchi.[19] cDNA was made from the total RNA using oligo dT primers. Amplification reactions were performed using Taq polymerase derived from *T. aquaticus*, 0·2 ml ^{32}P dCTP per reaction, and other reagents as directed by the manufacturer's instructions (GeneAmp kit; Perkin-Elmer Cetus, Norwalk, CT). Human LPL-specific primers (5′-GAG-ATT-TCT-CTG-TAT-GGC-ACC-3′ and 5′-CTG-CAA-ATG-AGA-CAC-TTT-CTC-3′) and glyceraldehyde-3 phosphate dehydrogenase primers (GADPH; 5′-CCC-TTC-ATT-GAC-CTC-AAC-TAC-ATG-G-3′ and 5′-AGT-CTT-CTG-GGT-GGC-AGT-GAT-GG-3′) were used in the PCR reactions to amplify the specific 277-bp fragment of LPL cDNA and the 456-bp fragment of GADPH cDNA, respectively. The reactions were performed using an automated thermal cycler (DNA Thermal Cycler; Perkin-Elmer Cetus). Briefly, samples were denatured at 94° for 5 min, and 25 cycles were performed. Each cycle consisted of denaturation at 94° for 40 seconds, annealing of primers at 60° for 40 seconds, and primer extension at 72° for 90 seconds. The RNA AW 109 (Perkin-Elmer Cetus), which contains sequences specific for LPL, was used as a control. The amplified products were analysed by electrophoresis in a 4% polyacrylamide gel followed by autoradiography with Kodak X-Omat films. The intensity of bands present in autoradiograms was measured using a Sciscan-5000 densitometer (USB, Columbus, OH).

NK cytotoxic assay

A short-term (4 hr) radiolabelled release assay using ^{51}Cr-labelled K562 cells as targets was performed[20] with minor modifications.[21] Briefly, 5×10^6 K562 cells were labelled with 150 μCi of Na^{51}Cr (200–500 μCi/mmol; Amersham International, Amersham, UK) for 1 hr at 37°. Labelled cells were washed three times in RPMI medium plus 5% FCS and resuspended at 5×10^4 cells/ml in RPMI containing 10% FCS. A fixed number (5×10^3 cells in 0·1 ml) of labelled K562 cells was mixed with 0·1 ml of effector cells at four different effector to target cell ratios (50 : 1, 25 : 1, 12 : 1 and 6 : 1, or 40 : 1, 20 : 1 and 10 : 1). The combination of target and effector cells was seeded in triplicate into 96-well U-bottomed microtest plates (Falcon Plastics, Lincoln Park, NJ). Five microlitres of either affinity-purified anti-LPL (1 mg/ml) antibody or control antibody (1 mg/ml total IgG from control rabbits) or RPMI–10% FCS was added to each well. In other experiments, the cells were preincubated with 2·5 μg of antibody/10^6 cells for 30 min with anti-MAC-1 (CD11b/CD18) or anti-CD56. Anti-LPL-was added in the assay at the same concentration, 2·5 μg/10^6, in order to monitor the possible additive effect of anti-LPL with each antibody.

^{51}Cr release was measured in 100-μl samples of supernatants using a γ-counter (Compugamma, Wallac, LKB, Sweden). Total release of radioactivity was determined by counting the radioactivity released from 5000 ^{51}Cr-labelled K562 cells. The percentage lysis was calculated by the following formula:

$$\% \text{ specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}}.$$

Statistical analysis

The paired Student's *t*-test was employed for analysing small numbers of samples ($n < 30$), and the *F*-test was used for analysing a large number of samples ($n > 30$).

RESULTS

LPL immunoprecipitation by anti-LPL

Purified NK cells ($CD3^- CD16^+$) were shown to synthesize LPL, as determined by immunoprecipitation of methionine-^{35}S metabolically labelled cells using anti-LPL antibody (Fig. 1). The immunoprecipitation of the LPL from unstimulated NK cells led to the resolution of a band of apparent MW 55 000, present in the supernatant and in the intracellular protein fraction. ANA-1, macrophages that produce high amounts of LPL, were used as controls. The antibody also recognized the dimer of LPL, of apparent MW 100 000, which is expressed by ANA-1 cells.

LPL expression on the cell surface

LPL expression was quantified by labelling the cells with anti-LPL–FITC and by analysing the staining of the cell population in an EPICS-753 flow cytometer. As shown in Fig. 2 d, 88% of $CD3^- CD16^+$ cells positively stained for LPL. No change in LPL expression was observed when the cells were cultured for 18 hr in RPMI-1640 medium supplemented with 10% FCS (see Fig. 5a, b). Only 8 ± 5% of purified T ($CD3^+ > 90\%$) or B ($CD5^+ > 90\%$; data not shown) lymphocytes expressed LPL in the surface ($P < 0{\cdot}01$; $n = 12$; Fig. 2c). A similar percentage, compared with $CD3^- CD16^+$ cells, of stained positivity with anti-LPL was observed in monocytes (Fig. 2b).

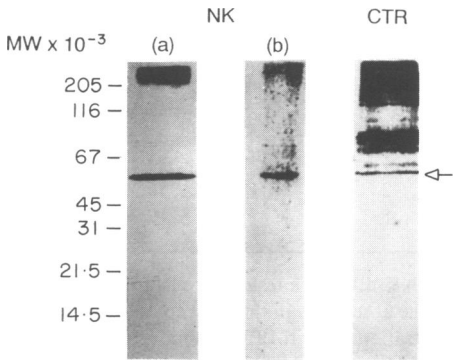

Figure 1. Immunoprecipitation of LPL from CD3$^-$ CD16$^+$ cells and from ANA-1 cells. Biochemical characterization of LPL recognized by anti-LPL antibody. CD3$^-$ CD16$^+$ cells were incubated overnight in the presence of 0·1 mCi/ml ^{35}S-methionine and LPL was immunoprecipitated as described in the Materials and Methods. (a) ^{35}S-methionine NK cell lysate. (b) ^{35}S-methionine supernatant from NK cells, secreted LPL. ANA-1, a murine macrophage line, was used as a control (CTR). Gel electrophoresis using 12% SDS–PAGE was performed as described in the Materials and Methods. The molecular markers used were: myosin (H chain) 205 000, β-galactosidase 116 000, BSA 67 000, ovalbumin 45 000, carbonic anhydrase 31 000, soybean trypsin inhibitor 21 500, and lysozyme 14 500 MW. Arrow represents 55 000 MW.

To test whether or not there was a correlation between the expression of the NK cell marker, CD16, and LPL expression in total fresh blood cells (using Q-Prep), we used both single- and double-labelling protocols with antibodies against the FcIII receptor and against LPL. By FACS analysis, we were able to quantify: (1) the total CD16$^+$ population; (2) the total LPL$^+$ population; and (3) the population of double-positive cells labelled by both antibodies. Using linear regression

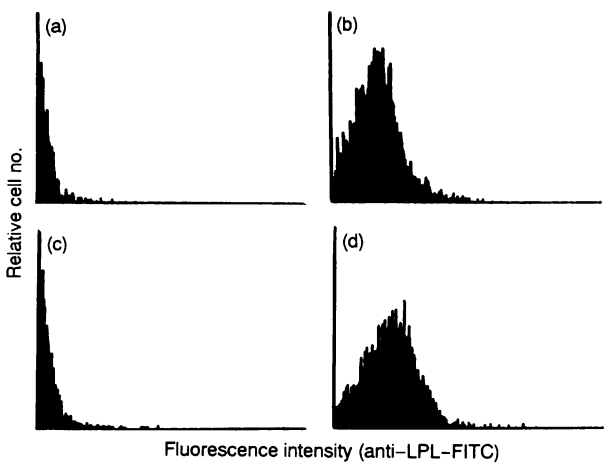

Figure 2. Expression of LPL in different populations of purified blood cells. The expression of LPL was determined in each purified cell population by FACS analysis using anti-LPL or the control IgG–FITC. Purified cells were labelled with the antibody as described in the Materials and Methods. Nonspecific binding was blocked by using human AB serum. (a) The background fluorescence from purified NK cells, B cells and monocytes labelled with the control rabbit anti-human IgG–FITC. (b) The LPL expression of monocytes (> 90% CD14$^+$) purified by Percoll. (c) The expression of LPL in T cells (> 90% CD3$^+$). (d) The expression of LPL in CD3$^-$ CD16$^+$ (NK cells).

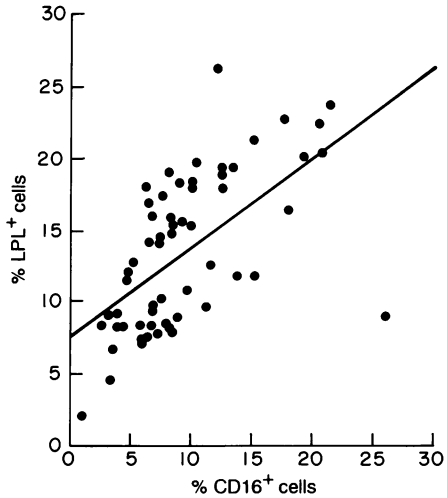

Figure 3. Simple regression analysis of LPL$^+$ cells versus CD16$^+$ cells. Linear regression analysis was performed in order to assess LPL and CD16 co-expression in heterogeneous blood cell populations isolated from 58 healthy individuals using the percentage of labelling of cells with a single antibody. Simple regression analysis in LPL$^+$ cells versus CD16$^+$ cells permits quantification of the relationship between LPL and CD16 expression using the percentage of positive lymphocytes for single labelling studies; $y = 7{\cdot}81 + 0{\cdot}6192x$; cor. = 0·50; $n = 58$; $P < 0{\cdot}001$. Note the high correlation between these two populations.

analysis, we found a good correlation ($r = 0{\cdot}50$; $P < 0{\cdot}001$; $n = 58$), between the percentage of CD16$^+$ cells and the percentage of LPL$^+$ cells, as shown in Fig. 3. Moreover, this correlation increased when bright stained CD16$^+$ populations were screened ($r = 0{\cdot}80$; $P < 0{\cdot}0001$; $n = 30$).

In order to further confirm the expression of LPL by NK cells, purified CD3$^-$ CD16$^+$ cells were sorted using anti-CD16 (Leu-11c–PE) and anti-CD3–FITC monoclonal antibodies. A highly purified (> 98%) CD3$^-$ CD16$^+$ population was obtained that expressed bright CD16, $> 10^2$ logarithmic units. The NK cells were then labelled with anti-LPL–FITC. As shown in Fig. 4, 99% of the CD16$^+$ cells expressed LPL on their surface. Moreover, anti-LPL was found to be cytolytic when added to the cells in combination with complement (68 ± 5% killing

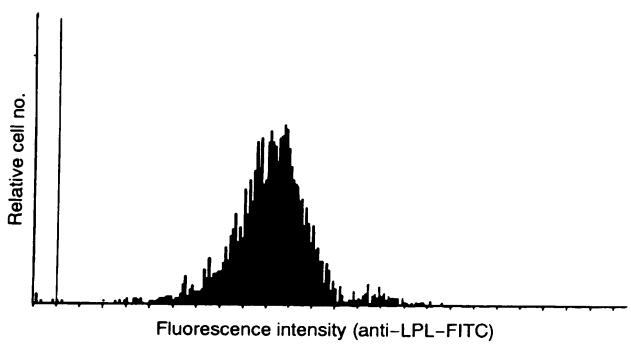

Figure 4. LPL expression on the surface of CD3$^-$ CD16$^+$ sorted cells. Sorted brightly fluorescent CD16$^+$ cells were washed extensively, cultured at 37° overnight and then labelled with anti-LPL–FITC antibody as described in the Materials and Methods. 99·8% of the cells were positive for LPL with high fluorescence intensity.

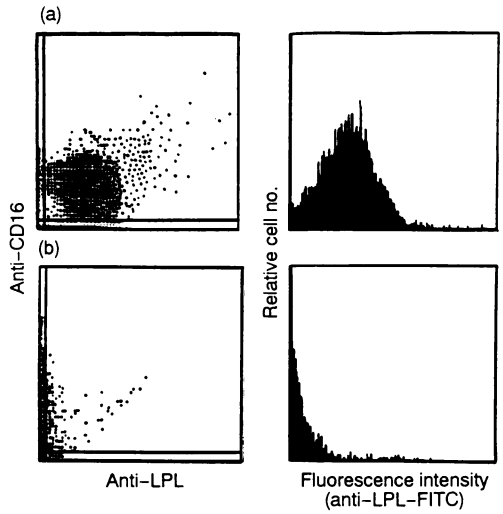

Figure 5. Single and double LPL expression in unstimulated and IL-2-stimulated $CD3^-CD16^+$. Purified $CD3^-CD16^+$ cells were cultured for 18 hr in RPMI–FCS 10% in the presence or absence of 50 IU IL-2/ml/10^6 cells. The cells were then labelled with anti-CD16 and with anti-LPL as described in Materials and Methods. (a) The expression of double-labelled CD16-LPL cells by anti-CD16–PE and anti-LPL–FITC, and single LPL^+ expression by unstimulated $CD3^-CD16^+$ cells. (b) The expression of double-labelled CD16-LPL cells by anti-CD16–PE and anti-LPL–FITC, and the expression of LPL^+ labelled IL-2-stimulated $CD3^-CD16^+$ cells.

compared with 72 ± 8% induced by anti-CD56) to unstimulated NK cells, as determined by ^{51}Cr release from labelled cells. In contrast, the viability of K562 tumour cells, and purified T cells, was not affected by treatment with anti-LPL antibody plus complement.

Incubation of NK cells ($CD3^-CD16^+$) in complete medium for 18 hr did not change the expression on the surface of NK cells (Fig. 5a, b); however, activation of NK cells with 50 IU IL-2/ml resulted in a marked reduction of the expression of LPL on the NK surface (Fig. 5c, d). This marked reduction in expres-sion of LPL was due to an increased release of the enzyme from the surface by IL-2 activation, as observed in Table 1.

Secretion of LPL

When NK cells ($CD3^-CD16^+$) were stimulated for 18 hr with 50 IU of IL-2, the expression of LPL on the surface of the cells decreased, compared to LPL expression on the surface of unstimulated NK cells (90 ± 5% for the unstimulated and 15 ± 5% for the IL-2-stimulated cells; $n = 6$; $P < 0.01$). As shown in Table 1, treatment of NK cells with IL-2 augmented the release of the enzyme into the media. The amount of secreted LPL increased more than threefold in IL-2-treated NK cells compared to untreated controls. Similarly, when the LPL enzymatic activity was assessed, an approximate threefold increase in LPL activity was detected in IL-2-treated NK cells compared to LPL activity observed in untreated NK cells. The data are consistent with the diminished expression of LPL in the IL-2-activated cells, as determined by flow cytometry (Fig. 5).

LPL enzyme activity was also assayed on the cell surface. Anti-LPL inhibited LPL catalytic activity in a dose-dependent manner, similar to that seen in the cell-free assay system (Table 2).

Table 1. LPL secretion by NK cells

Type of treatment	Activity (nmoles fa/min/ml)	Mass (ng LPL/ml)
Unstimulated	35 ± 3	12·5 ± 3·5
Stimulated	90 ± 8*	45·0 ± 12·0*

Supernatants of cultured unstimulated or IL-2-stimulated NK cells were tested for LPL activity and immunoreactive mass as described in the Materials and Methods. A significant difference was observed, $*P < 0.01$, $n = 10$, when NK cells were stimulated with IL-2.

Table 2. LPL activity on the surface of NK cells

Antibody (μg)	Activity (nmoles fa/min/ml)
0	35 ± 3
1	25 ± 2
2	15 ± 3*
5	9 ± 3*

NK cells were tested for surface LPL activity in the absence or in the presence of different concentrations of anti-LPL antibody, as described in the Materials and Methods. A significant decrease was observed, $*P < 0.01$, $n = 3$, when 2 or 5 μg of anti-LPL antibody was added to the cells.

LPL mRNA expression

cDNA templates prepared by reverse transcription of RNAs extracted from NK and T cells were amplified using primers specific for human LPL (Materials and Methods), and the amounts of LPL amplicons are shown in Fig. 6a. The same amounts mRNA expression in T cells either in the presence or in the absence of IL-2 were used in control amplification reactions in which GADPH-specific primers were employed, and the amounts of GADPH amplicons are shown in Fig. 6b. LPL mRNA expression in T cells and NK cells was normalized to the equal level of GADPH mRNA expression. As shown in Fig. 6c, NK cells expressed LPL mRNA constitutively and treatment of the cells with IL-2 did not cause significant alterations in the expression of LPL mRNA in NK cells. There were no detectable levels of LPL.

Cytotoxicity

As shown in Fig. 7, exposure of NK cells to anti-LPL antibody had a profound effect on the cytotoxic activity of the cells against the K562 tumour cell line. Significant inhibition of NK cell cytotoxic activity was observed at all effector: target (E : T) cell ratios. The inhibition of NK cytotoxic activity by anti-LPL antibody was not due to diminished binding of NK cells to the K562 targets, since the percentage of NK cell binding to K562 cells was not significantly altered upon treatment with anti-LPL (82 ± 5% NK : K562 complexes versus 80 ± 5% complexes following the addition of antibody). The addition of 50 IU

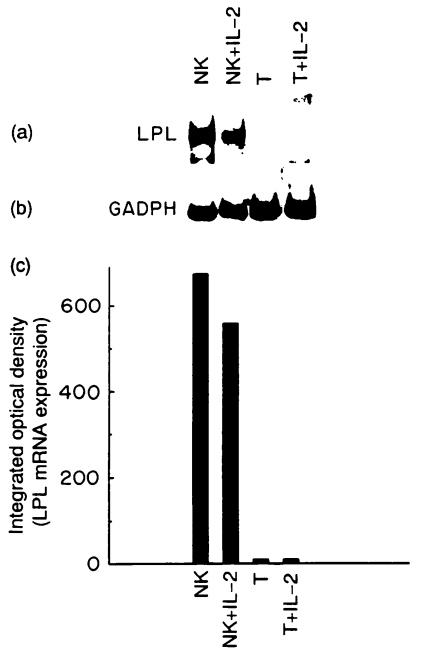

Figure 6. Expression of LPL mRNA. Total RNA was isolated from IL-2-stimulated and unstimulated NK cells, and from IL-2-stimulated and unstimulated T cells, cultured for 18 hr. cDNAs were made using oligo(dT) primers. RT–PCR reactions were performed using specific primers for LPL (a) and specific primers for GADPH (b), as described in the Materials and Methods. Expression of LPL mRNA was quantified by densitometric analysis of the autoradiograms obtained after the electrophoresis of PCR products shown in (a) and (b), and OD values for LPL mRNA expression were normalized to the levels of GADPH mRNA expression in the same experimental sample (c).

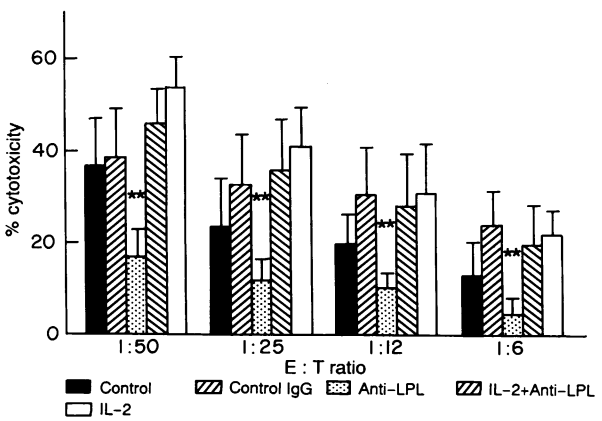

Figure 7. Effect of anti-LPL antibody treatment on the cytotoxic activity of $CD3^-$ $CD16^+$ cells against K562 tumour target cells. Cells were purified as described in the Materials and Methods and were cultured with ^{51}Cr-labelled K562 cells at various effector to target (E:T) ratios, 1:50, 1:25, 1:12, 1:6. Five microlitres (5 μg of the antibody) of either irrelevant or anti-LPL antibodies was added into different wells. IL-2 (50 IU/10^6 cells/ml) was added to cultures of the cells for 18 hr before the assay was performed. The results are expressed as percentage of cytotoxicity based on the formula described in the Materials and Methods.**$P < 0.001$ for E:T ratios of 1:50, 1:25, 1:12 and 1:6; $n = 10$.

Table 3. Effect of anti-CD56, anti-CD11b/CD18 and anti-LPL on NK cell cytotoxicity

Antibody	E:T	% cytotoxicity	% inhibition
None	40:1	40·7 ± 5·2	—
Anti-CD56		39·0 ± 2·5	0
Anti-CD11b/CD18		34·1 ± 0·6	16·2
Anti-LPL		28·3 ± 1·2	30·5
Anti-LPL + anti-CD56		24·6 ± 2·5	40·0
Anti-LPL + anti-CD11b/CD18		24·5 ± 1·9	40·0
None	20:1	34·3 ± 4·5	—
Anti-CD56		35·8 ± 5·8	0
Anti-CD11b/CD18		31·1 ± 1·9	9·0
Anti-LPL		22·3 ± 3·8	35·0
Anti-LPL + anti-CD56		21·4 ± 5·9	38·0
Anti-LPL + anti-CD11b/CD18		20·1 ± 3·2	41·5
None	10:1	28·3 ± 4·5	—
Anti-CD56		29·0 ± 2·5	0
Anti-CD11b/CD18		22·8 ± 2·4	19·4
Anti-LPL		16·7 ± 3·5	41·0
Anti-LPL + anti-CD56		17·5 ± 3·5	38·0
Anti-LPL + anti-CD11b/CD18		13·4 ± 5·0	47·5

NK cell cytotoxicity was assessed by the addition of different antibodies to surface molecules of the cells in the presence or absence of anti-LPL antibody. Samples without antibody (none) were used to monitor the percentage of inhibition obtained with the addition of antibodies. The table represents the mean ± SD of three different experiments.

rhIL-2 reversed the inhibition of NK cytotoxic activity, as evident in the presence of the anti-LPL antibody.

Table 3 shows the effect of the addition of anti-CD56 or anti-MAC-1 (anti-CD11b/CD18) antibodies to the cell cultures. In both cases, anti-LPL antibodies diminished the cytotoxicity of the unstimulated NK cells when added to the treated cells.

DISCUSSION

LPL is a key enzyme in the complex physiology of lipid metabolism. *In vivo* studies have shown the importance of LPL in different lipoprotein disorders, from the hyperchylomicronemia syndrome to atherosclerosis. In the hyperchylomicronemia syndrome, LPL is either absent or dysfunctional,[22] or is catalytically inactive due to the lack of the essential activator, apo CII.[23,24] On the other hand, over-production of LPL by smooth muscle cells and macrophages, or an increase in the amount of LPL in the vascular endothelium, generates lipoprotein remnants that seem to play an important role in the development of atherosclerosis.[5,25,26] Renier *et al.* have shown that an association exists between LPL production by macrophages and genetic predisposition to murine atherosclerosis.[13] The LPL mRNA expression, and the secretion of LPL, was found to be higher in strains of mice identified as being susceptible to atherosclerosis, compared to their resistant counterparts. Thus, the modulation of LPL gene expression may dramatically affect the equilibrium of lipoprotein anabolism and catabolism, leading to various metabolic disorders.

Specifically, the role of LPL in lipoprotein metabolism is to hydrolyse triglycerides from triglyceride-rich lipoproteins, including chylomicrons (QM) and very low-density lipoprotein (VLDL). The fatty acids generated by the action of LPL are adsorbed by the peripheral tissues, either forming an integral part of the cell membrane, or providing indispensable metabolites for signal transduction upon activation of the cell.

Fatty acid metabolites, including those generated by arachidonic acid metabolism, influence immune cell function in a variety of ways, including the regulation of cytokine transcription and secretion[8] and affecting cytotoxic functions.[8,10] A tentative explanation for the synthesis and secretion of LPL by NK cells is that LPL provides the fatty acids essential for NK cell metabolism and activation, crucial for different functions such as cytotoxic activity.

Experiments performed in rats have shown that an intravenous bolus of heparin plus 10% Intralipid®, a clinically used coconut fat emulsion for parenteral nutrition, increases NK cytotoxic activity.[27] These results suggest a link between LPL, lipoprotein metabolism and NK function. Specifically, heparin induces the release of LPL from the capillary endothelium, and macrophages and NK cells provide more LPL to degrade the Intralipid® emulsion and other triglyceride-rich lipoproteins. Augmented levels of fatty acids due to an increase in LPL secretion following intravenous heparin injection may be responsible for the modulation of NK cell functions, especially NK cytotoxicity, as described by Kurzer *et al.*[27] Moreover, heparin alone has been shown to inhibit NK cell cytotoxicity when added to assay systems,[28] although no clear explanation for this phenomenon has been encountered.

Recently, cytokines have been reported to be involved in disorders of lipoprotein metabolism.[29,30] Even though IL-2 has not been shown to be directly involved in disorders of lipid metabolism, *in vivo* studies in terminal cancer patients provide circumstantial evidence that the cytokine may play a role in natural lipoprotein metabolism.[31,32] In one study,[31] intravenous injection of IL-2 induced: (1) total cholesterol reduction [reduction in low-density lipoprotein (LDL) cholesterol by 62%, and in high-density lipoprotein (HDL) cholesterol by 77%]; (2) an increase in the triglyceride/cholesterol ratio by 352%; (3) a reduction in apolipoprotein B, AI and AII levels (26% reduction in Apo B, 55% reduction in Apo AI and 51% reduction in Apo AII levels); (4) an increase in VLDL and generation of two fractions of HDL. In the same study,[31] the authors also showed that the administration of LAK cells attenuated the effects described above and that the changes observed in response to IL-2 were reversible. The changes in lipoprotein profiles described above, the presence of two HDL fractions and a decrease in total cholesterol, seem to implicate an increased LPL activity in the patients treated with IL-2.[1]

In another study,[32] parenteral nutrition was shown to restore depressed NK cell activity in malnourished cancer patients, and the hypoproduction of IL-2 observed in these patients was not the cause of the impairment of NK cell cytotoxicity. The authors concluded that the malnutrition was responsible for the immune system dysfunction and that it was independent of the lack of cytokine synthesis. Although the addition of IL-2 to peripheral blood mononuclear cells (PBMC) isolated from those patients and cultivated *in vitro* rendered an improved lymphocyte activation parallel to an increase in NK activity, the observed effects were not comparable to the efficacy obtained with normal or hypercaloric parenteral nutrition in which lipids are included.[32] This report supports previous conclusions that lipids, especially fatty acids, are essential for immune function, including cytotoxic activity.

Our data, relating the expression of LPL mRNA and secretion of the enzyme by NK cells, suggest that LPL secretion may be fundamental in providing fatty acids essential for metabolism of NK cells and their cytotoxic activity against tumour cells. The inhibition of NK cell cytotoxicity in the presence of anti-LPL antibodies *in vitro* may be explained by the inability of LPL to hydrolyse triglycerides when the antibody is present, due to the fact that anti-LPL inhibits LPL catalytic activity (as shown in Table 2). Thus, it is likely that as soon as the catalytic activity of LPL is depressed, the level of fatty acids decreases and consequently the cytotoxic function of NK cells becomes less efficient.

It has been shown that the cytotoxic activity of NK cells is dramatically affected by a variety of cytokines.[7,8] Interferons, especially, interferon-γ, have been implicated as key activators of NK cytotoxicity by acting either directly on target cells or on NK cells.[7,8] IL-2 has also been shown to induce NK cytotoxicity.[7,8] IL-2 induces an increase of expression of a variety of cell-surface antigens by NK cells and stimulates the release of granules that contain other cytokines, enzymes or proteins responsible for the cytokine activity against target cells.[7,33] These processes require an enormous expenditure of energy, some of which is supplied by lipid metabolism.

Overall, we have shown that NK cells express LPL mRNA, express LPL on their surface and secrete the enzyme, and that the secretion of the enzyme may be dramatically augmented by IL-2. Therefore, our data suggest that LPL may play an important physiological role in regulating NK cell cytotoxic function against tumour targets.

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