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Lipoprotein lipase protects bovine endothelial cells from human NK cytotoxic activity

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Abstract

Human lipoprotein lipase (LPL), in a dose dependent fashion, significantly inhibited spontaneous human natural killer (NK) cells, but not lymphokine-activated killer (LAK) cytotoxic activity against bovine pulmonary endothelial cells. The effect was dependent on endothelial heparan sulfate (HS) sites, since heparitinase reverted it. When HS is added before LPL, NK and LAK cytotoxicity are markedly reduced. Endothelial and NK cell priming, with LPL and HS + LPL, significantly induced CD40 and CD154 expression, respectively. Furthermore, CD40 expression was inversely proportional to lytic units ($R^2 = 0.9$, $P < 0.001$). Treating endothelial cells simultaneously with indomethacin, CD154 fusion protein, and Wortmanin prevented the CD40 effect increasing xenograft rejection. LPL and HS + LPL protect bovine endothelial cells from NK cytotoxicity by inducing CD40, CD154 expression, and secretion of soluble factors. The high, non-modulated expression of adhesion receptors and the low number of HS sites account for the minor effect of CD40 in LAK cytotoxic responses against bovine endothelial cells.

Keywords: Xenograft rejection; Lipoprotein lipase; Endothelial cells; NK cells; Lymphokine-activated killer cells; CD40

1. 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 [1,2]. It has been established that LPL is secreted by the following tissues and cells: adipose (white/brown), heart, mammary gland (lactating), skeletal muscle, adrenal, ovary, thoracic aorta, spleen, small intestine, testis, lung, kidney, brain (hippocampus), neonatal liver, macrophages [1,2], and natural killer (NK) cells [3]. LPL is part of the lipase family of proteins that includes the recently identified endothelial lipase [4]. Despite their high protein homology, LPL and endothelial lipase are functionally different enzymes [5].

The cell surface receptors for LPL in endothelial cells have been proposed to be three proteoglycan proteins containing heparan sulfate of molecular weights 220, 116, and 85 kDa [1,2]. In similar fashion, one heparan sulfate proteoglycan in murine macrophages (76 kDa) and three in the human NK cell membrane (76, 57.2, and 27.2 kDa) are responsible for LPL effects on these cells [6]. LPL binding is thought to be important in the development of atherosclerosis since it generates lipoprotein remnants that are avidly taken up by macrophages [1,2,7–9], and it induces the transcription and secretion of TNFα [10]. The transcriptional activation of TNFα is dependent upon protein kinase C (PKC) activation [11]. This induction of TNFα by LPL may be related to the increased level of this cytokine as it has been reported in the atheroma [9]. Nevertheless, LPL is not only able to enhance directly or indirectly inflammatory responses [1,2,9], but also it induces proliferative responses, modulates cytokine secretion and cytotoxic responses [2,6,8].

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 tumor cells or virally infected cells [12,13]. The activity of NK cells is regulated by a concert of activating and inhibitory receptors and co-stimulatory molecules [13]. NK cells have been shown to

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express and secrete LPL, which modulates NK cytotoxic activity [6]. Even though LPL expression was decreased in IL-2 stimulated NK cells, the blocking effect of LPL on NK lymphokine-activated cell (LAK) cytotoxicity was maintained when LPL was added to the cell culture. The inhibition of LAK cytotoxic response was dependent on the target cell used; U937 cells are more resistant than K562 cells [6]. LPL effect is dependent upon heparan sulfate/LPL binding sites in the target cell membrane [6]. Moreover, cell proteoglycans that bind LPL have been shown to play an important role in plasma lipoprotein uptake [9], an event that has also been shown to modulate NK proliferative and cytotoxic responses [14].

NK cells have also been shown to be important in xenograft rejection [15–18]. In the porcine model, human NK binding to porcine endothelial cells involved receptor–ligand interactions between adhesion molecules [15–18]. Rolling or static adhesion of NK to endothelial cells recruits different adhesion molecules. The most important molecules for this interaction are CD2, CD11a, CD11b, CD18, CD29, CD49d, CD62L, and CD106 [15–18]. However, blocking adhesion receptors alone did not seem to be sufficient to protect in vivo porcine endothelial cells from NK cytotoxic activity [15].

Another set of co-stimulatory molecules that play an important role in the immune response is the CD40–CD154 complex [19]. In the literature, evidences suggesting that CD40 enhanced the NK cytotoxic response [20–22] contrast with reports proposing that signal transduction induced by CD40 is responsible for prolonging cell survival by blocking apoptosis [23–25]. This paradox may be due to the fact that several agonists, including pro-inflammatory cytokines, induce CD40 expression and that CD40–CD154 interactions themselves promote various cell responses: transdifferentiation, oxidative product formation, inflammation, and angiogenesis [19,23–27]. Thus, the conditions by which cells are activated may be responsible for the broad range of physiological responses.

Since LPL modulates cytotoxic responses of human NK cells against tumor cells [6], in the present report we investigated whether this enzyme may modulate NK and LAK xenograft rejection, using as target bovine pulmonary endothelial cells. We also investigated what molecules may affect this interaction.

2. Materials and methods

2.1. Chemicals

Fetal calf serum (FCS), L-glutamine, penicillin–streptomycin, and RPMI 1640 medium were purchased from Invitrogen (Gaithersburg, MD). Human recombinant interleukin 2 (IL-2) was kindly donated by Dr. Craig Reynolds from the Biological Response Modifiers Program, National Cancer Institute (Frederick, MD). Percoll and Ficoll-paque were purchased from Pharmacia LKB (Uppsala, Sweden). $Na^{51}Cr$, was purchased from New England Nuclear (Boston, MA). The fusion protein human CD154-muCD8, azide free, was purchased from Ancell (Bayport, MN). Heparitinase type III (1 U generates 0.1 μmol of saturated uronic acid/h at pH 7.5), and all other reagents were acquired from Sigma Chemical (St. Louis, MO).

2.2. Antibodies

Anti-human: CD16-FITC (FcγRIII receptor), CD49d-PE, and anti-mouse CD106 purchased from Becton–Dickinson (Mountain View, CA). Anti-P selectin and anti-human CD31 were purchased from R&D Systems (Minneapolis, MN). Anti-human: NKH-1 RD_1 (CD56, asialo GM_1), CD2-FITC, CD3, CD3-FITC, CD14-FITC, CD19-FITC, CD62L-RD_1, and CD40 and CD154 (unlabelled and PE labeled antibodies), anti-mouse IgG1 FITC, and anti-mouse IgG1-RD were purchased from Immunotech Beckman-Coulter Immunology (Hialeah, FL). Anti-murine CD40 was purchased from Serotec (Oxford, UK), and anti-von Willebrand factor, anti-CD54, anti-rabbit IgG-FITC, and anti-rabbit IgG-TRITC were purchased from Dako (Carpintería, CA).

Anti-LPL monoclonal and polyclonal antibodies were prepared in our laboratory as described previously [6,28]. The monoclonal is capable of binding LPL monomer and dimmer, it does not cross-react with any other lipase and it inhibits LPL catalytic activity from bovine, human, and mouse origin [28].

2.3. LPL purification

LPL was purified from human post-heparin plasma using Intralipid (Vitrus, Stockholm, Sweden) and two steps of heparin–Sepharose columns, as previously described [6]. Enzyme purity was assessed by polyacrylamide gel electrophoresis and Western blot analysis, respectively [6].

2.4. Determination of endotoxin concentrations

The endotoxin content of all media, LPL and anti-LPL preparations was determined by a quantitative limulus amebocyte lysate assay (Whittaker, Wakersville, MA). The endotoxin content in the LPL preparations and in the media were found to be lower than 0.007 ng/ml.

2.5. Labeling of LPL with FITC: enzyme binding to the endothelial cell surface

LPL was labeled using fluorescein isothiocyanate (FITC), as described in detail elsewhere [6], and adjusted to 1 mg/ml in PBS–0.02% Na azide. LPL–FITC conserved

LPL catalytic activity and anti-LPL retained the same reactivity for LPL–FITC, as determined by ELISA (results not shown).

The expression of membrane bound LPL was monitored by flow cytometry, LPL–FITC was added at different concentrations in PBS–0.02% sodium azide and incubated for 30 min at 37 °C. The cells were then washed twice with PBS and analyzed by flow cytometry. Non-specific binding was assessed by incubating the cells with 10 μg of LPL before adding LPL–FITC. A set of experiments was performed by adding LPL, and incubated for 30 min at 37 °C, and subsequently anti-LPL–FITC, obtaining similar results as previously reported [6].

In another set of experiments, the cells were treated, either before or after LPL–FITC incubation, with 10 U of heparitinase (type III) per million cells, at 37 °C for 30 min, and the bound LPL–FITC was quantified by flow cytometry.

2.6. Bovine endothelial cells

Three cloned bovine endothelial cell lines obtained from bovine pulmonary artery were used. The results for flow cytometry and cytotoxicity were similar with the three cell lines; however, only one of the cloned bovine pulmonary artery endothelial cell lines is represented in the histograms of Figs. 1–3. The endothelial nature of these cells was confirmed by their strong positivity for von Willebrand factor and anti-CD31, as determined by immunofluorescence and by flow cytometry [29]. No constitutive expression of LPL was observed in this cell line and no triglyceride lipase activity was detected (results not shown).

2.7. NK cell purification

Blood samples were taken from normal male healthy donors (age 32 ± 5 years, blood bank of the Central University Hospital). NK cells were purified, as described previously [6], by passage of the non-adherent mixed population of cells through nylon–wool columns and subsequent centrifugation on Percoll gradients [30]. The low density (mostly NK) cells isolated from the Percoll gradients were treated with anti-CD3 monoclonal antibody plus complement in order to deplete $CD3^+$ cells.

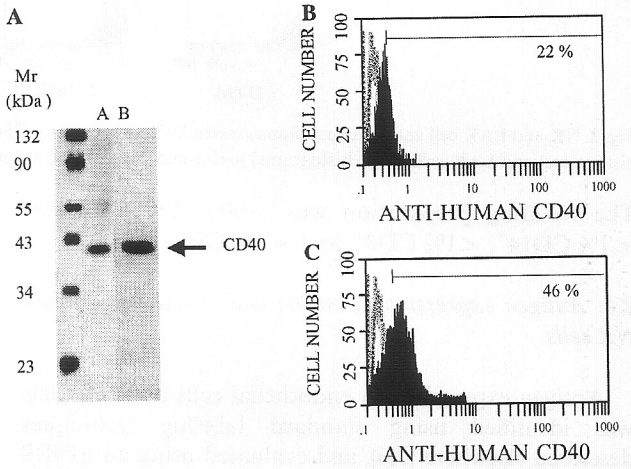

Fig. 2. Expression of CD40 in bovine endothelial cells using two monoclonal antibodies, one against the human and one against the murine molecule. Standard Western blot (A) and flow cytometry analysis (B and C) were performed using LPL activated (1 μg/ml) cells for 4 h, as described in Section 2. In (A) of the figure, the molecular weight of the standard is depicted and the band that corresponds to CD40 is pointed with an arrow. Lane A corresponds to the amount of antigen recognized by the antibody against the human molecule and lane B corresponds to the amount of antigen recognized by the antibody against the murine molecule. A typical flow cytometry result is depicted in the right side of the figure (B and C). In each histogram, background fluorescence is recorded with a dotted line and the dark filled histogram represents CD40 expression. The positiveness was recorded in the top right-hand side of the figure. A marked difference was obtained between the expression recorded among the two antibodies.

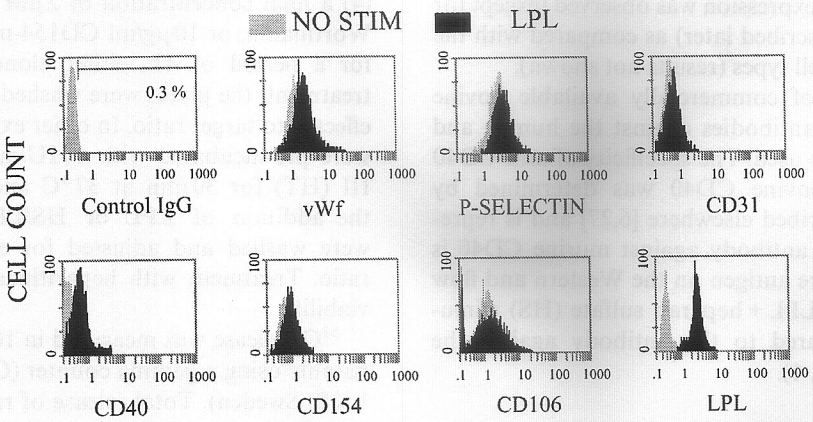

Fig. 1. Antigen expression of endothelial cells stimulated or not with LPL. The figure depicts the flow cytometry analysis of antigen expression in a cloned bovine pulmonary artery endothelial cell-line stimulated or not with LPL, as described in Section 2. The histograms in gray represent the values obtained with non-stimulated cells. The first histogram records the background fluorescence recorded with a control IgG. The results of the flow cytometry analysis are represented in Table 1.

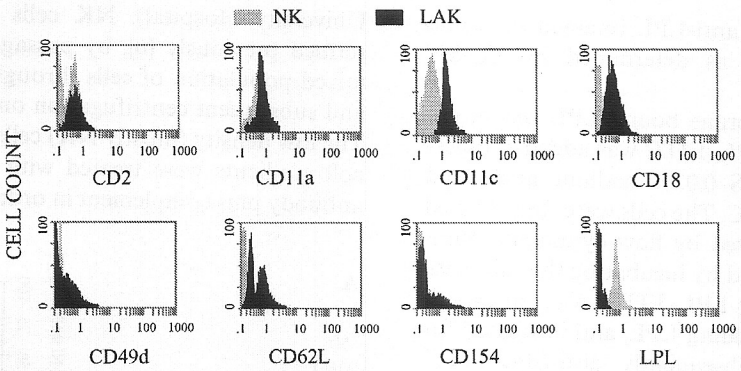

Fig. 3. NK and LAK cell antigen expression assessed by flow cytometry. The figure depicts the flow cytometry analysis of antigen expression in NK (gray histograms) and LAK cells (dark histograms) performed as described in Section 2. The results of the flow cytometry analysis are represented in Table 3.

The resulting preparation was >90% $CD56^+CD16^+$, <1% $CD14^+$, <1% $CD3^+$, and <1% $CD19^+$.

2.8. Antigen expression in bovine endothelial and human NK cells

Antigen expression on endothelial cells and NK cells was identified using standard labeling techniques described elsewhere [3,6], and evaluated using an EPICS ELITE flow cytometer (Coulter, Hialeah, FL). The instrument was calibrated for fluorescence and light scatter using DNA check calibrating beads (Beckman-Coulter). Excitation wavelength was set at 488 nm and all parameters except forward angle light scatter (FALS) were assessed using a logarithmic scale. The following antigens were assessed by flow cytometry: von Willebrand factor, P-selectin, CD2, CD11a, CD11c, CD18, CD31, CD40, CD49d, CD54, CD62L, CD106, CD154, and LPL. In order to ascertain the LPL effect on the bovine endothelial cell line, NK and LAK cells were stimulated with a single concentration of LPL (1 μg/ml) and antigen expression was analyzed after 4 h of stimulation. No major effect of heparan sulfate, alone or combined with LPL, on antigen expression was observed (except for CD40 and CD154 described later) as compared with the control, in the three cell types (results not shown).

Due to the lack of commercially available bovine CD40, two different antibodies against the human and murine molecule were used. The specificity of anti-CD40 antibodies against bovine CD40 was determined by Western blot as described elsewhere [6,27] and is represented in Fig. 2. The antibody against murine CD40 is able to recognize more antigen on the Western and flow cytometry assays of LPL + heparan sulfate (HS) stimulated cells as compared to the antibody against the human molecule (Fig. 1).

2.9. NK cytotoxic assay

A short term (4 h) radiolabeled release assay using ^{51}Cr labeled endothelial cells as targets was performed as described previously for K562 cells [6,30]. Briefly, 1×10^6 endothelial cells were gently scraped from petri dishes, labeled with 30 μCi of $Na^{51}Cr$ (200–500 μCi/mmol) for 1 h at 37 °C; then the 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 labeled endothelial cells was seeded in gelatin coated 96-well U-bottomed microtest plates (Falcon Plastics, USA) for a minimum period of 15 min before the addition of the defined stimuli. After the stimulus, the plates were washed with PBS and, finally, 0.2 ml of effector cells at different effector-to-target cell ratios (40:1, 20:1, and 10:1) were added to each well. In each assay the tests were performed in triplicate.

Generally, endothelial cells were previously incubated with: (1) different concentrations of LPL for a minimum of 30 min up to 24 h at 37 °C (the effect of LPL is independent of the time of incubation); (2) a fixed concentration of heparan sulfate 1 μg/100 μl for 30 min, prior the addition of LPL as described in (1); (3) different concentrations of LPL as described before, and then a fixed concentration of heparan sulfate 1 μg/100 μl for 30 min; (4) a final concentration of 2 μM Indomethacin, 10 nM Wortmanin, or 10 μg/ml CD154-muCD8 fusion protein, for a period of 4 h, either alone or combined. After treatment, the plates were washed and adjusted for each effector to target ratio. In other experiments, 1×10^6 cells were pre-incubated with 10 IU/ml of heparitinase type III (HT) for 30 min at 37 °C and then washed before the addition of LPL or HS/LPL. Finally, the cells were washed and adjusted for each effector to target ratio. Treatment with heparitinase did not modify cell viability.

^{51}Cr release was measured in 100 μl samples of supernatants using a gamma counter (Compugamma, Wallac, LKB, Sweden). Total release of radioactivity was determined by counting the radioactivity released from 5×10^3 labeled endothelial cells treated with 1% Triton X-100. The percent of lysis was calculated by the following formula:

$$\% \text{ Specific lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}}.$$

Lytic units (LU) were defined as the number of effector cells per 10^7 cells which causes 20% of lysis of 5×10^3 target cells [12].

2.10. Determination of LAK activity

Purified NK cells were stimulated for 72 h with 1000 IU of IL-2/10^6 cells to induce lymphokine activated killer activity (LAK) as previously described [6,12]. These cells were tested for cytotoxicity against the bovine endothelial cell line in a similar fashion as described above for NK cytotoxicity assays.

2.11. Statistical analysis

The paired and unpaired Student's t test were used as appropriate. A P value of <0.05 was considered to be significant. To determine both the correlation coefficients and the P values, Spearman and Pearson coefficients were employed for statistical analysis of CD40 and lytic units. A multiple regression analysis of all conditions assessed for CD40 expression and lytic units was also performed. One-way ANOVA analysis was used to assess the effect of COX, PI3K, and fpCD154 on LAK treated vs. non-treated cells.

3. Results

3.1. General characteristic of pulmonary artery endothelial cells

Fig. 1 illustrates a typical flow cytometry assessment of antigen expression on one of the cloned endothelial cells, either non-treated or stimulated with 1 μg/ml of LPL for 4 h. Antigen expression was similar in three stimulated vs. non-stimulated cell lines except for CD40 which was significantly increased in LPL treated cells (Table 1). Antigen expression was similar in the other three endothelial cell lines derived from bovine pulmonary artery. Fig. 2 represents the increased amount of CD40, determined with the antibody against the murine and human molecule by Western blot (A) and flow cytometry (B and C) analyses.

3.2. Antigen expression of NK and LAK cells

Fig. 3 illustrates the results obtained from a typical flow cytometry assessment of NK and LAK antigen expression. Table 2 presents the expression of the different antigens assessed. Major differences were observed between the two cell populations. The most significant differences were recorded in cell positiveness. Antigens that were poorly expressed in NK cells, CD49d, CD62, and CD154, were increased in LAK cells ($P < 0.001$). Other antigens that may play a role in cell binding such as CD11a, CD11c, and CD18 were also significantly enhanced ($P < 0.001$). On the contrary, LPL expression was almost absent in LAK cells as compared to NK cells ($P < 0.001$). The expression of CD2 positive cells, but not its density, was significantly increased ($P < 0.001$) in LAK cells.

In a set of experiments, NK and LAK cells were stimulated with LPL (1 μg/ml) for 4 h, as described for endothelial cells. The only major difference observed was a significant increase in CD154 expression in NK cells (from 7.3 ± 2.5 to $19.5 \pm 6.8\%$, $P < 0.01$, $n = 5$), but not in LAK cells (19.8 ± 4.8 vs. $26.5 \pm 8.9\%$). The expression of the other antigens was very similar. Moreover, the addition of up to 10 μg/ml of heparan sulfate before LPL treatment did not further enhance CD154 expression in NK and LAK cells (19.5 ± 6.8 vs. $23.9 \pm 5.8\%$ for NK and 26.5 ± 8.9 vs. $35.6 \pm 14.5\%$ for LAK cells). Treatment with HS or HT did not significantly affect CD154 expression on cell membrane (results not shown).

Table 1
Antigen expression of non-stimulated and LPL (1 μg/ml) stimulated endothelial cells

Antigen	Control		LPL	
	% Positive cells	MFI	% Positive cells	MFI
VWF	99.5 ± 1.5	1.8 ± 0.3	96.8 ± 3.6	2.4 ± 0.6
P-selectin	99.1 ± 0.8	3.4 ± 0.4	95.2 ± 4.0	3.9 ± 0.9
CD31	70.5 ± 6.2	0.8 ± 0.2	82.8 ± 6.3	1.1 ± 0.4
CD40	12.7 ± 2.1	0.7 ± 0.2	$49.0 \pm 4.5^{*}$	$1.4 \pm 0.3^{*}$
CD54	78.5 ± 7.6	1.2 ± 0.4	86.2 ± 9.5	1.4 ± 0.5
CD106	64.2 ± 3.9	2.2 ± 0.6	67.5 ± 9.8	2.5 ± 0.6
LPL	0.5 ± 0.5	0.3 ± 0.2	$76.5 \pm 11.8^{**}$	$3.8 \pm 1.8^{**}$

Antigen expression of non-stimulated and stimulated LPL (1 μg/ml) endothelial cells from three different cloned cell lines derived from bovine pulmonary artery. Endothelial cells were labeled as described in Section 2 for the different antigens analyzed. The numbers in the table represent meana ± SD of six different assays per cell line, performed in parallel. The percentage of positive cells represents the absolute number after subtracting background fluorescence assessed by a control labeled IgG; MFI corresponds to the mean channel fluorescence intensity in logarithmic units. Significance, using a paired Student's t test, was recorded as compared to non-treated control cells $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 2
Antigen expression on NK and LAK cells

Antigen	NK cells		LAK cells	
	% Positiveness	MFI	% Positiveness	MFI
CD2	62.5 ± 8.5	0.5 ± 0.1	89.5 ± 8.5***	0.8 ± 0.3
CD11a	77.2 ± 2.8	1.2 ± 0.4	95.5 ± 5.5***	2.2 ± 0.6*
CD11c	69.1 ± 4.8	1.1 ± 0.3	93.5 ± 4.5***	2.5 ± 0.5***
CD18	25.9 ± 2.9	0.7 ± 0.3	48.5 ± 8.7***	1.3 ± 0.3**
CD49d	5.5 ± 3.5	0.5 ± 0.2	39.9 ± 3.9***	1.1 ± 0.4*
CD62L	1.8 ± 0.3	0.3 ± 0.2	68.5 ± 5.1***	0.7 ± 0.3*
CD154	7.3 ± 2.5	0.4 ± 0.1	19.8 ± 4.8***	2.5 ± 0.3***
LPL	45.5 ± 9.5	0.9 ± 0.3	3.5 ± 1.7***	0.3 ± 0.1***

Antigen expression on NK and LAK cells. Antigen expression was assessed on purified NK and LAK cells from six different normal male controls (age 32 ± 5 years). The LAK cells generated are from the same controls. The results are expressed as means ± SD of the antigens recorded for each individual. Significance, using a paired Student's t test, was recorded as compared to non-treated control cells, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.3. Binding of LPL on endothelial cell surface

LPL binding on the endothelial cell surface was performed by using LPL–FITC. Endothelial cells avidly incorporated LPL–FITC on the cell surface (Fig. 4, CTR), an effect that was significantly ($P < 0.01$) blocked by treating cells with heparitinase (Fig. 4, HT) and that could be partially restored by adding 1 μg/ml heparan sulfate (Fig. 4, HS + HT). The addition of heparan sulfate prior to LPL significantly ($P < 0.05$) increased LPL–FITC binding to the cell membrane by approximately 25% (Fig. 4, HS).

Binding of LPL–FITC to NK cells and other cells used as the target has previously been reported [6]. The target cells K562 and U937 bind more LPL than monocytes and NK cells, no LPL binding was observed in T lymphocytes [6].

3.4. Cytotoxic activity of NK and LAK cells

Fig. 5A illustrates the effect of LPL on NK cell cytotoxicity against bovine endothelial cells. This effect was observed when purified NK cells or endothelial cells were incubated with LPL prior to the mixture. Addition of heparan sulfate prior to LPL stimulation (HS–LPL), but not after LPL (LPL–HS) further decreased NK cytotoxicity ($P < 0.01$). No direct effect was observed when HS (1 μg/ml) was added alone to the cells (results not shown). Treatment with heparitinase previous to the addition of LPL (HT–LPL) reverted the effect of LPL

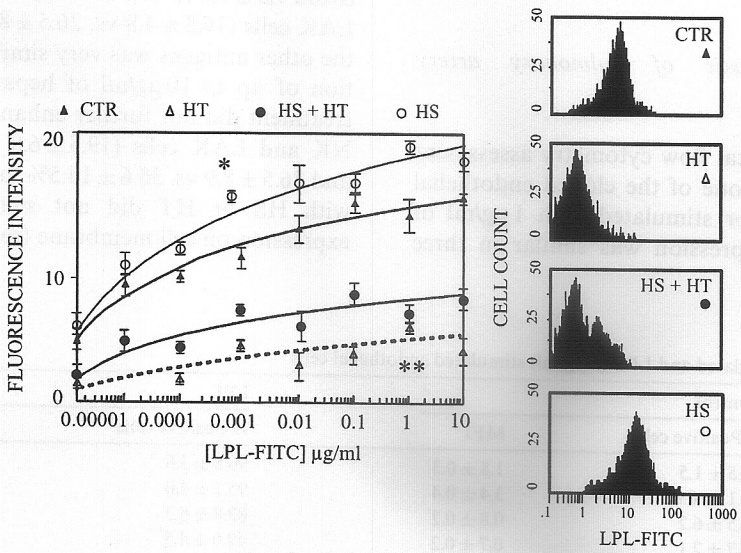

Fig. 4. Membrane binding of LPL–FITC. LPL–FITC binding was performed as described in Section 2. The figure on the left represents the mean channel fluorescence intensity, recorded in logarithmic units, of different concentrations of LPL–FITC binding on cell membrane. Each point represents the mean ± SD of six different assays. The four histograms on the right represent typical histograms for the different treatments reported, recorded at the maximum (10 μg) of LPL–FITC added to the cells. The first histogram represents LPL only (CTR), the second histogram represents the heparitinase (HT) effect, the third histogram represents the effect of cells treated with heparitinase and subsequently with heparan sulfate (HT-HS) (1 μg/ml) and, finally, the fourth histogram represents HS (1 μg/ml) treatment prior to the addition of LPL–FITC. Significant differences are recorded between treatments. Treatment with HT significantly decreased ($^{**}P < 0.01$) LPL binding as compared to control and to heparan sulfate treatment. Finally, heparan sulfate incubation prior to LPL–FITC significantly ($^{*}P < 0.05$) increases LPL–FITC binding.

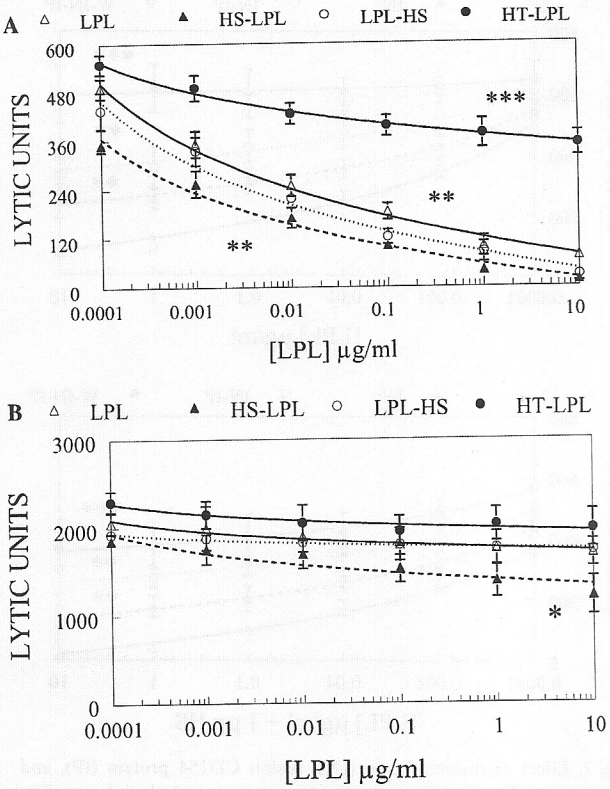

Fig. 5. Effect of LPL, HS, and HT on NK and LAK cytotoxic response against bovine endothelial cells. The experiments were performed and lytic units were calculated as described in Section 2. The results represent the mean ± SD of six different assays with all the conditions assayed in parallel. The figure represents the effect of LPL and the other compounds on the cytotoxic response represented by lytic units vs. LPL concentration (as described in Section 2); (A) NK spontaneous cytotoxic response and (B) LAK cytotoxic response. A marked reduction of NK cytotoxic response was recorded with LPL alone, LPL plus HS either added before (HS–LPL) or after LPL (LPL–HS). Treatment with HT significantly ($^{***}P < 0.001$) increased the cytotoxic response of NK cells. Significant differences were also recorded when HS + LPL was compared to LPL ($^{**}P < 0.01$). In (B), the only significant effect recorded corresponds to HS–LPL ($^{*}P < 0.05$) as compared to LPL and HT, at high LPL concentrations. Please note that the *Y* axis scale is fivefold higher in (B) as compared to (A) of the figure.

($P < 0.001$). The antibody against LPL (10 μg/ml) was able to inhibit the effect of LPL up to 1 μg/ml (505 ± 95 vs. 100 ± 25 lytic units, $n = 6$, $P < 0.001$).

In Fig. 5B, the effect of LPL and heparan sulfate on LAK cytotoxicity is depicted. The combination of HS 1 μg + LPL significantly ($P < 0.05$) decreased the cytotoxicity of LAK cells against endothelial cells, an effect that is significantly less ($P < 0.01$ when absolute differences were compared) than the one reported in Fig. 5A. Nevertheless, when endothelial cells were treated with 10 μg of HS prior to stimulation with 10 μg/ml LPL, a marked and significant reduction of LAK cytotoxicity was observed (1380 ± 185 vs. 420 ± 25 $n = 6$, $P < 0.001$). Contrasting with the results depicted in the above figure (A), no major effect of LPL alone and LPL + HS was observed. Addition of anti-LPL (10 μg/ml) to the culture stimulated with HS + LPL did significantly modify the cytotoxicity of LAK cells (1895 ± 390 vs. 1380 ± 185, $n = 6$, $P < 0.01$). HS alone, in concentrations ranging from 0.1 to 10 μg/ml, had no effect on LAK cytotoxic response.

The effect of LPL on NK and LAK cytotoxic responses was previously reported [6]. LPL affects both NK and LAK cytotoxicity and it was dependent on the target used [6].

3.5. Expression of CD40 on endothelial cells

LPL and LPL + HS, added either before or after LPL, induced a significant ($P < 0.01$, at 1 μg of LPL/ml) increase in CD40 expression on endothelial cells (Fig. 6A). This increase was not observed when endothelial cells were treated with heparitinase, in which case, neither LPL nor HS were able to significantly increase CD40 expression (results not shown). Moreover, when anti-LPL (10 μg/ml) was added prior to LPL stimulation (1 μg/ml) it was able to revert the effect of the enzyme (14.2 ± 6.3 vs. 38.5 ± 3.5, $n = 6$, $P < 0.005$).

A negative correlation was observed, independently of the conditions assayed, among CD40 expression and lytic units ($R^2 = 0.9$, $P < 0.001$, $n = 72$). Fig. 6B represents the different conditions assayed. Significant differences were observed between the different treatments: $P < 0.001$ when LPL was compared to HS–LPL and $P < 0.005$ when LPL was compared to LPL–HS and HT–LPL treatments.

3.6. Role of cyclooxygenase and phosphatidylinositol 3 kinase inhibitors in NK and LAK cytotoxicity against bovine endothelial cells

To assess the possible mechanism by which LPL induces endothelial cell resistance to NK and LAK cytotoxicity, three sets of experiments were designed. One in which indomethacin (2 μM) was used to inhibit cyclooxygenase (COX) activity, one in which Wortmanin was used to inhibit phosphatidylinositol 3 kinase (PI3K) activity, and one in which CD40–CD154 interaction was blocked by using the fusion protein CD154-muCD8.

Inhibition of COX products induced an increase in NK cytotoxic activity against endothelial cells; however, this increase although significant ($P < 0.05$), was not able to restore NK cytotoxic responses to the levels reported in the absence of LPL (Fig. 7A) or HS + LPL (Fig. 7B). CD40 involvement on endothelial cell survival was further analyzed using the CD154-muCD8 fusion protein (fP). The addition of the fP had a similar effect to that of indomethacin (IN) alone (results not shown); nevertheless, the combination of fP and IN significantly ($P < 0.01$) enhanced, in both LPL and HS–LPL, the cytotoxic response of NK cells. When endothelial cells

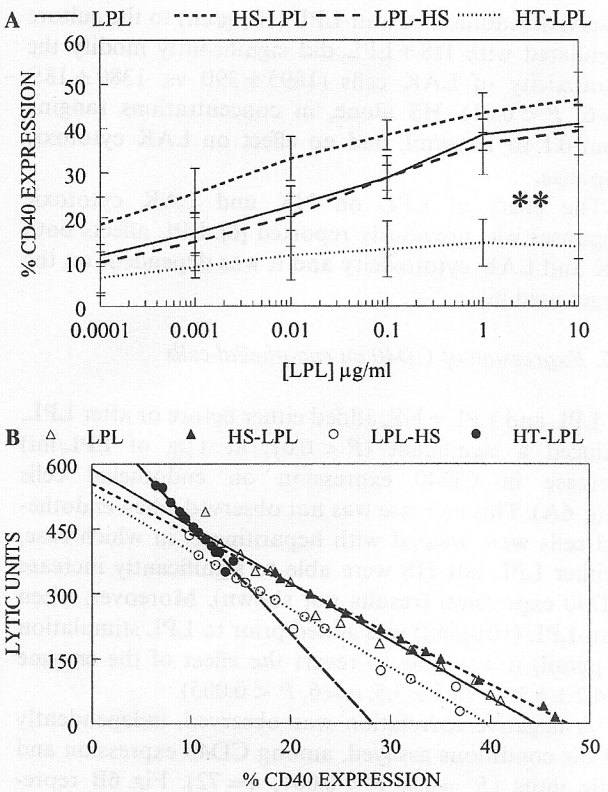

Fig. 6. Effect of LPL, HS, and HT on CD40 expression on endothelial cells. Regression analysis between lytic units and CD40 expression. (A) The effect of different concentrations of LPL, either alone or in combination with HS and HT treated cells, on CD40 expression recorded by flow cytometry as described in Section 2. A significant difference ($^{**}P < 0.01$) was recorded when LPL or HS–LPL or LPL–HS treated cells were compared to HT treated cells. In (B), the different regressions calculated for the diverse treatments assessed show, unequivocally, a negative correlation between lytic units and CD40 expression. The specific mathematical equations are: for LPL $y = 586.3 - 13x$ ($r^2 = 0.95$, $n = 18$), for HS–LPL $y = 555.1 - 11.6x$ ($r^2 = 0.95$, $n = 18$, $P < 0.001$ as compared to LPL alone), for LPL–HS $y = 534.1 - 13.5x$ ($r^2 = 0.96$, $n = 18$), $P < 0.05$ as compared to LPL alone, and $P < 0.001$ as compared to HS–LPL), for HT–LPL $y = 709.26 - 29.7x$ ($r^2 = 0.96$, $n = 18$, $P < 0.005$ as compared to LPL alone, $P < 0.001$ as compared to HS–LPL, and $P < 0.01$ as compared to LPL–HS).

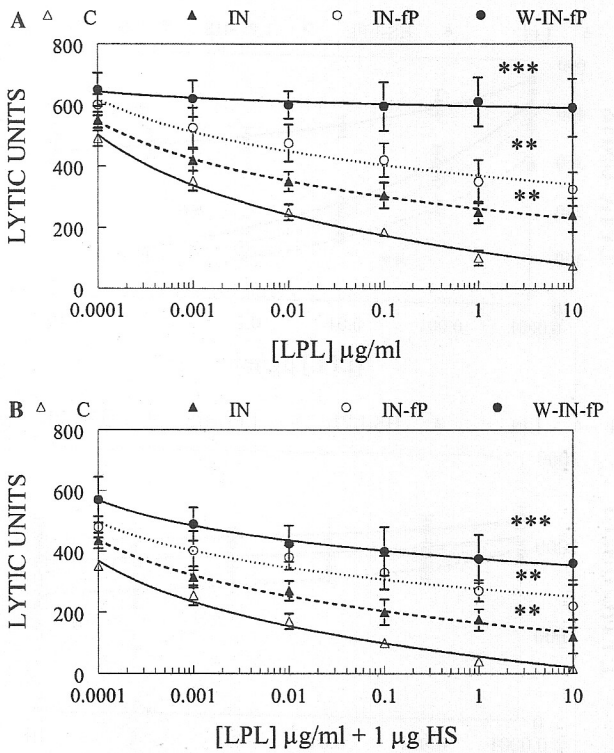

Fig. 7. Effect of indomethacin (IN), fusion CD154 protein (fP), and Wortmanin (W) on NK cytotoxic responses to endothelial cells. The figure represents the effect of the different inhibitors on cells stimulated either with LPL (A) or HS + LPL (B) on spontaneous NK cytotoxic response. The values represent means ± SD of six different experiments. The letter C represents control without inhibitor, the triangles represent indomethacin (IN) treated cells, the clear circles represent the mixture of IN and fusion protein CD154-muCD8 (fP) and, finally, the filled circles represent the three inhibitors simultaneously. Significant differences were recorded among treatment as compared to the control, $^{**}P < 0.01$, $^{***}P < 0.001$.

were incubated with Wortmanin alone (W) prior to the cytotoxicity assay, the outcome was similar to the one observed with the other inhibitors (results not shown). The combination of W with any of the two inhibitors IN or fP resulted in similar effects on cytotoxic responses (results not shown). On the other hand, a marked significant difference ($P < 0.001$) was observed when the three inhibitors were used in the presence of LPL (Fig. 7A) and HS + LPL (Fig. 7B).

Despite the fact that there is an increase in NK cytotoxic responses in the presence of two and three inhibitors, significant differences were observed when the cells were treated with HS + LPL as compared to LPL alone ($P < 0.05$ for two: IN-fP, and three: W-IN-fP inhibitors). No significant differences were recorded when the results using one inhibitor (either IN, fP, or W) were compared.

LAK activity is represented in Table 3. In similar a fashion as reported for NK cells, the addition of the three inhibitors at the same time significantly enhanced the LAK cytotoxic response in cells treated with LPL or LPL + HS. Interestingly, the effect of IN and IN-fP were significant only in the HS + LPL treated cells (1 and 10 μg). There were significant differences ($P < 0.001$ by ANOVA) when the three groups were compared in the absence of inhibitors. Significance ($P < 0.005$) was also observed when cells were treated with one inhibitor (IN, Table 3). No significance was observed when the results with the two or three inhibitors were compared.

4. Discussion

In xenograft rejection, NK cells play an important role; consequently, modulating NK cytotoxic activity is

Table 3
Effect of different inhibitors on LAK cytotoxic responses

Inhibitor	Lytic units [LPL] = 1 μg/ml	P	Lytic units [HS] = 1 μg/ml + [LPL] = 1 μg/ml.	P	Lytic units [HS] = 10 μg/ml + [LPL] = 10 μg/ml	P
None	1750 ± 259		1380 ± 185		420 ± 15	
IN	1995 ± 255	0.17	1785 ± 250	0.02	1260 ± 390	<0.001
IN + fP	1845 ± 315	0.63	1950 ± 350	0.01	1450 ± 520	<0.001
IN + fP + W	2275 ± 385	0.03	2325 ± 345	<0.001	1915 ± 320	<0.001

Effect of different inhibitors on LAK cytotoxic responses on endothelial cells. The effect of the different inhibitors on LAK cytotoxicity was assessed as described in Section 2. The values represent means ± SD of six different assays performed in parallel. Significance, using a paired Student's *t* test, was recorded as compared to non-treated control cells (specified in the table). The one-way ANOVA analysis between the three groups in the absence of inhibitors was significant $P < 0.001$ and in the presence of IN was also significant $P < 0.05$.

a major task [15–18]. Blocking several adhesion receptors have resulted only in a partial inhibition of NK–endothelial cell interactions [15] and, therefore, a search to encounter specific receptors to regulate this interaction are crucial in xenotransplant issues. LPL has been shown to play an important role in promoting the NK proliferative response and decreasing the cytotoxic response against tumor cell lines [6,8]. This enzyme binds to heparan sulfate proteoglycans, it is expressed but not produced in endothelial cells of major arteries, and its function and enzymatic activity differs from the recently cloned endothelial lipase [1,2,4,5]. A tentative hypothesis is that LPL modulates NK cytotoxic activity against undamaged endothelial cells by binding to its heparan sulfate receptors, which in consequence modulate receptors in target cells making them less susceptible to NK and LAK cell cytotoxicity.

Several factors affect NK–endothelial cell interactions: (1) adhesion antigens, expressed by endothelial cells, that are essential for cell recognition, binding, and rolling; (2) the expression of antigens involved in NK cell activation inhibition and/or; (3) production of soluble factors, like cyclooxygenase products and cytokines. Therefore, stimulated and non-stimulated cells respond differently. LAK cells seem to be more aggressive to xenograft rejection and tumor elimination due to the marked increase in adhesion and activating receptors as compared to fresh NK cells. LPL is able to modulate NK and LAK cytotoxic response against endothelial cells, probably through heparan sulfate rich receptors, which seem to be lower on the LAK cell membrane [6,8], and it may be the reason why only when high amounts of HS are added prior to LPL there is a decrease in LAK cytotoxic response against endothelial cells. On the contrary, LPL alone, without previous treatment with HS, affects both spontaneous and stimulated NK cytotoxic responses to K562 and U937 cell lines [6,8]. The inhibition shown depends on the binding of LPL to the target cell used, the number of sites available and the signal transduction generated by the enzyme (an increase in PKC translocation was described) [6]. Inhibition of PKC has been documented to be efficient in inhibiting NK cytotoxic responses to tumor cells [12]. In xenograft rejection, PKC inhibition seems to be less efficient than blocking adhesion receptors involved in NK–endothelial cell binding [12,15–18]. Thus, tumor cell survival may be dependent on factors that differ from those observed with endothelial cells.

Endothelial cells have been shown to survive apoptosis by CD40-receptor-triggered signal transduction upon CD154 binding [24]. The interaction CD40–CD154 in parallel stimulates angiogenesis [25]. Thus, it is not surprising that LPL, by inducing CD40 on endothelial cells and CD154 on NK cells, increases endothelial cell survival. The main difference between these conclusions as compared to the ones that propose an enhanced NK cytotoxicity upon CD40 expression [20–22], is the fact that endothelial cells treated with LPL or HS–LPL did not modify antigen expression (except CD40) as compared to controls, an indirect indication of the lack of a pro-inflammatory activation through this receptor [31]. Pro-inflammatory cytokines and oxidative products had been shown to increase the expression and density of adhesion receptors on endothelial cells [15,31], and proteoglycans and glycosaminoglycans appear to block this effect [15,16,32]. Dextran sulfate (MW 5000) seemed to modulate complement and NK cytotoxic responses through proteoglycan binding sites on porcine endothelial cells [16]. The incubation of endothelial cells with dextran sulfate prevented the cytotoxic effect of TNF involved in xenograft rejection [16]. These results, along of those of Lider et al. [32], further support the hypothesis that, in a normal endothelium, proteoglycans involved in LPL binding are crucial for cell survival, and that chronic activation with IL-2 and pro-inflammatory cytokines down-modulate these receptors and consequently affect cell endurance.

CD40 seems also to have been responsible for some of the soluble products and signal transduction molecules which promote endothelial cell survival. Deregibus et al. [24] have shown that CD40 stimulation enhances Akt phosphorylation through PI3K, which prolongs endothelial cell survival in serum free media, and Reinders et al. [25] have shown the proangiogenic role of CD40 in an engraftment model of human skin in SCID mice. These results contrast those reported with human HUVEC cells, in which there was an induction of COX-2 expression and consequently IL-6 synthesis in CD40

stimulated endothelial cells, resembling a pro inflammatory response [26]. Interestingly, the notorious effect on COX-2 expression was observed after stimulating endothelial cells with high concentrations of CD154, either recombinant or using the Jurkat cell line D1.1 that constitutively express CD154 (85–90% expression). In the experiments reported here, with a moderate expression of CD154 on LPL stimulated NK cells and LAK cells, indomethacin was able to increase the NK cytotoxic response, but not to the extent expected taking into account the aforementioned report [26,31]. Only when the three inhibitors were used (Wortmanin, CD40 blockage, and indomethacin) was there a significant increase in the cytotoxic response, which suggests that PI3K, as described previously probably through Akt activation, enhances endothelial cell survival, and even though PKC activation and phospholipase activity are activated in endothelial cells when binding occurs, other factors promoted by CD40–CD154 (indirectly monitored through fpCD154) are also important modulators of cell survival in a similar fashion as described in B cells [23].

The low effect of LPL, and consequently of the CD40–CD154 interaction in LAK cytotoxic response against endothelial cells, represents the other side of the coin. Since HS + LPL was able to modulate LAK cytotoxic response against endothelial cells, questions may arise on the possibility that endothelial cell survival upon HS + LPL stimulation may be dependent on the blockage of adhesion receptors. No changes in cell positiveness and density of the receptors was observed in our experiments. On the other hand, the three inhibitors used simultaneously on HS + LPL treated LAK cells suggests that CD40–CD154 still plays an important role in cell survival, although it seems less relevant than the adhesion and activating receptors overexpressed in LAK cells. Although inhibition of PKC with staurosporine is markedly effective in reducing the NK cytotoxic response against tumor cells [12,13], its effect on endothelial cells is markedly less than any one of the three inhibitors used here (results not shown). In addition, as reported previously [6], blocking CD44 did not affect tumor or xenograft rejection (results not shown). These results lead us to propose that the mechanisms involved in endothelial cell survival differ from those involved in tumor resistance even though similar receptors may play important roles in both responses.

Endothelial cell survival has been a major issue in different pathologies, from pulmonary hypertension to atherosclerosis, and CD40 plays an important role in these pathologies [31]. However, the impact of CD40–CD154 interaction on cell responses seems to be dependent upon co-stimulatory signals involved in either normal physiological responses or inflammatory reactions. In this regard, CD40 may have a protective effect on xenograft rejection, considering the results exposed in this report. A tentative conclusion may envision that LPL and heparan sulfate proteoglycans, along with the CD40–CD154 interaction, modulate NK and LAK cytotoxic responses, which in turn are responsible for different issues from normal tissue modeling, rejection, and pathology. Future studies should ascertain the importance of CD40 in in vivo xenograft cell survival.

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