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Lysophosphatidylcholine Induces Vascular Smooth Muscle Cell Membrane Vesiculation: Potential Role in Atherosclerosis through Caveolin-1 Regulation

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Abstract

Background: Microparticles are present in low concentrations in normal plasma, but increase in several diseases including atherosclerosis, mainly through membrane activation processes or during apoptosis. The number of circulating microparticles has been proposed as a marker of subclinical atherosclerosis, but their potential role in its progression has not been fully characterized.

Methods: Microparticles released by vascular smooth muscle cells treated with lysophosphatidylcholine were isolated and their composition characterized by proteomic techniques. Some hits were confirmed by western blot in this and other cell lines involved in atherosclerosis. Platelet poor plasma was obtained from patients suffering from carotid stenosis (>70%) by venipuncture and subsequential centrifugation. Platelet poor plasma was analyzed by flow cytometry and the microparticle fraction was analyzed by SDS-PAGE.

Results: Among the proteins identified as components of vascular smooth muscle cells-derived microparticles, caveolin-1 was confirmed by western blot. Caveolin-1 is detected in endothelial cell and fibroblast-derived microparticles but not in those released by other cell types involved in atherosclerosis progression. Caveolin-1 was detected in the microparticle fraction in 73.33% of patients compared to 15.38% of controls. The presence of Caveolin-1 did not correlate with the counts of circulating endothelial-derived microparticles.

Conclusions: Pro-atherogenic stimuli induce the release of microparticles containing Caveolin-1 by vascular smooth muscle cells and endothelial cells. Caveolin-1 is detected in microparticles isolated from plasma obtained from patients suffering from atherosclerosis. Due to its role in the regulation of smooth muscle cell proliferation we hypothesize that the release of microparticles in response to pro-atherogenic stimuli may be involved in the progression of the disease through the regulation of caveolin-1 levels in vascular smooth muscle cells.

Keywords: Microparticles; Atherosclerosis; Biomarkers; Smooth muscle cells; Endothelial cells

Abbreviations: AF: Atrial Fibrillation; AHT: Hypertension; Amu: Atomic Mass Units; AnxV: Annexin V; APC: Allophycocyanin; Cav-1: Caveolin-1; DL: Dyslipemia; DM: Diabetes Mellitus; DTT: Dithiothreitol; EC: Endothelial Cells; ECL: Enhanced Chemiluminescence; EMPs: Endothelial Cells-Derived Microparticles; eNOS: Endothelial Nitric Oxide Synthase; ERM: Ezrin Radixin Moesin Proteins; FBS: Fetal Bovine Serum; FITC: Fluorescein Isothiocyanate; FMO: Fluorescence Minus One; hBMEC: Human Bone Marrow-Derived Endothelial Cells; ICAM-1: Intercellular Adhesion Molecule-1; IMT: Intima-Media Thickness; LDL: Low Density Lipoproteins; LMW: Low Molecular Weight; lysoPC: Lysophosphatidylcholine; mAbs: Monoclonal Antibodies; MPs: Microparticles; oxLDL: Oxidized Low Density Lipoprotein; PAI-1: Plasminogen Activator Inhibitor-1; PE: Phycoerythrin; PMPs: Platelet-derived Microparticles; PPP: Platelet Poor Plasma; SEM: Standard Error of the Mean; SMA: Smooth Muscle Actin; SMC: Smooth Muscle Cell; TF: Tissue Factor; TNFα: Tumor Necrosis Factor Alpha; VCAM-1: Vascular Cell Adhesion Molecule-1; VSMC: Vascular Smooth Muscle Cells

Introduction

Atherosclerosis is a multicellular chronic inflammatory disease characterized by inflammation, lipid deposition, endothelial dysfunction and smooth muscle cell (SMC) proliferation [1]. The process of inflammation contributes to the progression of atherosclerosis. Several stimuli, such as oxidized LDL (oxLDL), tumor necrosis factor alpha (TNFα) or free radicals due to smoking, induce a pro-inflammatory phenotype of the endothelium, expressing adhesion molecules on its surface and cooperating in disease progression. Moreover, apoptosis plays a key role in atherogenesis and especially during plaque destabilization. In early stages, apoptotic vascular smooth muscle cells (VSMCs) or macrophages may delay atherosclerosis progression.

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But once the plaque is formed, VSMC apoptosis at the neointima contributes to the weakening of the plaque, EC apoptosis implies endothelium denudation, and these processes may lead to thrombosis and acute ischemic events [1].

Since circulating microvesicles have been detected in several diseases associated with inflammation, they may constitute a biomarker of the progression of atherosclerosis [2]. These are small membrane vesicles released by different cell types, including platelets, monocytes, and endothelial cells (EC) [2,3]. Microvesicles may be classified according to their size mainly into two groups: exosomes (less than 100 nm diameter) and microparticles (MPs, 0.1-3 μm). The molecular mechanism, by mean of which these vesicles are released has not been fully characterized, but it is a highly regulated process that varies depending on the stimuli-pro-inflammatory stimuli, cell activation or apoptosis - and is different from that observed during cell necrosis.

In fact, an increase in endothelial cells-derived MPs (EMPs) has been observed in patients suffering from myocardial infarction, in comparison with stable angina [4], and counts of CD31+/CD42- EMPs correlated with high-risk angiographic lesions in patients with acute coronary syndrome [5]. Besides their value as disease markers, MPs may play a role in disease progression. The components of MPs are responsible for their effects on other cell types [6]. Biological effects such as stimulation of blood coagulation [4-7], angiogenesis [8,9], bone cell proliferation [10] and hematopoietic stem cell engraftment [11] have been attributed to activated platelet-delivered MPs (PMPs). ECs shed MP with procoagulant activity in response to plasminogen activator inhibitor-1 (PAI-1) [7], whereas T-lymphocyte-derived MPs promote the decrease of endothelial nitric oxide synthase (eNOS) expression while inducing caveolin-1 (Cav-1) expression [12]. In fact, one of the main characteristics of cell MPs, the exposure of phosphatidylserine, confers pro-thrombotic properties to these vesicles. These and other evidences support the role played by MPs in the process of atherosclerosis and therefore prove to be of great interest.

Less is known regarding the potential role of MPs in the regulation of protein levels in the parental cell and therefore in the regulation of its function. In this study, we analyzed the protein composition of MPs released by VSMC in response to pro-atherogenic stimulus by qualitative proteomics in order to gain further insight into the possible role of MPs in the progression of the disease. This analysis has contributed to identify and characterize Cav-1 as a component of MPs released by VSMC and ECs in response to this stimulus, as well as in patients suffering from carotid atherosclerosis. Interestingly, decreased Cav-1 levels on these cell types are shown to correlate with increased VSMC proliferation [13-15].

Material and Methods

Cell culture

DPK-SMACR (VSMC) were obtained from Pharmakine (Vizcaya, Spain) and cultured according to manufacturer's instructions. hBMEC (human bone marrow-derived endothelial cells) were a generous gift from Dr. Weksler [16] and cultured on gelatin-coated plates in DMEM medium containing 5% heat-inactivated fetal bovine serum (FBS), RPMI vitamin mix, 2 mM glutamine and 10 mM Hepes. THP-1 cells were culture in RPMI supplemented with 5% heat-inactivated FBS. THP-1 derived macrophages were obtained and cultured as described [17]. 3T3 fibroblasts were cultured in DMEM supplemented with 5% FBS.

Patients and blood samples

Patients suffering from atherosclerotic carotid stenosis (>70% assessed by echography and an additional neuroimaging technique, mostly angioTC), irrespective of stroke occurrence, and healthy controls were enrolled in this study. The investigation conforms with the principles outlined in the Declaration of Helsinki. All patients and controls gave their written informed consent to the study, which was approved by the Ethics Committee of Hospital Universitario Central de Asturias.

Blood samples from 30 patients and 13 healthy controls were obtained as previously described [18]. Briefly, blood was obtained by venupuncture with a 21G needle and collected in sodium citrate tubes. The first 4 ml were discarded to avoid puncture artifacts. Platelet poor plasma (PPP) was obtained in the next 20 min after venipuncture by centrifugation for 20 min at 1550 g. Aliquots of plasma were maintained at -80°C until FACS analysis or further centrifuged to isolate MPs.

Intima-media thickness (IMT) measurement

IMT was determined by ultrasonography according to the Mannheim consensus [19] using a LOGIQ Book XP ultrasound machine (General Electric Medical Systems, Milwaukee, Wisconsin, USA).

Microparticle and exosome isolation

VSMC cells were grown to confluence and treated with lysophosphatidylcholine (lysoPC) (2.5, 6.25 or 12.5 μg/mL), 1 U/mL thrombin (both from Sigma Aldrich; St Louis, MO, USA) or 20 ng/mL TNFα (Insight Biotechnology; Wembley, UK) for 16 h in medium without serum. Media were collected and spun at 3200 g for 30 minutes to remove cell debris. The supernatants were further spun at 18000 g for 30 min to pellet the MPs. Pellets were washed three times in PBS. The same procedure was followed with hBMEC cells, 3T3 fibroblast, THP-1 cells, or THP-1 derived macrophages. Four ml aliquots of PPP from patients and healthy controls were processed by sequential centrifugation to isolate MPs (18000 g pellet) and exosomes (100000 g pellet).

Gel electrophoresis, protein digestion and mass spectrometry

MPs derived from VSMC cells were mixed with Laemmli buffer and protein samples were then subjected to SDS-PAGE and stained with Coomassie Blue. Protein bands were excised in 13 pieces of the gel. Proteins were reduced with 10 mM DTT, alkylated with 50 mM iodoacetamide and trypsin digested. The peptides were analyzed by LC-MS/MS in a Q-Trap Mass Spectrometer (Applied Biosystems, Foster City, CA, USA) coupled to a nano-HPLC (NanoLC Ultimate, LC Packings, Dionex, Sunnyvale, CA, USA). MS/MS spectra were matched to database entries using MASCOT software (Matrix Science Ltd. London UK). Trypsin was selected as the enzyme (one missed cleavage allowed). The peptide mass tolerance was set at ± 0.5 atomic mass units (amu), and the fragment mass tolerance was set at ± 0.3 amu. The MASCOT score obtained for each protein was greater than 28, indicating identity or extensive homology at a significance level of $p<0.05$. The proteins identified by MS were classified by cellular localization and biological function using PANTHER database (version 9.0) [20] and the UniProtKB Protein Knowledge database.

Western Blot

Isolated MPs or exosomes were mixed with Laemmli buffer and protein samples were run on 12.5% SDS-PAGE gels and blotted on

Immobilon-P membranes (Millipore, Billerica, MA, USA). Blots were developed with the enhanced chemiluminescence (ECL) detection system (Amersham; GE Healthcare, Munich, Germany). The following antibodies were used: anti-Cav-1 (1:1000 dilution), anti-ICAM-1 (1:1000), anti-alpha smooth muscle actin (SMA, 1:1000) and anti-RhoA (1:250 dilution, Santa Cruz Biotech; Santa Cruz, CA, USA); anti-Rac1 (1:1000 dilution, Upstate; Millipore Corporation, Billerica, MA, USA); anti-ERM (1:1000 dilution, Cell Signaling Technology; Danvers, MA, USA); anti-annexin-2 (1:2000 dilution, BD Biosciences; Erembodegem, Belgium). Secondary antibodies (horseradish peroxidase-conjugated, 1:2000 dilution) were from Dako (Glostrup, Denmark).

Flow cytometry

20 µl of PPP were incubated with the following monoclonal antibodies (mAb): APC-conjugated CD42b (BD Bioscience; Erembodegem, Belgium), PE-conjugated CD31 (eBioscience; San Diego, CA, USA), and FITC-conjugated AnxV (Immunostep; Salamanca, Spain). PPP from patients and healthy controls were incubated with the indicated mAbs or their respective isotype controls for 20 min at room temperature and under agitation (100 rpm). Subsequently, 1 ml of AnxV buffer was added and the samples analyzed in a FACSAria cytometer with the FACSDiva software (BD Bioscience, Erembodegem, Belgium). The concentration of MPs was estimated by counting a fixed number of 3 µm latex beads (Sigma) contained in the samples and expressed as MPs/µl of plasma. All solvents were 0.22 µm filtered.

Statistics

Results are expressed as mean ± S.E.M. Statistical analyses were performed by using the unpaired two-tailed Student's t-test. Differences were considered statistically significant at the $p<0.05$ level.

Results

Proteomic analysis of microvesicles released by lysoPC-treated vascular smooth muscle cells

Starved confluent VSMC were incubated with lysoPC (6.25 µg/ml), a pro-inflammatory lipid which plays an important role in atherosclerosis by altering the cell functions of smooth muscle cells, endothelial cells, monocytes, macrophages, and T cells [21]. The microvesicles released were then isolated by centrifugation. Proteins were separated by SDS-PAGE and thirteen bands were then excised for subsequent in-gel digestion and analysis by LC-MS/MS (Figure 1A). Our results showed a total of 297 proteins identified (Supplementary Table), some of which

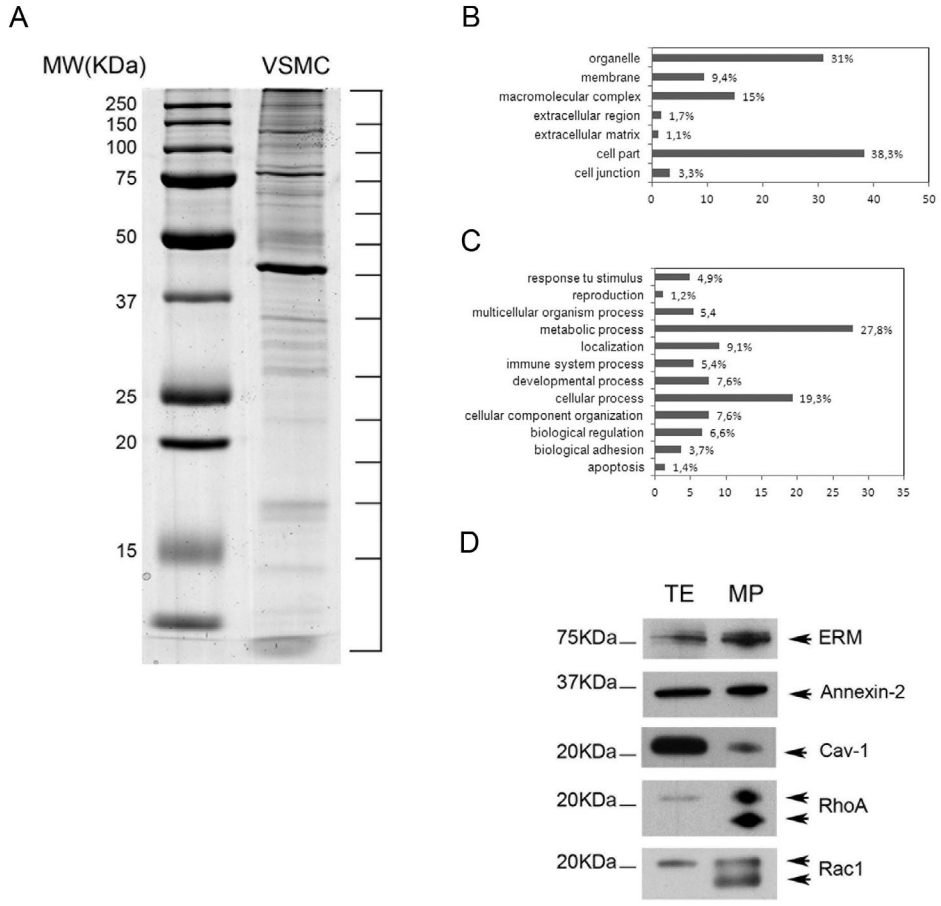

Figure 1: Analysis of composition of microvesicles released by vascular smooth muscle cells in response to lysoPC. Vascular smooth muscle cells were grown to confluence, starved in medium without serum for 4 hours and treated with lysoPC (6.25 µg/ml) for 16 hours. VSMC-derived MPs were then isolated and the proteins were separated by SDS-PAGE, subjected to trypsin digestion and analyzed by mass spectrometry. (A) Coomassie staining of gel. (B) Summary of cellular component and (C) biological function for each protein identified on lysoPC-derived MPs from a representative experiment. (D) The presence of proteins identified in the proteomic analysis (ERM, annexin-2, cav-1, RhoA, and Rac) was confirmed by western blot in total extracts (TE) and microparticles (MP) of lysoPC-treated VSMC. Blots shown are representative of at least 3 independent experiments.

Gene name	Protein name	Molecular weight kDa)	Score	Queries matched	% protein	Band
ACTB	Actin, cytoplasmic 1	41.710	210	13	48	7
ACTG	Actin, cytoplasmic 2	41.766	293	34	71	6
ARP2	Actin-related protein 2	44.705	48	2	5	6
	Actin-related protein 2/3					
ARPC2	complex subunit 2	34.370	172	6	29	8
	Actin-related protein 2/3					
ARPC5	complex subunit 5	16.810	62	2	20	10
ARP3	Actin-related protein 3	43.327	108	6	17	5
ANXA2	Annexin A2	38.654	289	13	44	6
CAV1	Caveolin-1	20.539	45	2	10	9
CD63	CD63 antigen	25.681	40	2	7	9
CD81	CD81 antigen	25.871	34	2	3	9
CD9	CD9 antigen	25.198	78	3	9	9
	Cell division control					
CDC42	protein 42 homolog	21.245	60	2	15	10
EZRI	Ezrin	69.348	223	16	22	3
ITA1	Integrin alpha-1	130.726	303	13	14	1
ITB1	Integrin beta-1	88.436	136	11	15	2
ICAM1	Intercellular adhesion molecule 1	60.104	213	10	15	4
MOES	Moesin	67.697	379	26	33	3
RAC1	Ras-related C3 botulinum toxin substrate 1	21.436	51	3	11	9
RHOA	Transforming protein RhoA	21.768	73	4	34	9
VCAM1	Vascular cell adhesion protein 1	81.194	142	9	12	2

Table 1: Identified proteins in MPs isolated from vascular smooth muscle cells treated with lysoPC. Selection of the most relevant proteins identified, indicating the molecular weight, score, number of queries matched, the percentage of the identified protein, and the band where the proteins were identified..

that we considered the most relevant are listed in Table 1. The proteins were classified by cellular localization (Figure 1B) and biological function (Figure 1C) using the PANTHER classification system [20]. Cell part and organelle groups comprised the biggest categories of proteins identified, which include intracellular, cytoskeleton or plasma membrane proteins. The major functional groups contained proteins involved in metabolic and cellular processes. It is worth noting that proteins commonly enriched in exosomes, tetraspanins CD9, CD63 and CD81, were identified. Integrins and cell adhesion molecules, such as ICAM-1, VCAM-1, integrin-alpha 1, and integrin-beta 1, were also found. The levels of these proteins are increased in response to inflammatory stimuli, including TNFα or oxLDL. As shown in Figure 1D, some of the proteins were additionally confirmed by western blot.

Pro-atherogenic stimuli induce Cav-1-positive MPs release by vascular smooth muscle cells and endothelial cells but not by monocytes or macrophages

To further confirm the results obtained by LC-MS/MS, we next isolated microvesicles released by VSMC in response to different concentrations of lysoPC (6.25 and 12.5 μg/mL). Cells were also treated with other pro-inflammatory stimuli, such as TNFα and thrombin to explore whether the results obtained were stimulus-dependent. The presence of different proteins previously identified was then analyzed by western blot (Figure 2A). Cav-1 was present in microvesicles released by VSMC in response to lysoPC, as in the previous analysis. However, MPs induced by TNFα and thrombin did not contain Cav-1. In addition, we analyzed MPs released by EC (hBMEC), which is other cell type implicated in the progression of atherosclerosis, in response to the same pro-inflammatory stimuli. We found that Cav-1 is present at very low levels after treatment with lysoPC and TNFα, and is absent from MPs isolated from thrombin-treated EC (Figure 2B). Interestingly, annexin-2, a protein present in VSMC-derived MPs, was not detected in the case of EC-derived microvesicles, whereas ICAM-1, an adhesion molecule involved in leukocyte extravasation, was detected in EC-derived MPs upon lysoPC stimulation and to a lesser extent after TNFα treatment.

Adventitial fibroblasts are activated and migrate into the intima, and contribute to atherosclerosis in early stages [22], whereas monocytes and macrophages are localized at the atherosclerotic plaques and contribute to plaque destabilization. Therefore, we explored whether Cav-1 was released by these cell types. As Figure 3 shows, while fibroblasts behaved as VSMC in terms of Cav-1 presence on MPs (Figure 3A), neither the monocytic cell line THP-1 (Figure 3B) nor THP-1-derived macrophages (Figure 3C) released Cav-1 in MPs upon stimulation by the different pro-inflammatory stimuli. Moreover, Cav-1 expression is decreased on macrophages treated with lysoPC compared to control cells.

Detection of Cav-1 in circulating MPs

On the basis of the results from *in vitro* experiments, we next studied plasma MPs in 30 patients suffering from carotid stenosis (>70%) and 13 healthy controls. Patients exhibited a high prevalence of hypertension (56.7%), atrial fibrillation (AF) and other cardiac diseases (46.67%), and diabetes mellitus (DM) (36.7%). The characteristics of both controls and patients are summarized in Table 2. First, the presence of EMPs and PMPs was determined by flow cytometry (Figure 4); thus, two populations of patients became apparent: one with low count of AnxV positive microparticles (PlowAnxV; Figure 4B) and a second one with high incidence of these vesicles (PhighAnxV; Figure 4C). The total number of EMPs and PMPs was then quantified

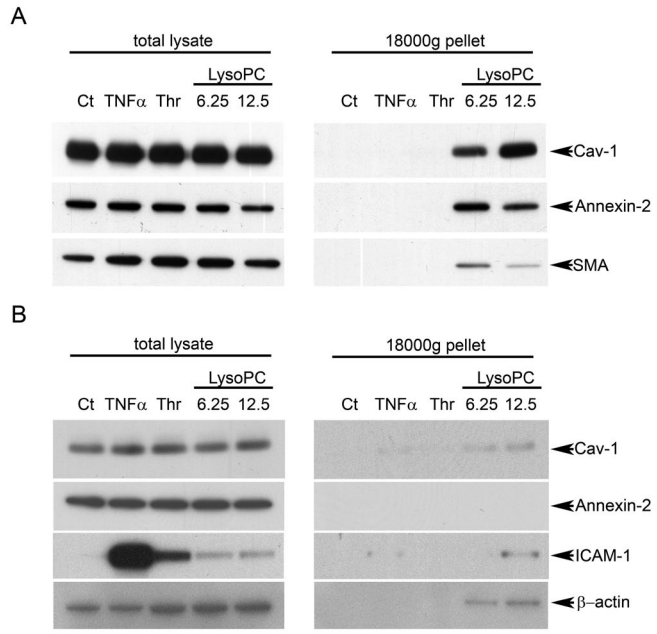

Figure 2: Analysis of microvesicles released by EC and VSMC in response to different stimuli. VSMC (A) and hBMEC (B) were treated with the indicated agents and the supernatants processed as described in methods. The presence of the indicated proteins in the microparticle fractions was analyzed by western blot. The blots shown are representative of at least three independent experiments.

	Controls	Patients
N	13	30
Mean age in years (range)	52.6 (35-77)	70.3 (52-85)
Male gender (%)	8 (61.5)	24 (80)
AHT (%)	2 (30.8)	17 (56.7)
DM (%)	1 (7.7)	11 (36.7) DL
(%)	-	9 (30)
AF&CP (%)	-	14 (46.7)
Heparin/LMW Heparin (%)	-	21 (70)
Statins (%)	-	23 (76.7)
Antiaggregants (%)	-	17 (56.7)
Mean IMT-mm (range)	0.56 (0.42-0.93)	0.88 (0.53-1.12)

AHT: Hypertension; DM: diabetes mellitus; DL: dyslipemia; AF&CP: atrial fibrillation and other cardiopathies; LMW: low molecular weight; ITM: intima-media thickness. Numbers in parentheses indicate the age range, the percentage for each parameter in healthy controls or patients or the IMT range.

Table 2: Patients' characteristics. Demographic and clinical characteristics of the patients enrolled in this study.

in healthy controls and in the two groups of patients (Figure 4D). We observed that the number of PMPs (CD31+/CD42+) significantly increased in the population with high incidence of AnxV+ vesicles compared with the control group. In contrast, a significant decrease was found in the PlowAnxV group. Regarding EMPs, no significant differences between the groups were found, although we observed a tendency to increase in the PhighAnxV group. Thus, although it has been proposed that the number of circulating microparticles could be a marker of atherosclerosis, the increase compared to control was statistically significant only in the population with high incidence of AnxV+ vesicles (Figure 4D). However, a decrease in CD31+/CD42b- MPs compared to healthy controls was observed in the PlowAnxV group. Therefore, this parameter cannot be considered a marker for this disease, at least by itself. Interestingly, AnxV- vesicles were increased in both groups of patients in comparison with healthy controls (Figure 4E) and were negative for CD31 and CD42 markers and therefore were not endothelial-or platelet-derived (data not shown).

Since the presence of Cav-1 was detected in MPs released in response to pro-atherogenic stimuli, we next investigated whether this protein was detected in plasma MPs and its correlation with the presence of atherosclerosis. An aliquot of the MP fraction corresponding to 600 µl of plasma was analyzed by western blot (Figure 5). 73.33% of patients were positive for Cav-1 compared to 15.38% of controls. The presence of this protein is detected exclusively in MPs, but not in exosomes. Annexin-2, a protein involved in vesicle formation, was detected in a lower percentage of samples (data not shown). The presence or absence of Cav-1 did not correlate with the number of EMPs or PMPs detected by flow cytometry in the samples, but most of the Cav-1 positive patients belonged to the PlowAnxV group. This observation suggests that another cell type was responsible for the presence of this protein in the samples. Cav-1 positivity did not correlate with any of the items recorded in the medical histories (Table 2) but it was clearly associated with the presence of atherosclerosis ($p<0.001$, Chi-square test).

Discussion

In this study we analyzed the proteome of MPs released by VSMC in response to lysoPC, a pro-atherogenic stimulus. This approach has contributed to identify Cav-1 as a protein present in VSMC-derived MPs *in vitro*, as well as in circulating MPs of patients suffering from atherosclerosis.

It has been suggested that circulating MPs and exosomes may be involved in several diseases including multiple sclerosis [18], cancer

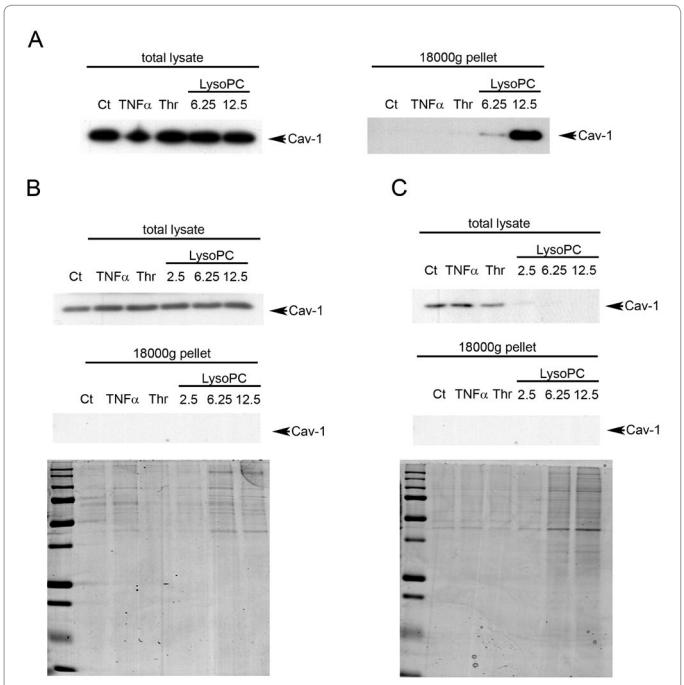

Figure 3: Analysis of Cav-1 in MPs released by different cell lines. The presence of Cav-1 was assessed by western blot in fibroblasts (A), macrophages (PMA-treated THP-1 cells, B) and monocytes (THP-1, C) in response to the indicated agents. The blots shown are representative of at least three independent experiments.

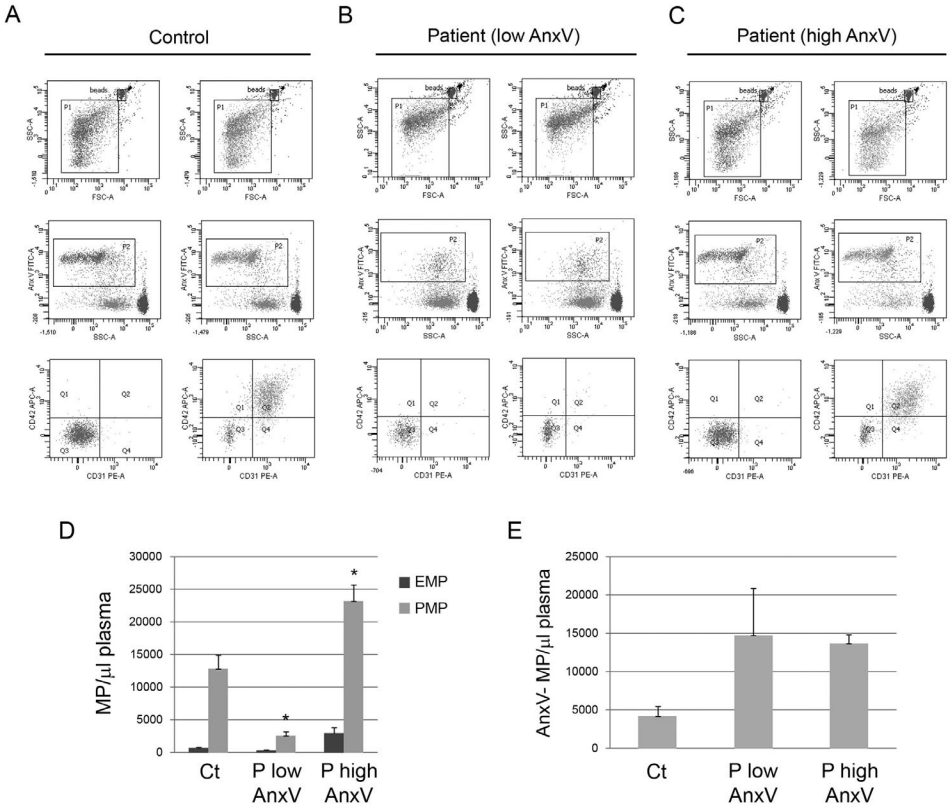

Figure 4: Analysis of microvesicles in plasma from healthy controls and atherosclerotic patients. Representative panel of the analysis of microparticles in a healthy control (A), a patient with low (B) and high (C) frequency of AnxV-positive microparticles. EMPs were positive for AnxV and CD31 but negative for CD42 (Q4). PMPs were positive for AnxV, CD31 and CD42 (Q2). The columns on the left show isotype controls for each sample. (D) Counts/µl plasma of EMPs and PMPs in controls and patients. The graph shows the mean ± SEM ($p<0.05$; Student's t test). (E) Counts/µl plasma of AnxV-negative microparticles in controls and patients. The graph shows the mean ± SEM.

[23], and atherosclerosis [6, 24]. Circulating MPs are of a great interest because of their potential value as biomarkers for atherosclerosis and their role in the progression of the disease [4,5,24,25]. Different authors have proposed the number of circulating EMPs as a marker for the presence of atherosclerosis [24,26]. Although it has been demonstrated that the amount of circulating MPs correlates with stroke severity in the acute stage of the stroke [27], the fact that this parameter cannot properly distinguish between acute ischemic strokes an d stroke mimics has also been reported [28]. In our study group, we found that the number of circulating AnxV+ EMPs was higher in atherosclerotic patients than in healthy controls, but without statistical significance. These results together with previous reports support the need to search for more specific markers for atherosclerosis.

The composition of MPs released by ECs and platelets in response to different stimuli has been previously characterized [29,30]. However, less is known regarding the composition and potential role of VSMC-derived MPs in atherosclerosis. Schechter et al. [31] described that SMCs release active tissue factor (TF) in microvesicles in response to different stimuli and proposed that at least part of the TF found in the injured arteries could be derived from this mechanism. More recently, it has been shown that apoptotic SMC-derived MPs enhance thrombus formation and that this correlated with the TF present on their surface [32]. These evidences support the potential role of SMC-MPs on atherosclerosis progression. Thus, MPs may contribute to the progression of different pathologies through their interaction with targets cells and the triggering of cell responses. The regulation of protein levels in the parental cell and therefore of their functions may constitute and additional mechanism.

Mayr et al. [33] published a study assessing the composition of the MPs present in atherosclerotic plaques and demonstrated the presence of SMC-derived MPs; however, no detailed information on the composition of circulating MPs in atherosclerotic patients is available, probably due to the limited amount that can be obtained.

In the present study we have addressed this problem by first analyzing the composition of VSMC-derived MPs in response to lysoPC, a pro-inflammatory and pro-atherogenic stimulus. Among the proteins identified by qualitative proteomics in this initial screening, Cav-1 was confirmed to be released in MPs. Moreover this protein is present in MPs released by EC and fibroblast, but not by monocytes and macrophages. Cav-1 was also detected in circulating MPs in plasma samples acquired from atherosclerotic patients, but not in exosomes. Interestingly, Cav-1 positivity neither correlated with EMPs or PMPs counts in PPP -the more abundant circulating MPs- nor with the presence of the different cardiovascular risk factors or the occurrence of stroke.

LysoPC, formed during the oxidation of LDL, is present in atherosclerotic plaques and it has been proposed to be responsible for the cellular effects of oxLDL, including VSMC proliferation [34-37]. Cav-1 is known to regulate proliferation of different cell types, including SMC [13-15]. Cav-1 deficiency stimulates neointima

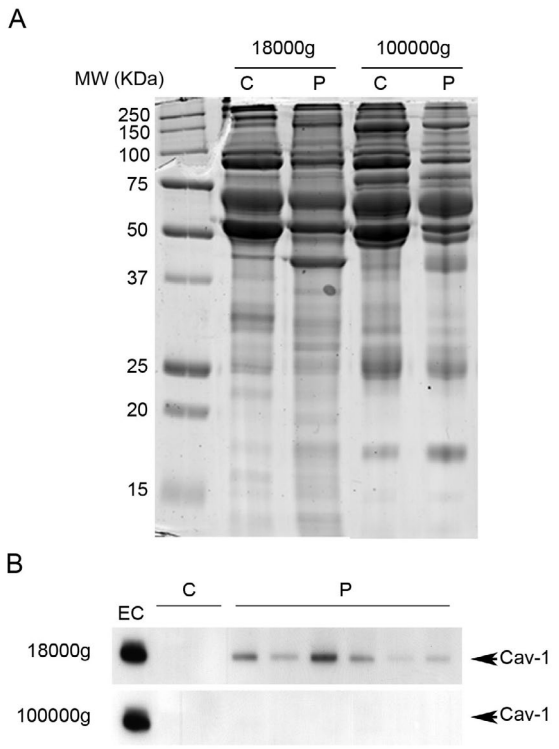

Figure 5: Cav-1 in MPs and exosomes isolated from healthy controls and patients. (A) Representative Coomassie staining of proteins present in microparticles (18000 g pellet) and exosomes (100000 g pellet) isolated from plasma from controls (C) and patients (P). (B) The presence of Cav-1 in microparticles was confirmed by western blot.

formation in a low-flow model of vascular injury [13]. Cav-1 levels decrease in VSMC in atheroma plaques [14]. Although Cav-1 is present in exosomes isolated from melanoma patients [23] and may not constitute a specific biomarker for atherosclerosis, these observations together with our results suggest that MP generation and release by VSMC may contribute to the regulation of Cav-1 levels on this cell type and therefore modulate their proliferative activity, contributing to the neointima formation and artery stenosis.

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