4. RESULTATS

I. INTEGRACIÓ RESULTATS ARTICLES *I* I *II*

L'objectiu dels primers treballs experimentals fou estudiar els mecanismes que porten a la retenció i/o modificació de les LDL infiltrades en la paret vascular, així com establir el/s mecanisme/s mitjançant el/s qual/s les CML uneixen i internalitzen les LDL modificades. En aquesta secció es pretén resumir i integrar els resultats obtinguts. Els articles originals, així com els corresponents annexes publicats *on-line*, s'adjunten a la ***secció III de resultats***.

La infiltració de LDL en la paret arterial porta a la seva interacció amb els PGs de la MEC. Com s'ha esmentat, els PGs que semblen tenir major rellevància en els processos desenvolupats a l'íntima arterial són els CSPG i els HSPG. Els CSPG són secretats per les cèl·lules de la paret vascular i tenen elevada capacitat d'interaccionar i/o modificar les LDL. Mentre que, els HSPG poden ésser secretats o restar units a la superfície cel·lular, i es caracteritzen per poder actuar com a receptors de lipoproteïnes aterogèniques i/o per facilitar l'aproximació de determinats lligants al seu receptor, incrementant d'aquesta manera l'eficiència d'internalització. Així doncs, s'estudià la contribució d'ambdós tipus de PGs en els processos de modificació de LDL i d'internalització cel·lular.

En primer lloc s'analitzà la importància d'un CSPG, el versicà, en la modificació de les LDL i conseqüentment en la seva internalització per les CML. S'escollí aquest PG doncs és el principal PG estructural de la MEC i és un dels PGs amb major afinitat per les LDL plasmàtiques.

I.1. Caracterització de lipoproteïnes modificades *in vitro*

Les LDLn es modificaren per agitació intensa (LDLag) i per incubació amb el versicà (versicà-LDL). Les fraccions precipitable i no precipitable, obtingudes de la centrifugació de les LDL modificades, es caracteritzaren en paral·lel juntament amb les LDLn. L'anàlisi de les diferents lipoproteïnes per electroforesi en gel d'agarosa i en gel en gradient d'acrilamida, així com la microscòpia electrònica, posaren de manifest la composició i tamany de les partícules de LDL.

La fracció precipitable de les LDLag es composava de partícules fusionades d'entre 77 i 160 nm de diàmetre (***fig 2AF, article I***), mentre que la fracció precipitable de les versicà-LDL estava formada de partícules fusionades de diàmetre entre 92 i 166 nm i de partícules monomèriques (***fig 2CG, article I***). Les partícules monomèriques que composaven el filtrat de

la fracció precipitable de les versicà-LDL, igual que les que formaven part de les fraccions no precipitables d'ambdós tipus de LDL modificades, presentaven un diàmetre de ≈26,2 nm (***fig 1B; fig 2BD, article I***) i mobilitat electroforètica semblant a les LDLn (***fig 1A, article I***).

I.2. Efecte de les versicà-LDL en el contingut de colesterol intracel·lular en les cèl·lules musculars llises

Les CML s'incubaren amb concentracions creixents (20, 40, 60 μg/mL) de les fraccions precipitables i no precipitables de les versicà-LDL i de les LDLag, i de les LDLn. Les partícules monomèriques de les fraccions no precipitables, malgrat tenir un tamany i mobilitat electroforètica semblant a les LDLn (***fig 1B, fig 2BDE, article I***), foren capaces d'induir una acumulació de CE intracel·lular superior a la que produien aquestes (***fig IVC annex, article I***). Mentre que, la captació de la fracció precipitable de les versicà-LDL portà a una acumulació intracel·lular de CE seguint un perfil dosi-resposta semblant al produït per les LDLag (***fig IVAB annex, article I***). Cap de les lipoproteïnes alterà els nivells de CL intracel·lular (***fig IV annex, article I***).

I.3. Internalització de les lipoproteïnes per les cèl·lules musculars llises

A fi d'establir el mecanisme d'internalització de les versicà-LDL es portaren a terme assajos de competència de lligant. Les CML, prèvia addició de les diferents lipoproteïnes (LDLn, LDLag, versicà-LDL), s'incubaren amb lligants de diferents receptors: lactoferrina (lligant pels receptors de la família del rLDL), àcid poliinosínic (lligant pels receptors *scavenger*), galactosa i fetuïna (lligants pel receptor asialoglucoproteic). D'aquests assajos es deduí la implicació d'un receptor de la família del rLDL, ja que l'únic lligant capaç d'inhibir l'acumulació de CE induïda per cadascuna de les lipoproteïnes, fou la lactoferrina (***taula I annex, article I***). Per concretar en el receptor implicat, es bloquejà l'activitat del rLDL i del receptor LRP amb un anticòs específic i amb ODNs antisentit, respectivament (control d'inhibició del LRP ***fig VI annex, article I)***. Anàlisis que posaren de manifest la implicació del rLDL en la internalització de les partícules monomèriques de LDLn, i de les partícules monomèriques que conformaven la fracció no precipitable i el filtrat de la fracció precipitable de les versicà-LDL, ja que el bloqueig amb l'anticòs contra el rLDL portà a una reducció ≈ 60-80 % de l'acumulació de CE induïda per aquestes partícules (***fig V annex, article I***). D'altra banda, els estudis d'inhibició de l'expressió del LRP evidenciaren la implicació d'aquest

receptor en la internalització d'una part de les partícules de les fraccions precipitables de les LDLag i de les versicà-LDL (***fig 3AB, article I***). La inhibició del LRP portà a una reducció de l'acumulació de CE induïda per les fraccions precipitables d'aquestes lipoproteïnes d'un ≈ 71% i ≈ 65%, respectivament; sense alterar l'acumulació de CE produïda per les fraccions no precipitables d'ambdós tipus de LDL modificades (***fig 3C, article I***). Resultats que concorden amb els obtinguts per microscòpia òptica amb la que s'observà que la inhibició del LRP portava a una menor adhesió de les versicà-LDL a la superfície de les CML (***fig 3D, article I***).

Coneguda la capacitat dels versicà (CSPG) de modificar les LDL, es passà a caracteritzar els PGs sintetitzats per les CML i pels fibroblasts. S'empraren fibroblasts com a cèl·lules control, ja que en nombrosos estudis previs s'havien emprat aquest tipus cel·lular per analitzar el paper dels HSPG i del LRP en la captació de diferents lligants. D'altra banda, s'estudià la possible implicació dels CSPG i dels HSPG, en el mecanisme de captació i internalització de les LDLag (s'escollí aquest model donada la semblança entre les LDLag i les versicà-LDL observada en l'***article I***).

I.4. Caracterització dels proteoglicans sintetitzats per les cèl·lules musculars llises i pels fibroblasts

Els estudis de caracterització de la MEC i de la síntesi de PGs es realitzaren en CML i en els fibroblasts MEF, ja que es considerà que els fibroblasts que expressen el LRP (MEF) i els que no l'expressen (PEA13) no presenten diferències pel què fa a la síntesi de PGs. Per microscòpia electrònica es posà de manifest que tant la matriu pericel·lular de les CML (***fig 2A, article II***) com la dels fibroblasts MEF (***fig 2B, article II***) estava constituïda per una malla de col·lagen entrellaçada amb filaments prims de PGs que quedava completament desestructurada per l'activitat de la HSI i de la ChABC (***fig 2CD, article II***).

Els HSPG i CSPG sintetitzats per les CML i pels MEF es caracteritzaren mitjançant marcatge metabòlic ($[^{35}S]Na_2SO_4$) i digestió selectiva amb HSI&III i ChABC, respectivament. El patró dels PGs sintetitzats (***fig I annex*** (tractament a les 2 h)***, article II***) no mostrà diferències significatives entre els tractaments enzimàtics de 2 i 18 h (en presència o no de LDLag) en cap dels tipus cel·lulars en estudi. D'aquest anàlisi es deduí que la major part de PGs sintetitzats per les CML i les MEF resten a la fracció cel·lular, 58% i 56%, respectivament (***taula I, article II***). Mentre que, la MEC conté un baix percentatge dels PGs sintetitzats, un 7% en les CML i un 4 % en les MEF, i la resta són secretats al medi de cultiu.

Tot i que, el patró d'elució dels PGs evidencià que ambdós tipus cel·lulars no secreten HSPG al medi de cultiu, la composició de PGs de les fraccions cel·lular i de la MEC presentà clares diferències entre CML i MEF. El percentatge de HSPG en les fraccions cel·lular i MEC de les cèl·lules MEF fou superior al trobat en les CML. D'altra banda, per SDS-PAGE, es posà de manifest que el perlecà semblava ser el HSPG majoritari en les CML, mentre que, en les MEF els HSPG trobats tenien el tamany propi del sindecà (***fig 1, article II***; control de càrrega proteica en el SDS-PAGE ***fig III annex, article II***). Pel què fa a la síntesi de CSPG, les fraccions de les CML presentaren un major contingut d'aquests PGs respecte les MEF (***taula I, article II***). El patró de bandes (per SDS-PAGE) dels CSPG, especialment de la fracció cel·lular, mostrà també diferències de tamanys entre CML i MEF (***fig 1, article II***).

I.5. Rellevància dels proteoglicans i del LRP en la internalització de les LDLag en les cèl·lules musculars llises i en fibroblasts

Per posar de manifest la contribució dels PGs i del LRP en la captació de les LDLag, es treballà amb cèl·lules que expressen el LRP (MEF i CML no tractades amb ODNs antisentit contra el LRP) i cèl·lules que no expressen el LRP (PEA13 i CML tractades amb ODNs antisentit contra el LRP), totes elles tractades o no amb HSI&III o amb ChABC. Les cèl·lules es preincubaren amb els diferents tractaments enzimàtics durant 2 h, prèvia addició de les lipoproteïnes, les quals es mantingueren en el medi de cultiu durant 18 h.

En les CML, els estudis d'acumulació de CE evidenciaren la capacitat de les LDLag d'induir altes concentracions intracel·lulars de CE de manera dosi-resposta (***fig 3, article II***). Acumulació que no s'observava en les CML tractades amb ODNs antisentit contra el LRP, en concordància amb el paper del LRP en la unió i internalització de les LDLag prèviament descrita en el nostre grup (Llorente-Cortés V 2000). Aquestes dades es corroboraren amb les imatges de microscòpia òptica que mostraren com la inhibició del LRP portava a una important davallada en l'adhesió de LDLag a la superfície cel·lular (veure ***fig 3D, article I***). D'altra banda, l'eliminació dels HSPG en les CML, portà a una reducció de l'acumulació de CE (derivada de les LDLag) de fins un ≈38 % (***fig 3, article II***), en concordància amb la lleugera davallada en l'adhesió de les LDLag a la superfície cel·lular (***fig IIC annex, article II***), permetent mantenir encara uns nivells considerables d'acumulació de CE (***fig 3, article II***). Mentre que, l'eliminació dels CSPG no modificà significativament els nivells intracel·lulars de CE (***fig 3, article II***).

Pel què fa als fibroblasts, l'absència de LRP en les PEA13 induia una davallada d'un ≈53 % en l'acumulació de CE derivada de la captació de les LDLag, a diferència de l'efecte de l'absència de LRP en les CML que produïa una davallada del 80 % (***fig 3, article II***). Resultats que concordaven amb l'adhesió de les LDLag observada en la superfície cel·lular, per microscòpia òptica (***fig II BE annex, article II***). Cal destacar que, l'eliminació dels HSPG inhibia completament l'acumulació de CE induïda per les LDLag tant en les MEF com en les PEA13 (***fig 3, article II***). Les imatges de microscòpia òptica corroboraven aquests resultats, ja que mostraven com la degradació dels HSPG en les MEF i PEA13 era capaç de bloquejar completament l'adhesió de les LDLag a la superfície cel·lular (***fig IIDF annex, article II***), portant a una completa inhibició de l'acumulació de CE (***fig 3, article II***). Mentre que, l'eliminació dels CSPG no modificava significativament els nivells intracel·lulars de CE en ambdós tipus de fibroblasts (***fig 3, article II***).

Analitzant les dades d'acumulació lipídica obtinguda dels diferents tractaments en les diferents línies cel·lulars es pot deduir que, els HSPG poden actuar com a receptors de LDLag en les MEF, però no en les CML. En les CML el LRP és imprescindible per la captació de les LDLag. Tot i això, els resultats posaren de manifest que tant en les CML com en les MEF existeix un mecanisme de cooperació entre LRP i HSPG. Les observacions esmentades es resumeixen en la taula que s'adjunta a continuació.

Taula I. Càlcul aproximat de la contribució dels HSPG i del LRP en la internalització de lipoproteïnes

CML			**Fibroblasts**		
Contribució dels HSPG	Cooperació HSPG i LRP	Contribució del LRP	Contribució dels HSPG	Cooperació HSPG i LRP	Contribució del LRP
1%	**26%**	**58%**	**41%**	**25%**	**12%**

II. INTEGRACIÓ RESULTATS ARTICLES *III* I *IV*

En els dos primers treballs (***articles I i II***) es posà de manifest la implicació del receptor LRP en la unió i internalització de LDL modificades (LDLag i versicà-LDL). Resultats que fan pensar que el LRP podria tenir un paper clau en la inducció d'acumulació lipídica intracel·lular en les CML, afavorint així la seva transformació en cèl·lules escumoses, esdeveniment clau pel desencadenament de l'aterogènesi.

S'ha descrit que el LRP té una elevada expressió tant en vasos sans com en lesió ateroscleròtica. Però, es coneix poc sobre la seva expressió i regulació en les CML presents durant la progressió de la placa ateroscleròtica en coronàries humanes. Així doncs, ens plantejàrem analitzar els nivells d'expressió del LRP en CML procedents d'artèries sanes i/o ateroscleròtiques. D'altra banda, a fi d'establir els mecanismes que regulen els nivells d'expressió i activitat del LRP en les CML, estudiàrem la possible implicació del lípid en la regulació del LRP, analitzant els nivells d'expressió del LRP en CML humanes en les que s'induïen elevats nivells de colesterol intracel·lular per incubació amb LDLag. Estudis que es contrastaren amb l'anàlisi dels nivells del LRP en la paret vascular d'animals amb hipercolesterolèmia induïda per dieta.

II.1. Efecte de la internalització de les LDL i LDLag en la regulació de l'expressió dels receptors LRP i del rLDL en cultius de cèl·lules musculars llises

La incubació durant 48 h de les CML amb LDLag induia uns nivells d'acumulació de CE d'unes tres vegades superior als produïts per les LDLn, les quals tant sols portaven a un lleuger augment en els nivells de CE intracel·lulars (***fig 1, article III***). L'estudi de d'acumulació lipídica en CML procedent d'artèries coronàries sanes i/o ateroscleròtiques, evidencià que la capacitat d'acumular CE (per la incubació amb LDLag) al llarg del temps era superior en les CML derivades de zones de placa (***fig 1, article IV***). Però no s'observaren diferències significatives entre ambdós tipus cel·lulars pel què fa a l'acumulació lipídica intracel·lular induïda per la incubació amb LDLn. Aquesta internalització incrementada de LDLag anava associada amb uns nivells d'expressió del LRP significativament més elevats, tant a nivell d'ARNm com de proteïna, en aquelles CML que derivaven de lesions ateroscleròtiques (***fig 2AC, article IV***). D'altra banda, la incubació de les CML amb LDLn o LDLag produí un increment en els nivells d'ARNm del LRP al llarg del temps i de manera

dosi-resposta (***fig 2AC, article III***). Cal dir però, que la inducció causada per les LDLn fou inferior a la produia per les LDLag a qualsevol dels temps estudiats. S'assolia un màxim d'inducció a les 24 h d'incubació, temps en el qual la síntesi proteica del LRP també es veia incrementada de manera diferencial pel tractament amb LDLn o LDLag (***fig 3, article III***).

Paral·lelament i com a control, s'estudiaren els nivells d'expressió del rLDL. Els estudis en CML derivades de coronàries sanes i/o ateroscleròtiques indicaren que l'expressió del rLDL era substancialment més baixa que la del LRP independentment de l'origen de les CML (***fig 2AB, article IV***). A més, es posà de manifest que l'expressió del rLDL era significativament més baixa en les CML procedents de lesions ateroscleròtiques respecte a les derivades d'artèries sanes (***fig 2B, article IV***). Resultats que concorden amb el fet que l'expressió del rLDL s'inhibia inclús per la incubació de les CML amb concentracions baixes d'ambdues lipoproteïnes (LDLn i LDLag) (***fig 2D, article III***). D'altra banda, tot i que, tant les LDLn com les LDLag eren capaces de reduir els nivells d'ARNm del rLDL al llarg del temps (***fig 2B, article III***), la reducció produïda per les LDLn era ja evident a les 6 h d'incubació, mentre que, l'efecte de les LDLag s'evidenciava a les 12 h. Amb tot, s'observà una lleugera reversió d'aquesta inhibició a partir de 24 h d'incubació amb ambdós tipus de lipoproteïnes.

II.2. Hipercolesterolèmia i expressió del LRP en la paret vascular

A fi de corroborar els resultats obtinguts en els cultius cel·lulars, que mostraven com l'entorn lipídic pot modular l'expressió del receptor LRP, es portaren a terme estudis d'hibridació *in situ* amb els que s'estudià si la hipercolesterolèmia (en el model porcí) pot modular l'expressió d'aquest receptor en la paret vascular.

Els animals hipercolesterolèmics presentaven un increment significatiu dels nivells plasmàtics de lípids respecte als normocolesterolèmics (***taula, article III***) i una incipient lesió aterosclerótica a la paret vascular (***fig 6AB, article III***). Cal destacar que, els anàlisis d'hibridació *in situ* demostraren un significatiu increment en l'expressió del receptor en la línia íntima-mitja (just per sota de les zones de desenvolupament de placa aterosclerótica) en els animals hipercolesterolèmics respecte als normocolesterolèmics (***fig 6, article III***).

Els resultats obtinguts en els animals hipercolesterolèmics es contrastaren amb estudis en mostres de coronàries humanes amb lesions ateroscleròtiques en diferents estadis de progressió. Les mostres de coronària es sotmeteren a tinció per Tricròmic de Masson (***fig 4AB, article IV***) per tal de classificar les lesions en: grup I (lesions inicials: baix engruiximent arterial), grup II (lesions de creixement mitjà), grup III (lesions avançades d'alt risc) i grup IV

(lesions severes). L'anàlisi immunohistoquímic del lípid present en la paret vascular (quantificant apoB100 i per tinció ORO) mostrà que, en progressar les lesions s'incrementava significativament la deposició lipídica en l'íntima (***fig 3A, article IV***). D'altra banda, els resultats obtinguts en el model porcí concordaven amb els obtinguts de mostres humanes on s'observà un increment de l'expressió del LRP amb la progressió de la lesió (***fig 3C i fig 4, article IV***).

II.3. Rellevància dels SREBPs en el control de l'expressió del LRP

S'ha descrit que el rLDL està altament regulat pels nivells intracel·lulars de colesterol. El rLDL, igual que la resta de gens involucrats en el metabolisme lipídic, conté una regió consens per SRE en el promotor, permetent ser regulat mitjançant proteïnes SREBPs. El receptor LRP conté un SRE en una regió inusual en l'extrem 5' de la regió no traduïda del gen que, tot i no trobar-se en el promotor, es podria pensar que intervingués en la regulació del LRP via les proteïnes SREBPs. Així doncs, ens plantejàrem analitzar la possible implicació d'aquests SREBPs en la regulació dels nivells d'expressió del LRP.

La incubació de les CML amb concentracions elevades d'ambdues lipoproteïnes (LDLn i/o LDLag) portà a una completa davallada de l'expressió de l'ARNm del SREBP-2 sense alterar els nivells del SREBP-1 (***fig 4A, article III***). Els estudis al llarg del temps mostraren que, seguint el patró de regulació del rLDL (***fig 2B, article III***), hi havia un retràs en la regulació a la baixa del SREBP-2 induïda per les LDLag (12 h) comparat amb l'efecte que produïen les LDLn (6 h) (***fig 4B, article III***). D'altra banda també s'observà una lleugera reversió en l'expressió del SREBP-2 a partir de les 24 h d'incubació amb ambdós tipus de lipoproteïnes, efecte que també s'apreciava en els nivells del rLDL (***fig 2B, article III***).

Per confirmar la implicació del SREBP-2 en el mecanisme de control d'expressió del LRP, s'analitzà l'efecte d'un inhibidor del catabolisme dels SREBPs (ALLN) en l'expressió del rLDL i en el LRP. L'increment en l'expressió del LRP produïda per les LDLn i/o LDLag es veié completament revertida, per efecte de l'ALLN, tant a nivell d'expressió (nivells d'ARNm i proteïna) com de funcionalitat del receptor (acumulació de CE intracel·lular) (***fig 5, article III***). Com a control, s'analitzà l'expressió del rLDL observant-se que el tractament amb ALLN impedia la regulació a la baixa del rLDL que exerceixen d'ambdues lipoproteïnes (***fig 5AC, article III***).

II.4. Efecte del contingut lipídic intracel·lular en la supervivència de les cèl·lules musculars llises

El fet que l'elevada acumulació de CE intracel·lular en les CML, facilitada per una positiva regulació del LRP, portaria a la seva transformació en cèl·lules escumoses, fa pensar en un possible canvi en la funcionalitat i viabilitat de les CML. Així, ens plantejàrem analitzar els efectes del contingut lipídic intracel·lular en la supervivència de les CML.

Els estudis immunohistoquímics en coronàries humanes mostraren una davallada en la presència de la CML en l'íntima arterial durant la progressió de la lesió (***fig 3B, article IV***) paral·lelament amb un increment en el contingut lipídic (***fig 3A, article IV***). Així doncs, es decidí estudià si l'entorn lipídic i/o el contingut lipídic intracel·lular podien condicionar la supervivència de la CML.

Els estudis de proliferació cel·lular (incorporació de [^{3}H]timidina) mostraren que la internalització de LDLn o LDLag no modificava el grau de proliferació de les CML procedent de coronàries sanes (***fig 5, article IV***). Mentre que, ambdós tipus de lipoproteïnes incrementaven significativament la síntesi d'ADN en les CML procedents de lesions ateroscleròtiques (***fig 5, article IV***). D'altra banda, la presència de PDGF-BB incrementava el grau de proliferació de les CML derivades de regions sanes, però no es veié modificada la proliferació de les CML procedents de regions ateroscleròtiques, independentment de la presència o no de lipoproteïnes.

Per corroborar els estudis de supervivència, s'analitzà si la internalització de lipoproteïnes modificava l'índex Bcl_2/Bax, marcador d'apoptosi. S'observà que, tot i que les CML de zones de placa tenien un índex Bcl_2/Bax més baix que les CML procedents de regions sanes, la presència de lipoproteïnes (LDLn o LDLag) no modificava aquest índex en cap dels dos tipus cel·lulars (***fig 6A, article IV***). D'altra banda, s'emprà com a control l'estudi del gen CPP32, gen que incrementa la seva expressió en estadis avançats d'apoptosi. No s'observaren diferències significatives en els nivells d'expressió de CPP32 ni a nivell d'ARNm (***fig 6B, article IV***) ni a nivell de proteïna (***fig 6C, article IV***) entre CML procedents de regions sanes o de regions amb lesió ateroscleròtica.

III. ARTICLES ORIGINALS

III.1. ARTICLE I.

Llorente-Cortés V, Otero-Viñas M, Hurt-Camejo E, Martínez-González J, Badimon L. Human coronary smooth muscle cells internalize versican-modified LDL through LDL receptor-related protein and LDL receptors.

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Human Coronary Smooth Muscle Cells Internalize Versican-Modified LDL Through LDL Receptor–Related Protein and LDL Receptors

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Abstract—Versican-like proteoglycans are the main component of the intimal extracellular matrix interacting with low density lipoprotein (LDL). The aim of this study has been to investigate the receptors involved in versican-modified LDL uptake by human vascular smooth muscle cells (VSMCs). We have found that versican-LDL interaction leads to the following: (1) monomeric LDL particles that are similar in size and electrophoretic mobility to native LDL but that have a higher capacity to induce intracellular cholesteryl ester (CE) accumulation and (2) fused LDL particles similar in size to those obtained by vortexing. The precipitable fraction of versican-LDL, composed of 50% monomeric and 50% fused LDL particles, induced a dose-response increase in the CE content of VSMCs. Anti–LDL receptor antibody decreased the CE accumulation derived from monomeric LDL particles by 88±3% and that derived from the total precipitable fraction by 45±3%. Inhibition of LDL receptor–related protein expression by antisense oligodeoxynucleotides reduced the CE accumulation derived from the precipitable fraction by 65±2.8%, whereas it did not produce any effect on the CE accumulation derived from monomeric LDL. These results suggest that versican-LDL induces CE accumulation in human VSMCs by the LDL receptor (monomeric particles) and LDL receptor–related protein (fused LDL). (***Arterioscler Thromb Vasc Biol.*** **2002;22:387-393.)**

Key Words: vascular smooth muscle cells ■ LDL receptor–related protein ■ versican ■ cholesteryl ester accumulation ■ antisense oligodeoxynucleotides

Vascular proteoglycans (PGs) have a common structure of a core protein to which glycosaminoglycan (GAG) chains are covalently attached. The content of versican-like PG, the main PG structuring the extracellular matrix, is high in regions prone to lesion development and increases with lesion progression.[1–3] Interestingly, it has been demonstrated that LDL binds and is retained by versican-like PGs secreted by human vascular smooth muscle cells (VSMCs)[3–5] and macrophages.[6,7] The binding between LDL and versican or biglycan seems to be enhanced in the presence of lipoprotein lipase[8] or phospholipase A_2.[9,10] The relevance of the interaction between LDL and PG in vivo has been demonstrated (1) by the isolation of complexes apoB-100–chondroitin sulfate (CS), which is rich in negatively charged GAGs from human arteries[3]; (2) by the colocalization of apoB in the intima with CS-rich regions[11,12]; and (3) by the formation of complexes ex vivo between LDL and CS-PGs extracted from arterial wall.[13,14] Versican is one of the PGs with the highest binding affinity for plasma LDL. It has been described that GAGs accelerate proteolytic and oxidative modification of the particles[3] and can also induce LDL aggregation and fusion of the LDL particle under certain incubation conditions.[15] Fused LDLs, remarkably similar to those found in the arterial wall,[16,17] can be obtained by vortexing.[18] We have recently demonstrated that modified LDL particles generated by vortexing are taken up through the LDL receptor–related protein (LRP) in human VSMCs.[19] LRP is also the receptor that mediates the binding and internalization of other modified lipoproteins, such as apoE-enriched VLDL,[20] lipoprotein lipase-triglyceride–rich lipoprotein complexes,[21,22] Lp(a),[23] and chylomicron remnants.[24] Although LRP is expressed in normal vessels and atherosclerotic lesions, LRP expression increases in rabbit atherosclerotic lesions; furthermore, LRP seems to play a role in the development of atherosclerotic lesions.[25,26] In the present study, we report on the characteristics of changes induced in the LDL particle by the interaction with versican, the effect of versican-LDL on the cholesteryl ester (CE) content of VSMCs, and the involvement of LRP in the uptake of versican-LDLs in human VSMCs. Our results indicate that versican interaction with LDL leads to the following: (1) monomeric LDL particles (similar to native LDL [nLDL] in electrophoretic mobility and electron microscopy size) that enter the cells through the LDL receptor but are able to induce CE accumulation and (2) fused LDL particles (similar in size to those particles obtained by vortexing) that are internalized through the LRP. These results indicate that versican increases LDL atherogenicity.

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Methods

Materials

For an expanded Methods section, please refer to the online supplement (which can be accessed at http://atvb.ahajournals.org).

VSMC Culture

Primary cultures of human VSMCs were obtained from human coronary arteries of explanted hearts at transplant operations performed at the Hospital de la Santa Creu i Sant Pau. VSMCs were obtained by a modification of the explant technique, as we have described previously.[27,28]

LDL Preparation

Human LDLs (density 1.019 to 1.063 g/mL) were obtained from pooled sera of normocholesterolemic volunteers, isolated by sequential ultracentrifugation, and dialyzed as previously described.[27] Vortexed LDL was prepared by vortexing LDL in PBS at room temperature.[27–29] The aggregates formed after vortexing (LDLs in fused form, precipitable fraction) can be separated from the nonaggregated LDLs (nonprecipitable fraction) by centrifugation at 10 000*g* for 10 minutes[16,29]

Preparation of Versican-LDL

Versican was isolated from pig aortas according to procedures previously described.[10,30,31] The GAG composition of the aortic PGs varied between 50% and 65% for chondroitin-6-sulfate, between 10% and 25% for chondroitin-4-sulfate, and between 10% and 20% for dermatan sulfate. This variation was more related to the original composition of GAGs in the aorta than to the isolation procedure. The GAG-to-protein ratio varied between 7:3 and 6:4. The molecular size of the CS PG preparation by high-performance liquid chromatography and the GAG composition indicated that this preparation of CS PGs consisted mainly of the versican type of PG. The interaction between versican and LDL was carried out after the equilibration of LDL and versican in a solution containing 5 mmol/L HEPES, 20 mmol/L NaCl, 4 mmol/L $CaCl_2$, and 2 mmol/L $MgCl_2$, pH 7.2. LDL and versican in the protein (proportion 100:1) were incubated for 2 hours at 37°C. Versican-LDL, like vortexed LDL, did not show any change in thiobarbituric acid–reactive substance content from nLDL (data not shown). The precipitable fraction was separated from the nonprecipitable fraction by centrifugation at 10 000*g* for 10 minutes[16,29] and resuspended in a solution containing 5 mmol/L HEPES, 150 mmol/L NaCl, 4 mmol/L $CaCl_2$, and 2 mmol/L $MgCl_2$, pH 7.2.

Characterization of Vortexed LDL and Versican-LDL

The precipitable and nonprecipitable fractions of versican-LDL, compared with nLDL and the precipitable and nonprecipitable fractions of vortexed LDL, were analyzed by agarose gel electrophoresis and electron microscopy.

For transmission electron microscopy, the different LDLs were negatively stained with 2% uranyl acetate for 1 minute and were observed in a Hitachi 600 AB transmission electron microscope. Images were digitalized with a Bioscan Gatan camera. The estimation of the particle diameter was performed by using a software program (IMAT) designed by the central Services of the University of Barcelona.

To eliminate large LDL aggregates, the precipitable fraction of versican-LDL was filtered through a 0.22-μm filter, and the particles in the filtrate were characterized by agarose gel electrophoresis and nondenaturing acrylamide gradient gel electrophoresis (GGE). GGE was performed according to Nichols et al,[32] with small modifications. Two solutions at 2% and 16% were prepared by using a stock solution of acrylamide and bis-acrylamide (30% total, 5% cross-linker) and mixed by using 2 P-1 peristaltic pumps (Pharmacia). The different fractions from versican-LDL (5 μL at 0.5 to 1 mg/mL) were preincubated for 15 minutes with 10 μL Sudan black (0.1% [wt/vol]) in ethylene glycol and 5 μL saccharose (50% [wt/vol]). Ten microliters of this mixture was electrophoresed at 4°C for 30 minutes at 20 V, 30 minutes at 70 V, and 16 hours at 100 V. Bands were scanned by densitometry at 595 nm, and LDL size was determined by using a plasma pool containing LDL particles of known size (22.9±0.5, 24.5±0.2, 26.2±0.2, and 28.4±0.4 nm) as a standard. The diameter of standard LDL particles was assessed by electron microscopy.

Determination of Free and Esterified Cholesterol Content

This procedure is discussed online (http://atvb.ahajournals.org).

RT-PCR and Western Blot

This procedure is discussed online (http://atvb.ahajournals.org).

LRP ODN Treatment

VSMCs were treated with antisense or sense LRP oligodeoxynucleotides (ODNs, 10 μmol/L), as previously described.[33] Then, nLDL (precipitable and nonprecipitable fractions) from vortexed LDL and versican-LDL (40 μg/mL) were added to nontreated and to antisense ODN– and sense ODN–treated VSMCs 12 hours before ending the second 24 hours of the arresting period. Then, the cells were exhaustively washed and harvested into 1 mL of 0.10 mol/L NaOH. The determination of free cholesterol (FC) and CE content was performed as previously described.[27,28]

Results

Characterization of Versican-LDL

After the incubation of LDLs with versican, the turbidimetry of the LDL preparation (680 nm) increased from undetectable levels to 0.20 relative units, indicating the presence of LDL aggregates. Versican-LDLs, vortexed LDLs, and nLDLs were centrifuged (10 000*g*, 10 minutes) in parallel, and the precipitable and nonprecipitable fractions were analyzed by agarose gel electrophoresis (Figure 1) and electron microscopy (Figure 2). Particles of the nonprecipitable fraction of vortexed LDL or versican-LDL had an electrophoretic mobility similar to that of nLDL (Figure 1A). The precipitable fraction of versican-LDL was composed of LDL particles with an electrophoretic mobility similar to that of nLDL and of particles that did not enter into the agarose gel (remaining at the gel origin), whereas the precipitable fraction of vortexed LDL was composed only of particles that did not enter into the agarose gel. To further characterize the monomeric particles of the precipitable fraction of versican-LDL, we filtered this fraction through a 0.22-μm filter and, thus, obtained a new fraction (filtrate). Agarose gel electrophoresis showed that the filtrate was composed of monomeric particles with an electrophoretic mobility that was the same as that for nLDLs (Figure 1A). GGE assays (Figure 1B) showed that the filtrate was composed of monomeric particles with a diameter of ≈26.2 nm. The relative proportion of LDL in the filtrate was estimated as 50±2% of the precipitable LDL (either in protein or cholesterol). Electron microscopic analysis (Figure 2) revealed nLDL as monomeric particles (particle diameters ranged between 17 and 30 nm, Figure 2E) and the precipitable fraction of vortexed LDL as fused LDL (particle diameters ranged between 77 and 160 nm; Figure 2A and 2F). In contrast, the precipitable fraction of versican-LDL contained a mixture of fused LDL (particle diameters ranged between 92 and 166 nm; Figure 2C and 2G) and monomeric particles that were similar to those in the nonprecipitable fraction of versican-LDL and vortexed LDL (Figure 2D and Figure 2B, respectively) and to nLDL (Figure 2E).

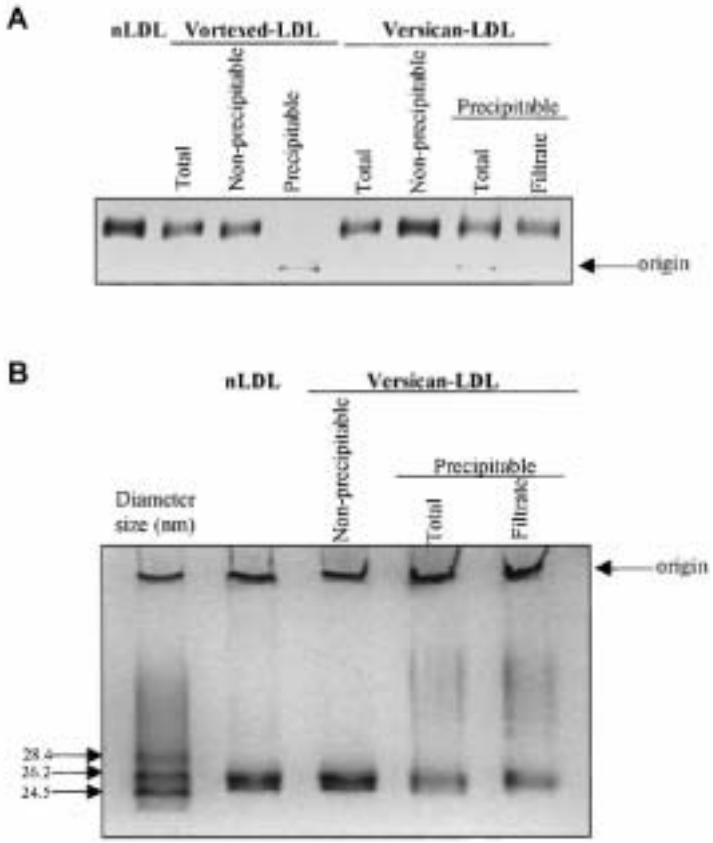

Figure 1. A, Agarose electrophoresis of nLDL, vortexed LDL, and versican-LDL. Vortexed LDL and versican-LDL were generated as described in Methods. After centrifugation at 10 000*g*, 1 aliquot from the precipitable fraction and 1 aliquot from the nonprecipitable fraction were applied to the electrophoresis gel. Arrow indicates electrophoretic origin. B, Nondenaturing acrylamide GGE of nLDL and versican-LDL. The precipitable fraction was filtered through a 0.22-μm filter, and the filtrate was applied beside the total precipitable and the nonprecipitable fraction into the GGE. The arrows indicate the position of bands corresponding to standard LDL of known diameter size.

Effect of Versican-LDL on VSMC Cholesterol Content

VSMCs were incubated in parallel with increasing concentrations of nLDL and the precipitable and nonprecipitable fractions of vortexed LDL and versican-LDL (20, 40, and 60 μg/mL). CE content increased in VSMCs incubated with the different types of LDL, whereas FC content of the VSMCs remained unaltered (see Figure I, which can be accessed online at http://atvb.ahajournals.org). nLDL induced a slight increase in CE content. However, monomeric particles that compose the nonprecipitable fraction of versican-LDL and vortexed LDL, although similar in size to nLDL, were able to induce a CE accumulation that was higher than that of nLDL. The precipitable fraction of versican-LDL induced a significant dose-dependent CE accumulation that was close to that induced by vortexed LDL (see online Figure I).

Cell Surface Binding of Versican-LDL

VSMCs were incubated with nLDL, with the precipitable and nonprecipitable fractions of vortexed LDL and versican-LDL (40 μg/mL), and simultaneously with different ligands, such as lactoferrin (ligand for the LDL receptor and the LRP), polyinosinic acid (ligand for the scavenger receptor), and galactose and fetuin (ligands for the asialoglycoprotein receptor. As shown in the online Table (which can be accessed at http://atvb.ahajournals.org), polyinosinic acid, galactose, and fetuin did not change CE accumulation levels. In contrast, lactoferrin produced a strong inhibition of the CE accumulation derived from all the lipoproteins tested. These results indicate that scavenger receptors or asialoglycoprotein receptors were not involved in versican-LDL uptake by VSMCs. To investigate whether the LDL receptor was involved in the uptake of the different fractions of versican-LDL, VSMCs were incubated with these fractions and increasing concentrations of anti–LDL receptor antibody. As shown in Figure II (which can be accessed online at http://atvb.ahajournals.org), CE accumulations induced by nLDL (27.21±4.5 μg CE per milligram protein), by the filtrate from the precipitable fraction of versican-LDL (47.36±5.2 μg CE per milligram protein), or by the nonprecipitable fraction of versican-LDL (44.52±2.2 μg CE per milligram protein) were almost abrogated by anti–LDL receptor antibody. However, the anti–LDL receptor antibody only partially inhibited (45±3% inhibition at 25 μg/mL) the CE accumulation induced by the precipitable fraction of versican-LDL (80.54±2 μg CE per milligram protein).

Effect of Antisense LRP ODNs on CE Accumulation From Versican-LDL

To determine the role of LRP on versican-LDL uptake, we tested the effect of versican-LDL on LRP ODN–treated VSMCs. The commercial anti-LRP antibody used to detect LRP in Western blot analysis was not able to inhibit LRP function (data not shown). Because other types of antibodies were not available, we used a molecular approach to test LRP function. LRP mRNA transcription was blocked by an antisense ODN previously designed by us.[19] LRP mRNA expression (see online Figure IIIA, which can be accessed at

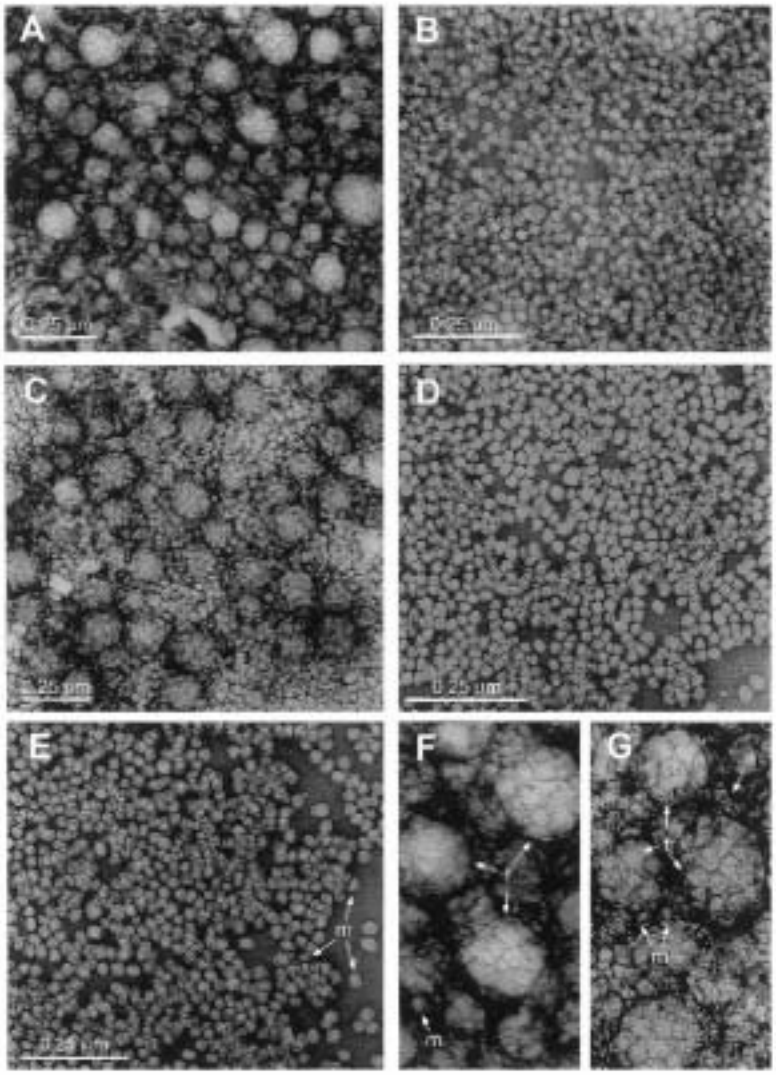

Figure 2. Electron microscopy of nLDL and nonprecipitable and precipitable fractions of vortexed LDL and versican-LDL. Samples were negatively stained and observed by electron microscopy as described in Methods. A, Precipitable fraction from vortexed LDL. B, Nonprecipitable fraction from vortexed LDL. C, Precipitable fraction from versican-LDL. D, Nonprecipitable fraction from versican-LDL. E, nLDL. F, Doubly magnified section from panel A. G, Doubly magnified section from panel C. m indicates monomeric; f, fused; and a, aggregated.

http://atvb.ahajournals.org) and LRP protein expression (see online Figure IIIB) decreased by 83±4.6% and 70±8.58%, respectively, in antisense ODN–treated VSMCs but not in sense ODN–treated VSMCs. LDL receptor mRNA expression (62.6±0.7 arbitrary units) was not altered by either antisense or sense LRP ODN treatment. As shown in Figure 3A and 3B, antisense LRP treatment reduced the CE accumulation derived from the precipitable fraction of vortexed LDL and versican-LDL by 70.8±1.4% and 65.3±3.5%, respectively, but it did not show any effect on CE accumulation derived from the nonprecipitable fraction of versican-LDL or vortexed LDL (Figure 3C).

Figure 3D shows microphotographs of representative cells after incubation of control and ODN-treated VSMCs with versican-LDL. Pictures were taken after the first PBS wash to eliminate free versican-LDL (not bound). As shown, untreated VSMCs (Figure 3D, a) had versican-fused LDL bound (arrows) on the cell surface, whereas antisense LRP ODN–treated VSMCs did not (Figure 3D, b). Sense ODN–treated VSMCs (Figure 3D, c) also had versican-fused LDL bound to the cell surface. Treatment of VSMCs with ODNs did not induce morphological changes in VSMCs.

Discussion

Secreted versican molecules occupy the tridimensional network of the intimal extracellular space and are highly expressed in human arteries with high susceptibility to atherosclerosis.[2] Our results demonstrate that LDL-versican interaction produces structural changes in the LDL particle. Versican was able to induce LDL fusion in a very short incubation time (2 hours), with few molecules of versican (protein versican–to–protein LDL ratio 1:100) and under physiological conditions as reported by Camejo and colleagues,[10,11] who described structural alterations in the apoB-100 surface structure by interaction with CS-PGs. The obtained fused particles are similar in size to those obtained by vortexing[18] and those described in atherosclerotic lesions.[16,17] The high capacity of versican to induce LDL fusion seems to be due to the high affinity of VSMC-secreted versican for LDL.[3] Fused LDLs generated by incubation with

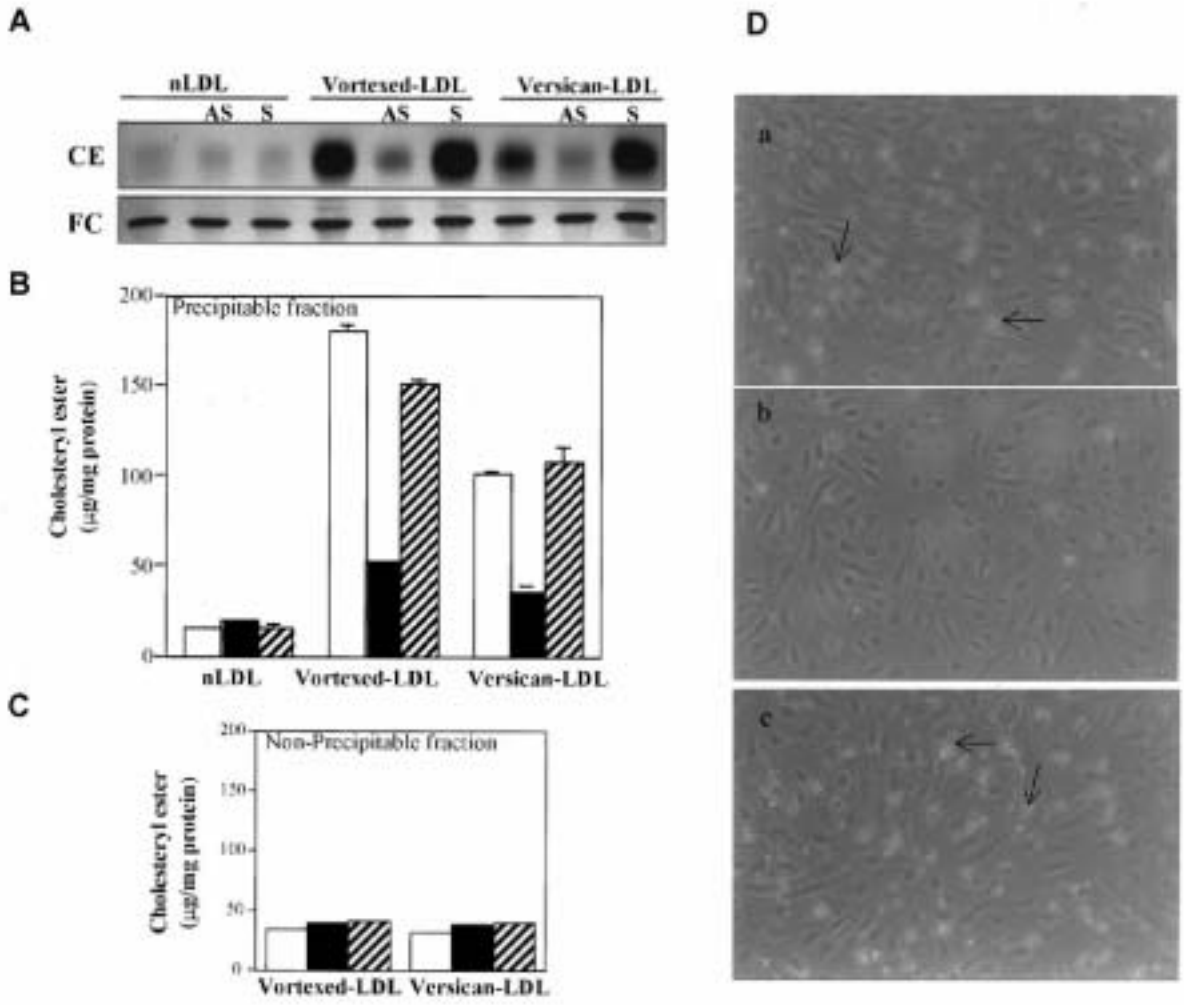

Figure 3. Effect of LRP ODN treatment on the CE accumulation derived from vortexed LDL and versican-LDL in VSMCs. VSMCs were treated with ODNs as explained in Methods. Then, VSMCs were exhaustively washed and harvested for measurement of FC and CE. A, Thin-layer chromatography showing the FC and CE bands corresponding to VSMCs treated with antisense ODN (AS) or sense ODN (S) against nontreated VSMCs (control) and incubated with the precipitable fraction. B, Bar graphs showing quantification of the CE bands in untreated VSMCs (open bars) and in antisense (solid bars) and sense (hatched bars) LRP ODN–treated VSMCs incubated with the precipitable fraction. C, Bar graphs showing the quantification of the CE bands in VSMCs incubated with the nonprecipitable fraction. Results are expressed as micrograms cholesterol per milligram protein and are shown as mean±SEM of 3 independent experiments. D, Effect of ODN anti-LRP antisense on versican-fused LDL binding by VSMCs. VSMCs were treated with LRP ODN, as explained in Methods (a, untreated VSMCs with versican-fused LDL bound [arrows] to the cell surface; b, antisense LRP ODN–treated VSMCs with no versican-fused LDL; and c, sense ODN–treated VSMCs with versican-fused LDL bound [arrows] to the cell surface). During the final 12 hours of the arresting period, versican-LDL (40 μg/mL) was added to the incubation medium. Then, VSMCs were washed with PBS and photographed (original magnification ×100). Arrows indicate versican-fused particles bound to the cell surface.

versican, contrary to those generated by vortexing, were not completely separated from monomeric LDLs by low-speed centrifugation. In fact, LDL vortexing induced such strong surface changes on LDLs that most LDL particles underwent fusion,[18] allowing a clear separation of monomeric and fused LDL particles. In contrast, the LDL modification induced by versican depends on the strength of versican-LDL interaction, which seems to be driven by the structural characteristics of the LDL.[3] In our experimental conditions, the formation of versican-LDL complexes was facilitated by using a low ionic strength buffer. Afterward, the precipitable fraction was resuspended in a physiological buffer with increased ionic strength, favoring separation of versican from LDL. The reversibility of the aggregation process, facilitated by the incubation conditions, and LDL heterogeneity[3,33] might explain the presence of monomeric and a few small LDL aggregates besides fused LDL particles in the precipitable fraction of versican-LDL.

The monomeric particles that precipitate are similar to those that remain in the nonprecipitable fraction, according to their electrophoretic mobility, size, and capacity to induce CE accumulation. Although monomeric particles were able to induce higher CE accumulation than were nLDLs, they were similar in size and electrophoretic mobility to nLDLs. In fact, the uptake of monomeric LDL particles was a saturable process that could be completely inhibited by LDL receptor

antibodies, indicating that these particles enter the cell through LDL receptors. Our results are in agreement with those obtained by Hurt-Camejo et al[34] and Hurt et al[35] and differ from those of Vijayagopal and colleagues,[36,37] who proposed that the LDL receptor is not involved in the uptake of PG-LDL complexes. The difference could be due to the nature of the complexes formed in the different studies; Vijayagopal et al, different from Hurt-Camejo et al, Hurt et al, or the present study, used a high ratio of PG to LDL and a buffer that stabilizes the complexes LDL-PG in the incubation media. The increase of CE accumulation induced by monomeric versican-LDL over nLDL could be explained by the selectivity of versican for small dense LDL particles.[38]

In contrast to the uptake of the nonprecipitable fraction, the uptake of the precipitable fraction, composed of monomeric and fused particles, involved a nonsaturable process. Because monomeric particles represent 50% of the LDL in the precipitable fraction, they are responsible for ≈40% of the CE accumulation induced by the precipitable fraction. Furthermore, the percentage of CE inhibition by LDL receptor antibodies fully corresponds to that induced by monomeric LDLs. According to our results, scavenger and asialoglycoprotein receptors are not involved in the CE accumulation derived from versican-LDL uptake. In contrast, we have demonstrated the involvement of LRP on versican-LDL internalization by the marked decrease of the LDL binding and CE accumulation derived from versican-LDL in antisense LRP ODN–treated VSMCs. Because this treatment has no effect on the CE accumulation derived from monomeric LDL, the observed 65% reduction would correspond to the CE accumulation induced by fused LDL. These results are in agreement with the high capacity of LRP to bind and internalize fused LDLs generated by vortexing,[19] which have a size similar to that of versican-fused LDLs.

In summary, we demonstrate that versican PGs have a very high capacity to induce fusion of LDL particles that are internalized through LRP in VSMCs. The LRP involvement on the internalization of versican-fused LDLs further enhances the importance of LRP as a lipoprotein receptor involved in VSMC foam cell formation. Although monomeric LDLs are also able to induce CE accumulation after interaction with versican with use of the LDL receptor, this receptor is only moderately expressed in the vascular wall cells, whereas LRP is highly expressed in normal and atherosclerotic lesions.[25,26] Because versican is one of the main PGs interacting with LDL, and LRP is one of the main lipoprotein receptors in the arterial wall, the uptake of versican-fused LDL through LRP is likely one of the main mechanisms contributing to VSMC LDL internalization.

Acknowledgments

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References

1. Hurt-Camejo E, Olsson U, Wiklund O, Bondjers G, Camejo G. Cellular consequences of the association of apoB lipoproteins with proteoglycans. *Arterioscler Thromb Vasc Biol*. 1997;17:1011–1017.
2. Wagner WD, Salisbury BG, Rowe HA. A proposed structure of chondroitin 6-sulfate proteoglycans of human normal and adjacent atherosclerotic plaque. *Arteriosclerosis*. 1988;6:407–417.
3. Camejo G, Fager G, Rosengren B, Hurt-Camejo E, Bondjers G. Binding of low density lipoproteins by proteoglycans synthesized by proliferating and quiescent human arterial smooth muscle cells. *J Biol Chem*. 1993; 268:14131–14137.
4. Olsson U, Camejo G, Hurt-Camejo E, Elfsber K, Wiklund O, Bondjers G. Possible functional interactions of apolipoprotein B-100 segments that associate with cell proteoglycans and the apoB/E receptor. *Arterioscler Thromb Vasc Biol*. 1997;17:149–155.
5. Pentikäinen MO, Lehtonen EMP, Öörni K, Luoa S, Somerharjus P, Jauhiainen M, Kovanen PT. Human arterial proteoglycans increase the rate of proteolytic fusion of low density lipoprotein particles. *J Biol Chem*. 1997;272:25238–25288.
6. Maor I, Aviram M. Macrophage released proteoglycans are involved in cell-mediated aggregation of LDL. *Atherosclerosis*. 1988;142:57–66.
7. Maor I, Hayek T, Hirsh M, Iancu TC, Aviram M. Macrophage-released proteoglycans enhance LDL aggregation: studies in aorta from apolipoprotein E-deficient mice. *Atherosclerosis*. 2000;150:91–101.
8. Olin K, Chait A, Wight T. Lipoprotein lipase enhances the binding of native and oxidized low density lipoproteins to biglycan and versican. *Circulation*. 1997;96(suppl I):I-40. Abstract.
9. Sartipy P, Camejo G, Svensson L, Hurt-Camejo E. Phospholipase A_2 modification of low density lipoproteins forms small high density particles with increased affinity for proteoglycans and glycosaminoglycans. *J Biol Chem*. 1999;274:25913–25920.
10. Hakala JK, Öörni K, Pentikäinen MO, Hurt-Camejo E, Kovanen PT. Lypolysis of LDL by human secretory phospholipase A_2 induces particle fusion and enhances the retention of LDL to human aortic proteoglycans. *Arterioscler Thromb Vasc Biol*. 2001;21:1053–1061.
11. Frank JS, Fogelman AM. Ultrastructure of the intima WHHL and cholesterol-fed rabbit aortas prepared by ultra rapid freezing and freeze-etching. *J Lipid Res*. 1989;30:967–978.
12. O'Brien KD, Olin KL, Alpers CE, Wimie Ch, Ferguson M, Hudkins K, Wight TN, Chait A. Comparison of apolipoprotein and proteoglycan deposits in human coronary atherosclerotic plaques. *Circulation*. 1998; 98:519–527.
13. Steele R, Wagner W. Lipoprotein interaction with artery wall derived proteoglycan: comparisons between atherosclerosis-susceptible WC-2 and resistant Show Racer pigeons. *Atherosclerosis*. 1987;65:63–73.
14. Camejo G, Olofsson S-O, Lopez F, Carlsson P, Bondjers G. Identification of apoB-100 segments mediating the interaction of low density lipoproteins with arterial proteoglycans. *Arteriosclerosis*. 1988;8: 368–377.
15. Tirzin D, Jinga VV, Serban G, Simonescu M. The effects of low density lipoprotein modified by incubation with chondroitin 6-sulfate on human aortic smooth muscle cells. *Atherosclerosis*. 1999;147:155–166.
16. Guyton JR, Klemp KF, Mims PM. Altered ultrastructural morphology of self-aggregated low density lipoproteins: coalescence of lipid domains forming droplets and vesicles. *J Lipid Res*. 1991;32:953–961.
17. Aviram M, Maor Y, Keidar S, Hayek T, Oiknine J, Bae EIY, Adler Z, Kertzman V, Milo S. Lesioned low density lipoprotein in atherosclerotic apolipoprotein E-deficient transgenic mice and in humans is oxidized and aggregated. *Biochim Biophys Res Commun*. 1995;216:501–513.
18. Pentikäinen MO, Lehtonen EMP, Kovanen PT. Aggregation and fusion of modified low density lipoprotein. *J Lipid Res*. 1996;37:2638–2649.
19. Llorente-Cortés V, Martínez-González J, Badimon L. LDL receptor-related protein mediates uptake of aggregated LDL in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2000;20: 1572–1579.
20. Kowal RC, Herz J, Goldstein JL, Esser V, Brown MS. Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived form apolipoprotein E-enriched lipoproteins. *Proc Natl Acad Sci U S A*. 1989;86:5810–5814.
21. Chappel DA, Fry GL, Waknitz MA, Iverius PH, Williams SE, Strickland DK. The low density lipoprotein receptor-related protein/α_2 macroglobulin receptor binds and mediates catabolism of bovine milk lipoprotein lipase. *J Biol Chem*. 1992;267:25764–25767.
22. Chappel DA, Fry GL, Waknitz LE, Muhonen LE, Pladet MW, Iverius PH, Strickland DK. Lipoprotein lipase induces catabolism of normal triglyceride-rich lipoproteins via the low density lipoprotein receptor-

related protein/α2-macroglobulin receptor in vitro. *J Biol Chem*. 1992; 268:14168–14175.
23. Rebiln T, Niemeier A, Meyer N, Willnow TE, Kronenberg F, Dieplinger H, Greten H, Beisiegel U. Cellular uptake of lipoprotein (a) by mouse embryonic fibroblasts via the LDL receptor and the LDL receptor-related protein. *J Lipid Res*. 1997;38:2103–2110.
24. Fujioka Y, Cooper AD, Fong LG. Multiple processes are involved in the uptake of chylomicron remnant by mouse peritoneal macrophages. *J Lipid Res*. 1998;39:2339–2349.
25. Luoma J, Hiltunen T, Särkioja T, Moestrup SK, Gliemann J, Kodama T, Näkari T, Ylä-Herttuala S. Expression of α2-macroglobulin receptor/low density lipoprotein receptor-related protein and scavenger receptor in human atherosclerotic lesions. *J Clin Invest*. 1994;93:2014–2021.
26. Hiltunen TP, Luoma JS, Nikkari T, Ylä-Herttuala S. Expression of LDL receptor, VLDL receptor, LDL receptor-related protein, and scavenger receptor in rabbit atherosclerotic lesions. *Circulation*. 1998;97: 1079–1086.
27. Llorente-Cortés V, Martínez-González J, Badimon L. Differential cholesteryl ester accumulation in two human vascular smooth subpopulations exposed to aggregated LDL: effect of PDGF-stimulation and HMG-CoA reductase inhibition. *Atherosclerosis*. 1999;144:335–342.
28. Llorente-Cortés V, Martínez-González J, Badimon L. Esterified cholesterol accumulation induced by aggregated LDL uptake in human vascular smooth muscle cells is reduced by HMG-CoA reductase inhibitors. *Arterioscler Thromb Vasc Biol*. 1998;18:738–746.
29. Khoo JC, Miller E, Loughlin Mc, Steinberg D. Enhanced macrophage uptake of low density lipoprotein after self-aggregation. *Arteriosclerosis*. 1988;8:348–358.
30. Camejo G, Hurt E, Wiklund O, Rosengren B, López F, Bondjers G. Modification of low-density lipoproteins induced by arterial proteoglycans and chondroitin-6-sulfate. *Biochem Biophys Acta*. 1991;1096: 253–261.
31. Camejo G, Hurt-Camejo E, Rosengren B, Wiklund O, López F, Bondjers G. Modification of copper-catalyzed oxidation of low density lipoprotein by proteoglycans and glycosaminoglycans. *J Lipid Res*. 1991;32: 1983–1991.
32. Nichols AV, Krauss RM, Musliner TA. Nondenaturing polyacrylamide gradient gel electrophoresis. In: Segrest JP, Albers JJ, eds. *Methods in Enzymology: Plasma Lipoproteins*. New York, NY: Academic Press; 1986:417–431.
33. Nigon F, Lesnik P, Rouis M, Chapman MJ. Discrete subspecies of human low density lipoproteins are heterogeneous in their interaction with the cellular LDL receptor. *J Lipid Res*. 1991;32:1741–1753.
34. Hurt-Camejo E, Camejo G, Rosengren E, López F, Ahlström C, Fager G, Bondjers G. Effect of arterial proteoglycans and glycosaminoglycans on low density lipoprotein oxidation and its uptake by human macrophages and arterial smooth muscle cells. *Arterioscler Thromb*. 1992;12:569–583.
35. Hurt E, Bondjers G, Camejo G. Interaction of LDL with human arterial proteoglycans stimulates its uptake by human monocyte-derived macrophages. *J Lipid Res*. 1990;31:443–454.
36. Vijayagopal P, Srinivasan SR, Jones KM, Radhakrishnamurthy B, Berenson GS. Metabolism of low-density lipoprotein-proteoglycan complex by macrophages: further evidence for a receptor pathway. *Biochim Biophys Acta*. 1988;960:210–219.
37. Vijayagopal P, Srinivasan SR, Radhakrishnamurthy B, Berenson GS. Human monocyte-derived macrophages bind low-density-lipoprotein-proteoglycan complexes by a receptor different from the low-density-lipoprotein receptor. *Biochem J*. 1993;289:837–844.
38. Hurt-Camejo E, Camejo G, Rosengren B, López F, Wiklund O, Bondjers G. Differential uptake of proteoglycan-selected subfractions of low density lipoprotein by human macrophages. *J Lipid Res*. 1990;31: 1387–1398.

III.2. ANNEX ARTICLE I (*On-line*)

Methods

Materials

Cell culture medium and reagents were from Gibco Laboratories (Renfrewshire, UK). ECL chemiluminiscent detection system was purchased from Amersham. Anti-LDL receptor antibodies (LP02) and anti-LRP β chain (clone 8B8 RDI 61067) were purchased from Oncogene and Research Diagnostics, Inc, respectively. Bovine lactoferrin and polyinosinic acid (5'), D(+) galactose and fetuin were obtained from Sigma Chemical Co. Bicinchoninic acid protein assay were from Pierce (Rockford, Illinois). Tripure™ isolation reagent, oligodeoxynucleotides, PCR DIG labelling mix and Expand ™ High Fidelity DNA polymerase were from Roche Molecular Biochemicals. Nytran TM-plus membranes were from Schleicher & Schuell. M-MLV-RT was from Life Technologies.

Determination of free and esterified cholesterol content

In order to know the effect of versican-LDL on the cholesterol content of VSMC, arrested cells (48 hours) were incubated with increasing concentrations of LDL (20, 40 and 60 μg/mL) for 18 hours. All the experiments were performed in parallel with the precipitable and non-precipitable fractions from versican-LDL and vortexed-LDL. Different ligands such as lactoferrin (50 μg/mL), polyinosinic acid (50 μg/mL), galactose (50 mM), fetuin (100 μg/mL) and the anti-LDL receptor antibody (12.5, 25 μg/mL) were tested on the CE accumulation derived from versican-LDL and vortexed-LDL (40 μg/mL). Then, cells were exhaustively washed, twice with PBS, twice with PBS/1% BSA and twice with PBS/1% BSA/heparin 100 U/mL before harvesting into 1 mL of 0.10 mol/L NaOH. Lipid extraction and TLC was performed as previously described.[27,28]

RT-PCR

Total RNA and protein were isolated by using the Tripure Reagent™ according to the manufacturer. LRP mRNA levels were analyzed by RT-PCR. 0.5 μg RNA was reverse transcribed in a 15 μL reaction mixture containing 0.02 μg/μL oligo-dT, 20 U RNAsin, 1 mmol/L dNTPs, 20 mM DTT, 200 U M-MLV-RT, 50 mmol/L Tris-HCl pH 8.3, 75 mmol/L KCl and 3 mmol/L $MgCl_2$. Reaction was performed at 42ºC for 1 hour. The cDNA obtained was diluted 1:2. An aliquot of 1 μL of this dilution was amplified in a 25 μL reaction mixture containing: 2.5 μL PCR DIG labelling mix, 1.3 U Expand ™ High Fidelity DNA polimerase and 100 ng of each specific oligonucleotides in 1xbuffer and 1.5 mmol/L $MgCl_2$. The specific oligonucleotides selected were: LRP: 5'-ccagaagattgatggcactg-3' (upper primer), 5'-tagacactgccactccgata-3'(lower primer). Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalize results using the following primers: 5'-ttcaccaccatggagaaggc-3' (upper primer), 5-gcagggatgatgttctgggc-3'(lower primer). Amplification was carried out by 18 (GAPDH) or 20 (LRP) cycles of 94ºC 30'', 53ºC 40'' and 72ºC 1 min followed by a final extension of 72ºC 7 min. PCR products (10 μL) were resolved by electrophoresis in 1.5%-2% agarose gels and transferred onto nylon membranes by standard capillary technique. Blots were UV cross-linked. Detection of digoxigenine (DIG)-labelled nucleic acids was performed with an anti-DIG antibody linked to alkaline phosphatase and CSPD was used as substrate.

Western blot

Between 15-20 μg of total protein were separated on 7.5% polyacrylamide gels under non-reducing conditions. Proteins were blotted onto nitrocellulose membranes at 40 mA for 2 h at 4ºC. The residual binding capacity of the membranes was blocked with 5% non-fat milk in 10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20. Blots were incubated with monoclonal antibodies against human anti-LRP β chain (RDI-PRO61067, Research Diagnostics). Bound antibody was detected by using the appropiate horseradish peroxidase-conjugated antibody. Signals were detected with the ECL chemiluminiscent detection system on a standard X ray system.

Results

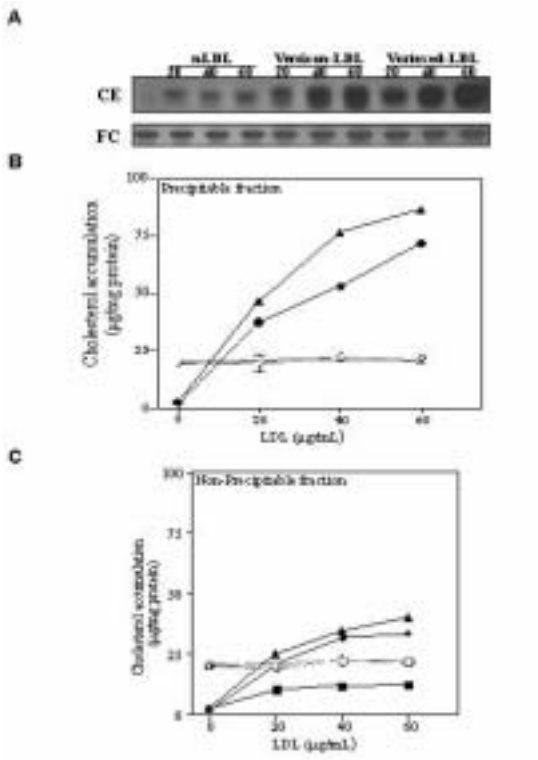
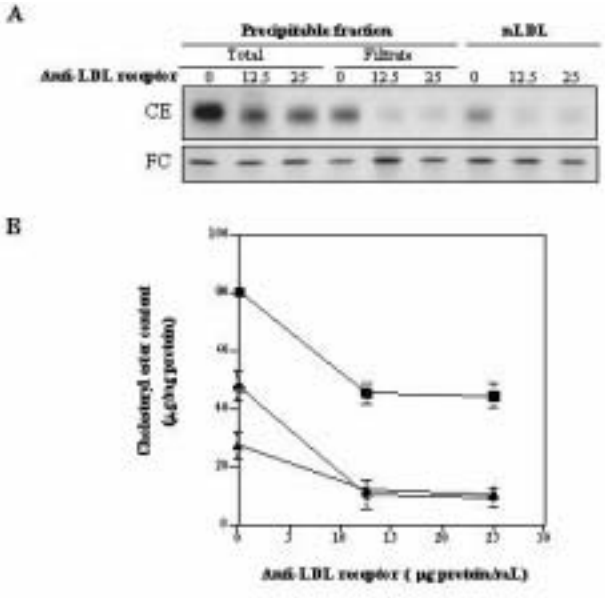


Figure IV.
Effect of increasing concentrations of nLDL, versican-LDL and vortexed-LDL on the cholesterol content of human VSMC. VSMC were incubated with increasing concentrations (20, 40, 60 µg/mL) of nLDL (squares), precipitable and non-precipitable fractions from versican-LDL (circles) and vortexed-LDL (triangles). After this period, VSMCs were exhaustively washed and harvested for measurament of CE (solid symbols) and FC (open symbols) A, TLC showing the FC and CE bands corresponding to VSMC incubated with the precipitable fractions. B, Line graph showing the quantification of CE bands and FC bands in VSMC incubated with the precipitable fraction. C, Line graph showing the quantification of CE and FC from VSMCs incubated with the non-precipitable fraction. Results are expressed as µg cholesterol per milligram protein and are shown as mean ± SEM of one representative experiment performed in triplicate.

Figure V.
Effect of the antibodies anti-LDL receptor on the CE accumulation induced by nLDL and versican-LDL. VSMC were incubated with nLDL, with the total precipitable fraction and the filtrate (40 µg/mL) from versican-LDL and with the non-precipitable fraction of versican-LDL and simultaneously with increasing concentrations of monoclonal anti-LDL receptor antibodies (12.5, 25 µg/mL) for 18 hours. A, TLC showing the bands corresponding to FC and CE of VSMC incubated with the precipitable fraction from versican-LDL and nLDL. B, Line graph showing the quantification of CE bands of VSMC incubated with nLDL (triangles), total precipitable fraction (squares), filtrate (cicles) and non-precipitable fraction from versican-LDL (diamond). Results are expressed as µg cholesterol per milligram protein and are shown as mean ± SEM of three independent samples from one representative experiment.



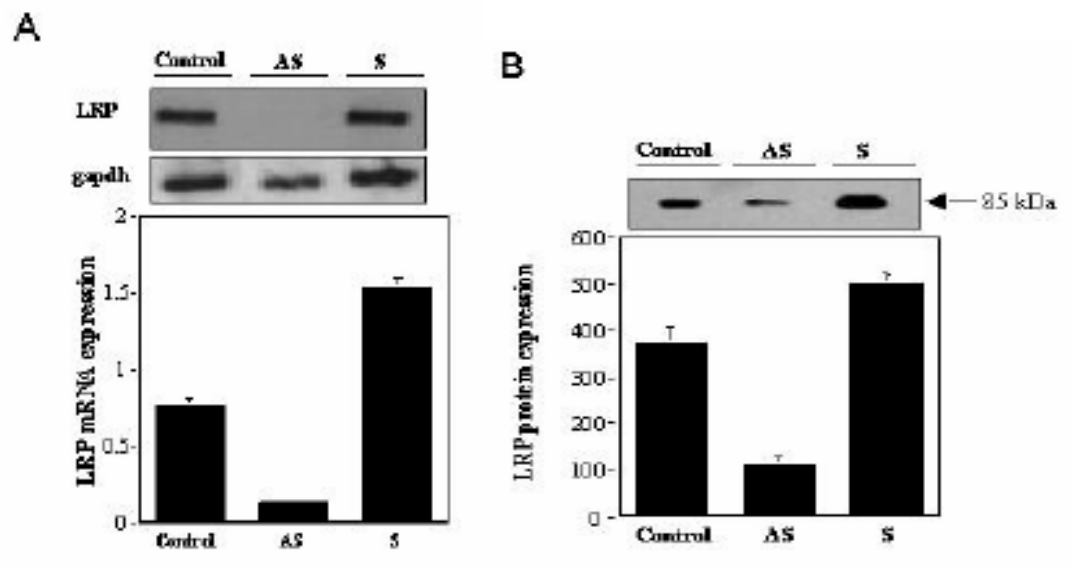


Figure VI. Effect of LRP ODN treatment on LRP expression by VSMC. VSMC were treated with LRP ODN as explained in Methods. RNA and protein were isolated after collecting the cells in Tripure^{TM} isolation reagent. A, RT-PCR showing LRP mRNA expression in VSMC incubated with antisense LRP ODN (AS) or sense LRP ODN (S) against nontreated VSMC (control). The bar graph represent normalized values against GAPDH bands intensities (arbitrary units) of three independent experiments. B, Western blot analysis using specific anti-LRP β chain (85 kDa) antibodies. The bar graphs represent the quantification of the band intensities (arbitrary units) of two different experiments.

Table I. Effect of different ligands on the CE accumulation derived from versican-LDL and vortexed-LDL uptake in human VSMC.

	nLDL	Versican-LDL		Vortexed-LDL	
Ligand		**precipitable**	**Non-precipitable**	**precipitable**	**Non-precipitable**
None	17.06±2	80.54±3.7	29.90±2.5	176.65±14.9	26.81±13.5
Lactoferrin (50 μg/mL)	4.02±1.2*	38.17±2.8*	9.63±1.4*	40.35±11.2*	8.6±1.5*
Polyinosinic acid (50 μg/mL)	20.64±1.3	76.02±5.7	33.10±2.2	174.52±10.70	30.80±5.6
Galactose (50 mM)	19.32±5.0	87.73±5.9	34.10±2.0	180.62±13.2	38.45±2.5
Fetuin (100 μg/mL)	17.35±6	95.05±3.2	33.60±1.6	185.40±6.5	24.80±6.1

Cells were incubated for 18 hours with nLDL, the precipitable and non-precipitable fractions of versican-LDL and vortexed-LDL and simultaneously with the different ligands tested. Data are expressed as μg CE per mg protein and are shown as the mean±SEM of triplicate samples of one representative experiment. *$p<0.05$, vs CE in untreated cells.

III.3. ARTICLE II

Llorente-Cortés V, Otero-Viñas M*, Badimon L. Differential role of heparan sulfate proteoglycans on aggregated LDL uptake in human vascular smooth muscle cells and mouse embryonic fibroblasts.*

**Ambdós autors han contribuït per igual a l'article.*

Arterioscler Thromb Vasc Biol. 2002;22:1905-1911. SCI: 6,35.

Differential Role of Heparan Sulfate Proteoglycans on Aggregated LDL Uptake in Human Vascular Smooth Muscle Cells and Mouse Embryonic Fibroblasts

Vicenta Llorente-Cortés,* Marta Otero-Viñas,* Lina Badimon

Objective—Low density lipoprotein (LDL) receptor–related protein (LRP) binds and internalizes aggregated LDL (agLDL) in human vascular smooth muscle cells (VSMCs). To analyze the contribution of proteoglycans (PGs) to agLDL uptake in human VSMCs, in wild-type mouse embryonic fibroblasts (MEF line), and in LRP-deficient mouse embryonic fibroblasts (PEA13 line).

Methods and Results—PGs in the medium and cellular and extracellular matrix have been isolated by metabolic radiolabeling with [^{35}S]Na_2SO_4 and characterized by selective digestion with heparinase I and III (4 U/mL each) and chondroitinase ABC (2 U/mL). To examine the contribution of PGs and LRPs to agLDL internalization, nonexpressing and LRP-expressing cells, treated or not with polysaccharidase, were incubated with agLDL (25, 50, and 100 μg/mL) for 18 hours. In human VSMCs, agLDL was unable to induce cholesteryl ester (CE) accumulation in antisense LRP-oligodeoxynucleotide–treated cells, and heparan sulfate (HS)-PG depletion leads to a reduction of the CE accumulation. In mouse fibroblasts, PEA13 compared with MEF showed lower, but still considerable, CE accumulation, and HS-PG depletion almost completely inhibited CE accumulation.

Conclusions—In MEF, HS-PGs can function alone as receptors that bind and internalize agLDL in the absence of LRP, but in human VSMCs, although HS-PGs facilitate agLDL binding to the cells, LRP is essential for agLDL internalization. **(*Arterioscler Thromb Vasc Biol.* 2002;22:1905-1911.)**

Key Words: heparan sulfate proteoglycans ■ human vascular smooth muscle cells ■ mouse embryonic fibroblasts ■ aggregated LDL ■ LDL-related protein

In the intima, proteoglycans (PGs) are in the pericellular space of the endothelial and smooth muscle cells and are also the major constituents of the extracellular matrix (ECM).[1] Vascular smooth muscle cells (VSMCs) secrete most chondroitin sulfate (CS)-PGs associated with hyaluronan, a glycosaminoglycan (GAG). Indeed, versican (a CS-like PG) is the main PG structuring the ECM.[2–4] Several studies demonstrate that CS-PGs act as sites for apoB-100 lipoprotein retention by the interaction between positively charged heparin-binding domains on apoB-100 or apoE and negatively charged GAG chains of the PGs.[2,4] The GAGs of versican induce alterations in the LDL particle that lead to the formation of fused and aggregated LDL (agLDL).[5,6] VSMCs also synthesize heparan sulfate (HS)-PGs, which can be secreted (perlecan) or shed from the cell surface (perlecan, syndecan, or glypican).[7,8] In contrast to CS-PGs, which play a major role in LDL retention and modification in arterial intima, HS-PGs may act as potential receptors for atherogenic lipoproteins[9–11] or facilitate the uptake of ligands by a process called ligand transfer to lipoprotein receptors, such as the LDL receptor–related protein (LRP).[12–16] We previously demonstrated that LRP binds and internalizes agLDL in human VSMCs.[6,17] The aim of the present study was to analyze the contribution of PGs and LRPs on agLDL internalization by human VSMCs and fibroblasts. HS-PGs and CS-PGs from the medium and from cellular and ECM fractions have been characterized by metabolic radiolabeling and selective digestion of PGs with heparinase I and III (HSI&III)[18,19] and chondroitinase ABC (ChABC),[20] respectively. To examine the contribution of PGs and LRPs on agLDL internalization, human VSMCs, which do not express LRP (antisense LRP-oligodeoxynucleotide [ODN]–treated VSMCs),[6,17] and LRP-deficient mouse embryo fibroblasts (PEA13 line)[21] in parallel with LRP-expressing cells (either treated or not with polysaccharidase) were incubated with increasing concentrations of agLDL (25, 50, and 100 μg/mL). We have found that in VSMCs, CS-PGs are the major component of cellular matrix and ECM. In contrast, HS-PGs are more abundant than CS-PGs in cellular and ECM fractions of wild-type mouse embryonic fibroblasts (MEF line). Although HSI&III and ChABC treatment completely degrades HS-PGs and CS-PGs, respectively, only HS-PG cleav-

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age has consequences for agLDL internalization in both cell types. However, there are marked differences in the role of HS-PGs between human VSMCs and fibroblasts. In fibroblasts, HS-PGs alone can function as receptors that bind and internalize agLDL in the absence of LRP. In contrast, in human VSMCs, although HS-PGs facilitate the agLDL binding to the cells, LRP is essential for agLDL internalization.

Methods

Materials

Cell culture medium and reagents were from GIBCO Laboratories. MEF (CRL-2214) and PEA13 (CRL-2216) fibroblasts were from American Type Culture Collection. Benzamidine-HCl, Triton X-100, ε-amino caproic acid, guanidinium-HCl, BSA, cetylpyridinium chloride, heparinase I (heparin lyase I, EC 4.2.2.7), heparinase III (heparin lyase III, heparitinase I; EC 4.2.2.8), and ChABC (ChABC lyase, EC 4.2.2.4) were from Sigma Chemical Co. HiTrap Q ion exchange columns and [^{35}S]Na_2SO_4 (100 mCi/mmol) were from Amersham Pharmacia Biotech. Bicinchoninic acid protein assays were from Pierce. Four percent to 12% Tris-glycine gels and Sypro Ruby protein gel staining were from Bio-Rad, and EN^{3}HANCE (NEF981G) was from NEN Life Sciences.

Cell Culture

Primary cultures of human VSMCs were obtained from human coronary arteries of explanted hearts at transplant operations performed at the Hospital de la Santa Creu i Sant Pau. VSMCs were obtained by a modification of the explant technique that we described previously.[5,17] Explants were incubated at 37°C in a humidified atmosphere of 5% CO_2. Outgrown cells were suspended in a solution of trypsin/EDTA and subcultured. They grew in monolayers in DMEM supplemented with 20% FCS, 2% human serum, 2 mmol/L L-glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin. VSMCs were used between passages 2 and 6. Nonexpressing LRP human VSMCs were obtained by treatment of the cells with antisense LRP-ODNs as previously described.[5,17]

MEF and PEA13 were grown in DMEM supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin, as previously described.[23]

ECM Ultrastructure

Cells grown on coverslips were treated or not with heparinase I (4 U/mL) or ChABC (2 U/mL) at 37°C for 2 hours. They were then fixed with glutaraldehyde (1.6%), washed, cryoprotected in 10% methanol, and cryofixed by projection against a copper block cooled by liquid nitrogen (−196°C) with the use of Cryovacublock de Reichert-Jung (Leica). The frozen samples were stored at −196°C in liquid nitrogen until subsequent use. Samples were freeze-dried and coated with platinum and carbon by using a freeze-etching unit (model BAF 060, BAL-TEC). A rotatory shadowing of the exposed surface was made by evaporating 10 nm of carbon evaporated at a 75° angle. The replica was separated from the coverslip by immersion in 38% hydrofluoric acid, washed twice in distilled water, and digested with 5% sodium hypochlorite for 5 to 10 minutes. Finally, the replicas were washed several times in distilled water, broken into small pieces, and picked up on copper grids coated with plastic for electron microscopy. All electron micrographs were obtained by using an electron microscope (Hitachi HU-600), operating at 75 kV.

Radiolabeling and Digestion of PGs

Cells were synchronized in medium containing 0.2% FCS for 2 days. Then the medium was removed, and fresh DMEM (10% FCS) containing 20 μCi/mL [^{35}S]Na_2SO_4 was added and maintained for 3 days to biosynthetically label PGs as previously described.[22] CS-PGs and HS-PGs were digested by adding a mixture of HSI&III (4 U/mL each) or ChABC (2 U/mL), respectively, to the incubation media for 2 or 18 hours. Control cells without PG enzymatic digestion were processed in parallel.

Isolation of PGs

After the 3 days of labeling, the culture medium from cells treated or not with enzymes for 2 or 18 hours in the absence or presence of agLDL (100 μg/mL) were transferred to tubes. Protease inhibitors were added to a final concentration of 10 mmol/L EDTA, 10 mmol/L ε-amino caproic acid, and 1 mg/mL benzamidine-HCl, and the medium was stored at −20°C until use.

Cells were washed with PBS containing 50 μg/mL heparin for 30 minutes at room temperature. The heparin-containing buffer was removed, and the cells were washed 3 times with PBS without heparin and dissolved by 2 extractions (5 mL each) of buffer containing 1% Triton X-100, 0.15 mol/L NaCl, 10 mmol/L Tris, 5 mmol/L $MgCl_2$, 2 mmol/L EDTA, 0.255 mmol/L dithiothreitol, and 1 μmol/L AEBSF, pH 7.2. After incubation for 30 minutes under gentle shaking, the cellular extract was removed and stored at −20°C until use. The remaining matrix was washed with PBS and solubilized by 2 extractions (5 mL each) of 8 mol/L urea, 2 mmol/L EDTA, 0.5% Triton X-100, and 20 mmol/L Tris-HCl (pH 7.5) containing protease inhibitors (1 mg/mL benzamidine-HCl and 10 mmol/L ε-amino-ε-caproic acid). The wells were left overnight at 4°C before the ECM extract was collected with a cell scraper.[23]

The culture media and cellular and ECM extracts were dialyzed against binding buffer (8 mol/L urea, 2 mmol/L EDTA, 0.5% Triton X-100, and 20 mmol/L Tris-HCl, pH 7.5) for 48 hours at 4°C and were then chromatographed on a HiTrap Q (5-mL) column equilibrated with binding buffer at a flow rate of 5 mL/min. The ^{35}S-labeled PG-containing fractions were collected after elution with a linear NaCl gradient (0.25 to 3 mol/L NaCl) and dialyzed at 4°C against water.

PG Characterization by SDS-PAGE

Equal amounts of protein from VSMCs and fibroblasts were loaded on a precasted 4% to 12% Tris-glycine gels, and SDS-PAGE was run for 2 hours at 60 V. The proteins were then fixed with methanol/acetic acid, stained with Sypro Ruby protein gel stain, and observed under UV light to control the equal protein loading.

The gels were impregnated with EN^{3}HANCE before drying. The dried gels were placed for autoradiography at −80°C for 14 days before they were developed.

LDL Preparation and Modification

Human LDLs (density 1.019 to 1.063 g/mL) were obtained from pooled sera of normocholesterolemic volunteers, isolated by sequential ultracentrifugation, and dialyzed. The model system of agLDL was generated by vortexing as previously described.[5,19,24]

Determination of Intracellular Cholesterol Content

Arrested VSMCs or fibroblasts were untreated or treated with HSI&III (4 U/mL each) or ChABC (2 U/mL) for 2 hours before the addition of increasing concentrations of agLDL (25, 50, 100 μg/mL) to the incubation medium containing the enzymes. After 18 hours, cells were exhaustively washed and harvested into 1 mL of 0.10 mol/L NaOH. Lipid extraction and thin-layer chromatography (TLC) were performed as previously described.[5,17,24] The spots corresponding to free cholesterol and cholesteryl esters (CEs) were quantified by densitometry against the standard curve of cholesterol and cholesterol palmitate, respectively, by using a computing densitometer (Molecular Dynamics).

Results

Characterization of PGs Synthesized by Human VSMCs and MEF

Human VSMCs and MEF were metabolically labeled with [^{35}S]Na_2SO_4 for 3 days, and PGs from the medium and cellular and ECM fractions were purified from untreated or

HS-PGs and CS-PGs Content of the Medium, Cell, and ECM Fraction From Human VSMCs and MEF

	Untreated Cells	HSI&III-Treated Cells		ChABC-Treated Cells	
Fraction	PG (Total)	No HS-PGs (Remaining)	HS-PGs (Digested)	No CS-PGs (Remaining)	CS-PG (Digested)
Human VSMCs					
Medium					
a	16.78±2.80	17.23±1.30	0.00±0.40	7.18±0.71	9.60±2.10
b	100	100±8	0±5	43±3	57±3
Cell					
a	27.48±1.65	25.93±2.63	1.56±0.97	6.63±0.44	20.87±2.06
b	100	94±4	7.5±2.5	24±3	76±3
ECM					
a	3.32±0.42	2.52±0.22	0.80±0.20	1.19±0.07	2.12±0.49
b	100	74.5±1.5	24±3.5	36.5±6.5	63±7
MEF					
Medium					
a	15.02±0.49	16.63±0.93	0.00±0.54	8.89±0.35	6.77±0.20
b	100	100±6	0±6	75.5±11.5	34.5±8.5
Cell					
a	20.55±4.26	13.96±3.24	6.59±1.02	13.78±4.46	3.30±2.0
b	100	68±1.5	32.5±1.5	65.5±8.5	24.5±11.5
ECM					
a	1.46±0.05	0.60±0.05	0.86±0.04	0.69±0.09	0.77±0.02
b	100	41±2	59±2	47±3.5	52.5±4.5

a, Results are expressed as the area of the peaks in cpm ($\times 10^3$) and are shown as the mean of one experiment performed in duplicate.

b, Results are expressed as a percentage of the peak areas obtained from every fraction of untreated cells.

enzyme-treated cells. Enzymatic treatment was performed by incubating the cells with HSI&III (4 U/mL each) or ChABC (2 U/mL) for 2 hours. To test the maintenance of the enzymatic activity during the agLDL incubation period, we also tested the pattern of PGs after 18 hours in the absence or presence of agLDL. Online Figure I (available at http://www.ahajournals.org) shows the elution patterns of PGs synthesized by untreated cells and cells treated enzymatically for 2 hours. In human VSMCs and MEF, PGs eluted as a single peak between 1 and 1.5 mol/L NaCl. The majority of synthesized [^{35}S]GAGs were cell-associated (58% in human VSMCs and 56% in MEF). Approximately 36% and 40% of the newly synthesized [^{35}S]GAGs were secreted in the cellular medium in human VSMCs and MEF, respectively, and the remaining newly synthesized GAGs were in the ECM (7% in human VSMCs and 4% in MEF). As shown in the Table, HS-PGs seem to be absent from the cell medium of both cell types. The percentage of HS-PGs in the cell and ECM fractions is higher in MEF compared with human VSMCs (32.5±1.5% versus 7.5±2.5%, respectively, in the cell fraction; 59±2% versus 24±3.5%, respectively, in the ECM). On the contrary, the percentage of CS-PGs is higher in all the fractions from human VSMCs compared with those obtained from MEF (57±3% versus 24.5±11.5%, respectively, in medium; 76±3% versus 34.5±8.5%, respectively, in the cell fraction; and 63±7% versus 52.5±4.5%, respectively, in the ECM). These results indicate significant differences in the GAG composition of PGs synthesized by human VSMCs and MEF. No statistically significant differences in GAG chromatographic patterns were observed between 2 and 18 hours of enzymatic treatment (in the absence or presence of agLDL) in any cell type.

The autoradiographic analysis of the eluted PGs (Figure 1) revealed differences in the pattern of bands susceptible to being degraded by HSI&III and ChABC between human VSMCs and MEF. In human VSMCs, there is a defined band at the beginning of the polyacrylamide gel in the cell and ECM fractions that was degraded by HSI&III treatment and that, according to its size, could be perlecan.[25,26] In MEF, the main bands degraded by HSI&III seem to be mostly syndecans.[27] Additionally, there are high amounts of bands that are susceptible to being degraded by ChABC; these bands were especially abundant in the cell fraction and were different in size for human VSMCs and MEF. Protein loading was determined to be equal for enzymatically treated and untreated cells.

As shown in Figure 2, the pericellular matrix was observed as a tangled network of thin filaments in both VSMCs (Figure 2A) and MEF (Figure 2B). Heparinase I treatment disrupted the pericellular matrix in human VSMCs (Figure 2C) and

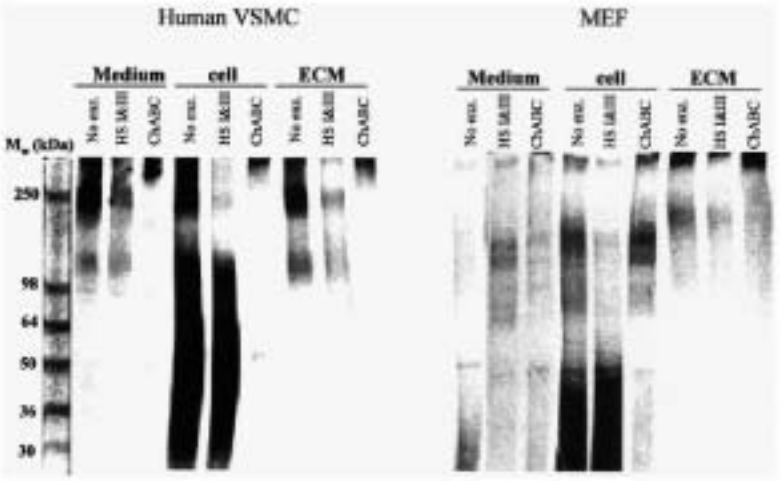

Figure 1. Characterization of PGs by SDS-PAGE. Representative autoradiography of the PG in the medium and in cellular and ECM fractions from untreated, HSI&III-treated, and ChABC-treated cells.

MEF (Figure 2D). Similar photographs were obtained by ChABC treatment of the cells.

Role of PGs and LRPs on agLDL Internalization in Human VSMCs and MEF

To reveal the role of HS-PGs and CS-PGs on agLDL internalization, HS-PGs and CS-PGs were selectively degraded in human VSMCs and MEF. It has been previously demonstrated that heparinase treatment did not influence LRP-binding capacity in fibroblasts.[25] agLDL internalization experiments were performed by incubating LRP-expressing VSMCs and MEF and non–LRP-expressing cells (antisense LRP-ODN–treated VSMCs and PEA13, respectively), either enzymatically treated or not, with increasing concentrations of agLDL for 18 hours.

We have previously demonstrated that the increase in CE content observed in VSMCs reflects the cholesterol that enters the cell as LDL.[24] An initial period of prolonged cell surface contact, facilitated by cell PGs, in which CE hydrolysis exceeds protein degradation (selective uptake), has previously been described in macrophages.[26] A similar process cannot be excluded in CE accumulation induced by agLDL in human VSMCs and fibroblasts. As shown in Figure 3A, although agLDL induced a high intracellular cholesterol accumulation in a dose-dependent manner in human VSMCs (from 44.87±1.77 μg CE per milligram protein at 25 μg/mL

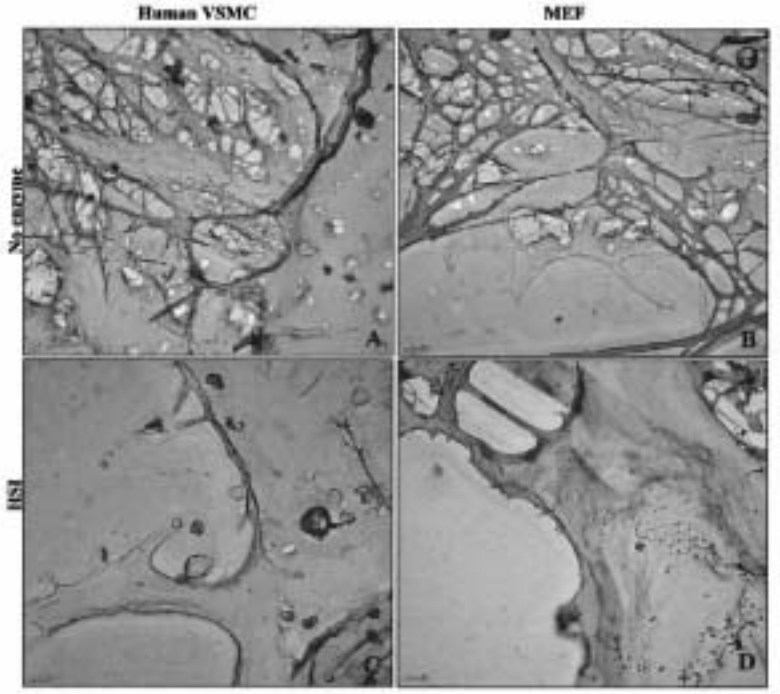

Figure 2. Effect of heparinase treatment on the ECM ultrastructure in human VSMCs and MEF. Nonenzymatically treated human VSMCs (A) and MEF (B) and heparinase I (HSI)-treated VSMCs (C) and MEF (D) were grown on coverslips. Cells were then washed, fixed, and processed for freeze-drying and replication in electron microscopy as described in Methods. Photographs were taken by electron microscopy (Hitachi HU-600).

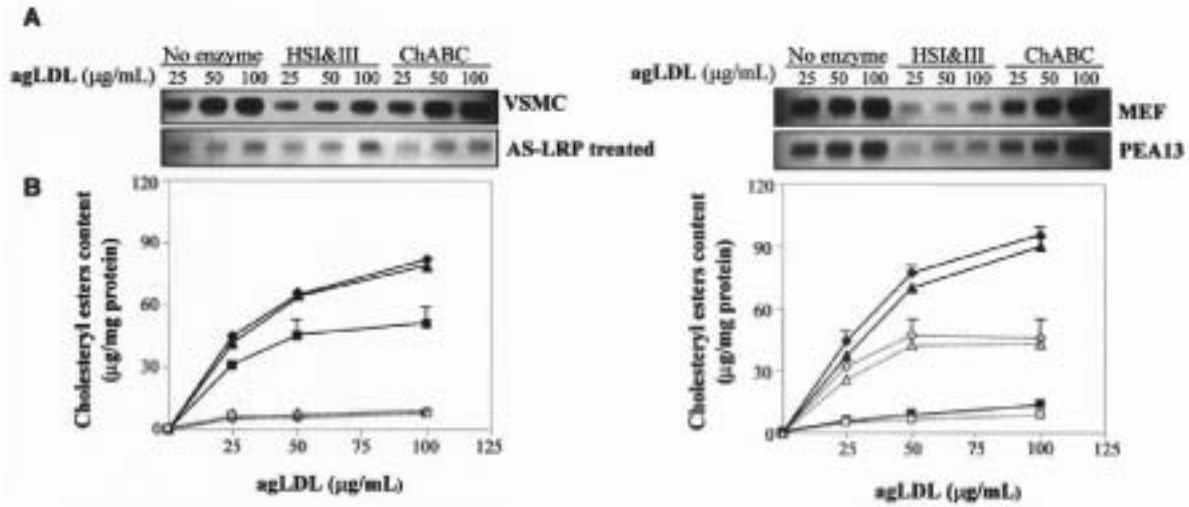

Figure 3. Role of PGs and LRPs on CE accumulation derived by agLDL in human VSMCs and MEF. Human VSMCs/antisense LRP-ODN–treated VSMCs and MEF/PEA13 were nonenzymatically treated or incubated with HSI&III (4 U/mL) or ChABC (2 U/mL) for 2 hours before addition of agLDL. After 18 hours, cells were exhaustively washed and harvested, and the lipid extract was analyzed by TLC. A, TLC showing the effect of increasing concentrations of agLDL (25, 50, and 100 μg/mL) on the CE accumulation induced by agLDL in VSMCs/antisense LRP-ODN–treated cells (left) and MEF/PEA13 (right). B, Line graphs showing quantification of CE in VSMCs/MEF (solid symbols) and antisense LRP-ODN–treated VSMCs/PEA13 (open symbols) either nonenzymatically treated (rhombuses) or treated with HSI&III (squares) or ChABC (triangles). Results are expressed as micrograms CE per milligram protein and are shown as a mean±SEM of 3 samples from 2 different experiments.

to 81.81±1.6 μg CE/mg protein at 100 μg/mL), agLDL was unable to induce CE accumulation in antisense LRP-ODN–treated VSMCs, in agreement with previous results.[8,17] HS-PG depletion leads to a reduction in the CE accumulation derived from agLDL at each analyzed concentration (31.68±2 versus 44.87±1.77 μg CE/mg protein at 25 μg/mL, 45.9±3 versus 64.72±2.82 μg CE/mg protein at 50 μg/mL, and 51.1±3 versus 81.81±1.6 μg CE/mg protein at 100 μg/mL). Taken together, these results indicate that LRP is essential for agLDL internalization in VSMCs and that HS-PGs facilitate the process. In mouse fibroblasts, the mechanism seems to be different, inasmuch as PEA13 showed significantly lower, but still considerable, CE accumulation compared with MEF (32±6 versus 44.58±5 μg CE/mg protein, respectively, at 25 μg/mL; 47±8 versus 77.5±3.06 μg CE/mg protein, respectively, at 50 μg/mL; and 46±9 versus 95.51±4 μg CE/mg protein, respectively, at 100 μg/mL; Figure 3B). In addition, HS-PG depletion almost completely inhibited the CE accumulation induced by agLDL in MEF and PEA13. CS-PG depletion, in contrast to HS-PG depletion, did not show any significant effect on CE accumulation derived from agLDL in any cell type.

As previously shown, agLDL (100 μg/mL) induced a CE accumulation of 81.81±1.6 μg CE/mg protein in human VSMCs and 95.51±4 μg CE/mg protein in MEF. Considering the contribution of pathways independent of LRPs and HS-PGs in human VSMCs (4±0.75 μg CE/mg protein) and MEF (7±1.5 μg CE/mg protein), LRP alone is responsible for intracellular CE accumulation in HSI&III-treated VSMCs (51.1±2.46 μg CE/mg protein) and in HSI&III-treated MEF (18±7 μg CE/mg protein), ≈58% and 12% of CE accumulation in human VSMCs and MEF, respectively. In the same way, considering the role of HS-PGs alone as responsible for intracellular CE accumulation in antisense LRP-ODN–treated VSMCs (4.59±1.0 μg CE/mg protein) and PEA13 (46±9 μg CE/mg protein), HS-PGs would account for ≈1% and 41% of the CE accumulation in human VSMCs and MEF, respectively.

By subtracting LRP-mediated CE accumulation and HS-PG–mediated CE accumulation from the total CE accumulation, a percentage of 26% in human VSMCs and 25% in MEF can be ascribed to an accumulation that is accomplished by a cooperative mechanism (both pathways).

Online Figure II (available at http://www.ahajournals.org) shows photomicrographs of representative untreated and HSI&III-treated human VSMCs and MEF incubated with agLDL. Pictures were taken after the first wash with PBS to eliminate agLDL that was not bound. As shown, untreated human VSMCs (online Figure IIA) or untreated MEF (Figure IIB) had many aggregates of LDL bound (arrows) on the cell surface. In contrast, HSI&III-treated VSMCs (Figure IIC) had less aggregate bound, and HSI&III-treated MEF did not show any aggregate bound (Figure IID). As observed in the photographs, HSI&III treatment did not induce changes in the morphology of any cell type.

Discussion

We recently demonstrated that LRP is responsible for agLDL uptake in human VSMCs. These cells have very high levels of LRP expression and are unable to accumulate cholesterol from agLDL in the absence of LRP.[8,17] Because it has been proposed that PGs may play a role in the internalization of certain LRP ligands in hepatic and arterial cells,[8–16] we explored the role of HS-PGs and CS-PGs on agLDL inter-

nalization in human VSMCs and MEF by degrading PGs with specific polysaccharidase. Chromatographic and autoradiographic results indicate some differences in HS-PG and CS-PG composition between VSMCs and MEF, especially in the cellular and ECM fractions. The amount and molecular weight of CS-PGs were higher in VSMCs than in MEF. HS-PGs were more abundant in MEF compared with human VSMCs. Additionally, although perlecan was the main HS-PG associated with VSMCs, syndecans were most abundant in MEF. Our results are in agreement with the pattern of PGs previously described in fibroblasts[21] and VSMCs.[30,31] For further analysis, we have assumed that synthesized PGs by the parental MEF line and the selected PEA13 line are identical. HSI&III and ChABC treatment completely degraded HS-PGs and CS-PGs, respectively, and also disrupted the pericellular matrix in both cell types, but only HS-PGs cleavage has consequences on agLDL internalization by the cells. These results support a specific role of HS-PGs on cell uptake mechanisms, in agreement with previous studies describing the role of HS-PGs in the arterial wall.[8] However, there are differences in the role of HS-PGs on agLDL internalization in VSMCs and fibroblasts. According to our results, HS-PGs can bind and internalize agLDL in the absence of LRPs, as demonstrated in PEA13 fibroblasts. HS-PGs seem to be essential for CE accumulation in fibroblasts, although LRPs can facilitate agLDL internalization. Our results are in agreement with the presence of an LRP-independent pathway involving HS-PGs as receptors in fibroblasts[10,12] and in macrophages.[9,10,14] However, in human VSMCs, HS-PGs do not play a role as receptors for agLDL because they do not internalize agLDL in the absence of LRPs. LRPs alone internalize most of the agLDLs, although as in fibroblasts, there is a certain synergism between LRPs and HS-PGs. The HS-PG and LRP cooperation that we observed for agLDLs in human VSMCs and fibroblasts has been previously described for a wide variety of LRP ligands.[12–16] The differential role of HS-PGs on agLDL internalization by fibroblasts and human VSMCs could be partially related to the higher amount and species of HS-PGs associated with fibroblasts compared with human VSMCs. In fibroblasts, the main HS-PGs associated with the cell fraction are syndecans, which are very efficient in internalization processes, whereas the main HS-PG associated with VSMCs is perlecan, which has been described to be fairly inefficient at internalizing bound material, but it cooperates with LDL receptor family members.[25] Additionally, in the fibroblast membrane, with a sparser LRP distribution, HS-PGs seem to be indispensable for the binding of agLDL, a multimeric ligand that likely requires extensive binding to many cell surface molecules at once. In contrast, the sheer quantity of LRPs on the VSMC cell membrane[17] may be sufficient for agLDL binding. Smaller ligands, such as tissue factor pathway inhibitor, can be internalized through the LRP independently of HS-PGs in fibroblasts,[32] suggesting that the nature of the ligand might also be important in determining the relative role of LRPs and HS-PGs in ligand internalization.

Therefore, an important role is played by HS-PGs in intracellular cholesterol accumulation in human VSMCs and MEF. However, the differences in HS-PG contribution to agLDL internalization make extrapolation from one cell type to another not suitable for target-specific cell internalization mechanisms. The main mechanism for agLDL internalization in human VSMCs is mediated by LRPs.

Acknowledgments

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References

1. Wight TN. The extracellular matrix and atherosclerosis. *Curr Opin Lipidol*. 1995;6:326–334.
2. Camejo G, Hurt-Camejo E, Wiklund O, Bondjers G. Association of apoB lipoprotein with arterial proteoglycans: pathological significance and molecular basis. *Atherosclerosis*. 1998;139:205–222.
3. Camejo G, Fager G, Rosengren B, Hurt-Camejo E, Bondjers G. Binding of low density lipoproteins by proteoglycans synthesized by proliferating and quiescent human arterial smooth muscle cells. *J Biol Chem*. 1993; 268:14131–14137.
4. Williams KJ. Arterial wall chondroitin sulfate proteoglycans: diverse molecules with distinct roles in lipoprotein retention and atherogenesis. *Curr Opin Lipidol*. 2001;12:477–487.
5. Tirzitis D, Jinga VV, Serban G, Simionescu M. The effects of low density lipoprotein modified by incubation with chondroitin-6-sulfate on human aortic smooth muscle cells. *Atherosclerosis*. 1999;147:155–166.
6. LLorente-Cortés V, Otero-Viñas M, Hurt-Camejo E, Martínez-González J, Badimon L. Human coronary smooth muscle cells internalize versican-modified LDL through LDL receptor-related proteins and LDL receptors. *Arterioscler Thromb Vasc Biol*. 2002;22:387–393.
7. Iozzo RV. The biology of the small leucine-rich proteoglycans: functional network of interactive proteins. *J Biol Chem*. 1999;274:18843–18846.
8. Williams KJ, Fuki IV. Cell-surface heparan sulfate proteoglycans: dynamic molecules mediating ligand catabolism. *Curr Opin Lipidol*. 1997;8:253–262.
9. Halvorsen B, Aas MA, Kulseth MA, Drevon CA, Christiansen EN, Kolset SO. Proteoglycans in macrophages: characterization and possible role in the cellular uptake of lipoproteins. *Biochem J*. 1998;331:743–752.
10. Sehayek E, Wang XX, Vlodavsky I, Avner R, Levkovitz H, Ilivecrona T, Olivecrona G, Willnow TE, Herz J, Eisenberg S. Heparan sulfate-dependent and low density lipoprotein receptor-related protein independent catabolic pathway for lipoprotein lipase in mouse embryonic fibroblasts. *Isr J Med Sci*. 1996;32:449–454.
11. Al-Haideri M, Goldberg IJ, Galeano NF, Gleeson A, Vogel T, Gorecki M, Starley SL, Deckelbaum RJ. Heparan sulfate proteoglycan-mediated uptake of apolipoprotein E-triglyceride-rich lipoprotein particles: a major pathway at physiological particle concentrations. *Biochemistry*. 1997;36: 12766–12772.
12. Sarafanov AG, Ananyeva NM, Shima M, Saenko EL. Cell surface heparan sulfate proteoglycans participate in factor VIII catabolism mediated by low density lipoprotein receptor-related protein. *J Biol Chem*. 2001;276:11970–11979.
13. Kounnas MZ, Chappell DA, Wong H, Argraves WS, Strickland DK. The cellular internalization and degradation of hepatic lipase is mediated by low density lipoprotein receptor-related protein and requires cell surface proteoglycans. *J Biol Chem*. 1995;270:9307–9312.
14. Mahley RW, Ji ZS, Brecht WJ, Miranda RD, He D. Role of heparan sulfate proteoglycans and the LDL receptor-related protein in remnant lipoprotein metabolism. *Ann N Y Acad Sci*. 1994;737:39–52.
15. Beisiegel U, Krapp A, Weber W, Olivecrona G. The role of alpha 2 M receptor/LRP in chylomicron remnant metabolism. *Ann N Y Acad Sci*. 1994;737:53–69.
16. Rohlmann A, Gotthardt M, Hammer RE, Herz J. Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnant. *J Clin Invest*. 1998;101:689–695.

17. Llorente-Cortés V, Martínez-González J, Badimon L. LDL receptor-related protein mediates uptake of aggregated LDL in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2000;20:1572–1579.
18. Desai UR, Wang H, Lindhart RJ. Specificity studies on the heparin lyases from *Flavobacterium heparinum*. *Biochemistry*. 1993;32:8140–8145.
19. Lindhart RJ, Turnbull JE, Wang HM, Loganathan D, Gallagher JT. Examination of the substrate specificity of heparin and heparan sulfate lyases. *Biochemistry*. 1990;29:2611–2617.
20. Brown CT, Nugent MA, Lan FW, Trinkaus-Randall V. Characterization of proteoglycans synthesized by cultured corneal fibroblasts in response to transforming growth factor β and fetal calf serum. *J Biol Chem*. 1999;274:7111–7119.
21. Willnow TE, Herz J. Genetic deficiency in low density lipoprotein receptor-related protein confers cellular resistance to Pseudomonas exotoxin A: evidence that this protein is required for uptake and degradation of multiple ligands. *J Cell Sci*. 1994;107:719–726.
22. Sartipy P, Johansen B, Camejo G, Rosengren B, Bondjers G, Hurt-Camejo E. Binding of human phospholipase A2 type II to proteoglycans. *J Biol Chem*. 1996;271:26307–26314.
23. Sartipy P, Bondjers G, Hurt-Camejo E. Phospholipase A2 type II binds to extracellular matrix byglican. *Arterioscler Thromb Vasc Biol*. 1998;18:1934–1941.
24. Llorente-Cortés V, Martínez-González J, Badimon L. Esterified cholesterol accumulation induced by aggregated LDL uptake in human vascular smooth muscle cells is reduced by HMG-CoA reductase inhibitors. *Arterioscler Thromb Vasc Biol*. 1998;18:738–746.
25. Fuki IV, Iozzo RV, Williams KJ. Perlecan heparan sulfate proteoglycan. *J Biol Chem*. 2000;275:25742–25750.
26. Iozzo RV, Cohen IR, Grassel S, Murdoch AD. The biology of perlecan: the multifaceted heparan sulfate proteoglycan of basement membranes and pericellular matrices. *Biochem J*. 1994;302:625–639.
27. Fuki IV, Kuhn KM, Lomazov IR, Rothman VL, Tuszynski GP, Iozzo RV, Swenson TL, Fisher EA, Williams KJ. The syndecan family of proteoglycans. *J Clin Invest*. 1997;100:1611–1622.
28. Ji ZS, Brecht WJ, Miranda RD, Hussain MM, Innerarity TL, Mahley RW. Role of heparan sulfate proteoglycans in the binding and uptake of apolipoprotein E-enriched remnant lipoproteins by cultured cells. *J Biol Chem*. 1993;268:10160–10167.
29. Button X, Mamdouh Z, Ghosh R, Du H, Kuriakose G, Bentini N, Grabowski GA, Maxfield FR, Tabas I. Unique cellular events occurring during the initial interaction of macrophages with matrix-retained or methylated aggregated low density lipoprotein (LDL). *J Biol Chem*. 1999;274:32112–32121.
30. Lemire JM, Braun KR, Maurel P, Kaplan ED, Schwartz SM, Wight TN. Versican/PG-M isoforms in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 1999;19:1630–1639.
31. Cizmeci-Smith G, Stahl RC, Showalter LJ, Carey DJ. Differential expression of transmembrane proteoglycans in vascular smooth muscle cells. *J Biol Chem*. 1993;268:18740–18747.
32. Warshawsky I, Herz J, Broze GJ, Schwartz AL. The low density lipoprotein receptor-related protein can function independently from heparan sulfate proteoglycans in tissue factor pathway inhibitor endocytosis. *J Biol Chem*. 1996;271:25873–25879.

III.4. ANNEX ARTICLE II (*On-line*)

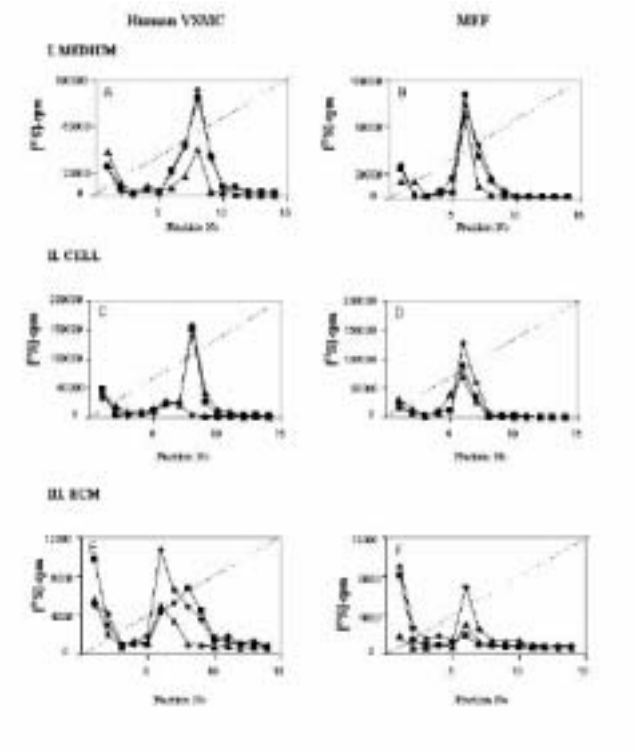

Figure I. Representative chromatography of PG synthesized by human VSMC and MEF. PG of the medium, cellular and ECM fractions from untreated (romboid), HSI&III-treated cells (squares) and ChABC-treated cells (triangles) were applied onto an anion-exchange column equilibrated in a buffer containing urea 8 mol/L, Tris 20 mmol/L, 0,5% Triton X-100, pH 7,5. PG were eluted with a linear NaCl gradient at 0,5 mL/min and fractions (2,5 mL) were collected. Total counts in each fraction were determined. Y-axis scales are adjusted to the counts obtained in each fraction.

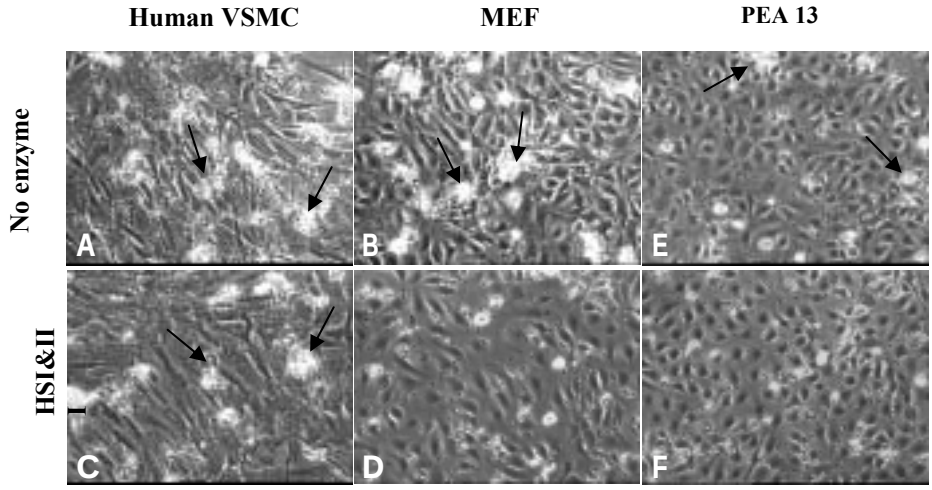

Figure II. Effect of HS-PG depletion on agLDL binding by human VSMC and MEF. Non-enzymatically treated VSMC (A), MEF (B) and PEA 13 (E) or HSI&III (4 U/mL)-treated VSMC (C), MEF (D) and PEA 13 (F) were incubated with agLDL (100 µg/mL) for 18 hours. VSMC were then washed with PBS and photographed (magnification x 100). Arrows indicated LDL aggregates bound to the cell surface.

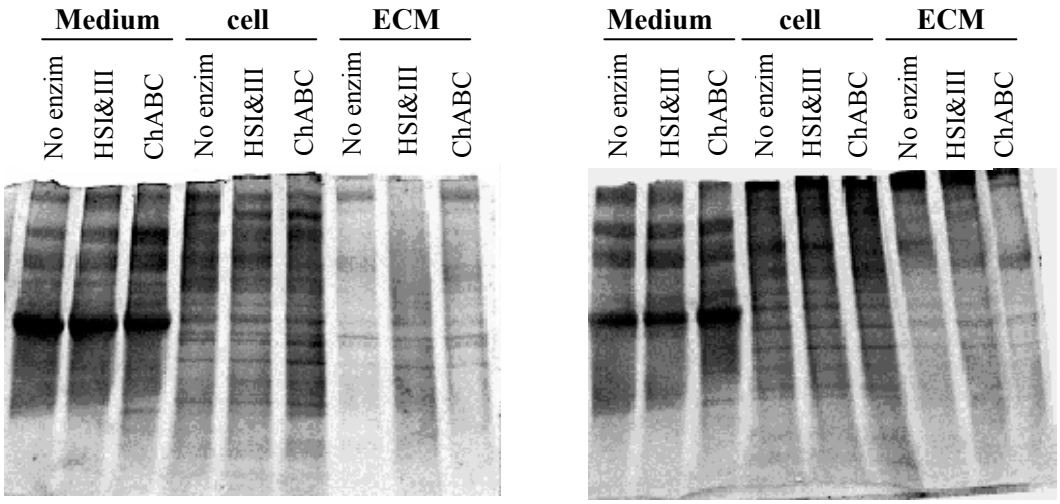

Figure III. Characterization of PG by SDS-PAGE. Representative stained gel of the proteins in the medium, cell and ECM fractions from untreated, HSI&III-treated cells and ChABC-treated cells.

III.5. ARTICLE III

Llorente-Cortés V, Otero-Viñas M, Sánchez S, Rodríguez C, Badimon L. Low-density lipoprotein receptor-related protein expression in vascular smooth muscle cells. Possible involvement of sterol regulatory element binding protein-2-dependent mechanism.

Circulation. 2002;106:3104-3110. SCI: 10,25.

Low-Density Lipoprotein Upregulates Low-Density Lipoprotein Receptor–Related Protein Expression in Vascular Smooth Muscle Cells

Possible Involvement of Sterol Regulatory Element Binding Protein-2–Dependent Mechanism

Vicenta Llorente-Cortés, PhD; Marta Otero-Viñas, MS; Sonia Sánchez, MS; Cristina Rodríguez, PhD; Lina Badimon, PhD, FESC

Background—Low-density lipoprotein (LDL) receptor–related protein (LRP) is highly expressed in vascular smooth muscle cells (VSMCs) of both normal and atherosclerotic lesions. However, little is known about LRP regulation in the vascular wall.

Methods and Results—We analyzed the regulation of LRP expression in vitro in human VSMCs cultured with native LDL (nLDL) or aggregated LDL (agLDL) by semiquantitative reverse transcriptase–polymerase chain reaction, real-time polymerase chain reaction, and Western blot and in vivo during diet-induced hypercholesterolemia by in situ hybridization. LRP expression in human VSMCs is increased by nLDL and agLDL in a time- and dose-dependent manner. Maximal induction of LRP mRNA expression was observed after 24 hours of exposure to LDL. However, agLDL induced higher LRP mRNA expression (3.0-fold) than nLDL (1.76-fold). LRP mRNA upregulation was associated with an increase on LRP protein expression with the greatest induction by agLDL. VSMC-LRP upregulation induced by nLDL or agLDL was reduced by an inhibitor of sterol regulatory element binding protein (SREBP) catabolism (*N*-acetyl-leucyl-leucyl-norleucinal). In situ hybridization analysis indicates that there is a higher VSMC-LRP expression in hypercholesterolemic than in normocholesterolemic pig aortas.

Conclusions—These results indicate that LRP expression in VSMCs is upregulated by intravascular and systemic LDL. **(*Circulation*. 2002;106:3104-3110.)**

Key Words: arteriosclerosis ■ lipoproteins ■ receptors ■ hypercholesterolemia ■ vasculature

Low-density lipoprotein receptor–related protein (LRP) is a type I membrane protein (600 kDa) that has a short C-terminal subunit (LRP-85) and a large N-terminal subunit (LRP-515) that contains most of the extracellular portion of the molecule and binds to all known LRP ligands.[1–3] LRP has been shown to act as an endocytosis-mediating receptor for several ligands, including trombospondin,[4] protease–anti-protease complexes,[5] tissue factor pathway inhibitor (TFPI),[6] and plasma lipoproteins such as apolipoprotein E–enriched VLDL,[7,8] lipoprotein lipase, hepatic lipase and lipoprotein lipase-triglyceride-rich lipoprotein complexes,[9,10] chylomicrons remnant,[11] and lipoprotein a.[12] We recently demonstrated that in human vascular smooth muscle cells (VSMCs), LRP mediates the internalization of aggregated LDL (agLDL) generated either by vortexing[13] or by incubation with versican,[14] one of the main proteoglycans interacting with LDL in the arterial wall.[15] LRP-mediated agLDL uptake could be one of the main mechanisms for intracellular lipid accumulation in VSMCs. Indeed, an increase in LRP expression has been described in advanced atherosclerotic plaques[16] and in patients with coronary obstruction.[17] These results indicate that LRP might play a role in atherosclerosis progression, although little is known about how LRP is upregulated. In macrophages, it has been described that LRP mRNA levels are increased by colony stimulating factor-1 and insulin,[18,19] whereas they were decreased by transforming growth factor-β and LPS.[20,21] Despite the strong sequence homology of LRP and LDL receptor,[22] they have little similarity in the promoter region.[23] LDL receptor, like other genes involved in lipid metabolism, contains the consensus sterol regulatory elements (SRE) in the promoter, allowing its regulation through SRE-1 binding proteins (SREBPs).[24] SRE-1 can bind 3 different transcription factors: SREBP-1a, SREBP-1c, and SREBP-2.[25] Although LRP does not contain

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an SRE in the promoter, an intriguing SRE-1 site has been found in the unusually long 5′-untranslated region of LRP.[26]

The aim of this work was to analyze the effect of agLDL on LRP expression in vitro in cultured human VSMCs and in vivo in a hypercholesterolemic porcine model. Here, we show that agLDL upregulates LRP expression in a dose- and time-dependent manner in human VSMCs. In agreement, LRP expression was significantly enhanced in the vascular wall of hypercholesterolemic animals.

Methods

VSMC Culture

Primary cultures of human VSMCs were obtained from nonatherosclerotic areas of macroscopically healthy ascending aortas of explanted hearts removed from nonischemic cardiomyopathy patients transplanted at the Hospital de la Santa Creu i Sant Pau with the permission of the ethics committee of the Hospital. Histological analysis (Masson Trichromic stain) of these arteries did not show atherosclerotic changes, although preatherosclerotic molecular abnormalities in these human arteries cannot be excluded. VSMCs were obtained by a modification of the explant technique as we described previously.[13,14] VSMCs were identified morphologically using light microscopy and by their growth behavior and were characterized by immunological staining of cytoskeleton proteins. Primary and subcultured VSMCs with a low in vitro age had a spindle-shaped appearance. Confluent VSMC cultures showed the characteristic "hill-and-valley" growth pattern. Mouse monoclonal antibodies specific for human α-SM actin (clone 1A4), human von Willebrand factor (clone F8/86), and human fibroblast surface protein (clone 1B10) were used. VSMCs were used between passages 2 and 4. Cells showed positive immunostaining with anti-SMC α-actin. No staining was observed with antibodies against a human fibroblast cell-surface antigen or von Willebrand factor. Cells were arrested 48 hours in M199 supplemented with 0.2% FCS and incubated with different concentrations of nLDL and agLDL for the indicated times (0 to 48 hours). In some experiments, cells were treated for 24 hours with *N*-acetyl-leu-leu-norleucinal (ALLN) (25 μmol/L).

Animals

Female pigs (body weight at initiation, 32±4 kg) were divided into two groups: normocholesterolemic animals (n=6), which were fed a normal chow diet, and hypercholesterolemic animals (n=10), which were fed a cholesterol-rich diet (2% cholesterol, 1% cholic acid, 20% beef tallow) for 100 days.[27] After 100 days, the animals were killed with a thiopental overdose. Plasma cholesterol levels and hematological parameters were measured at baseline and at death. Because the porcine model of atherosclerosis initially develops lesions in the abdominal aorta, rings from this vessel were collected and fixed in 4% paraformaldehyde in PBS 0.1 mol/L (pH 7.4), cryoprotected in 30% saccharose in PBS 0.1 mol/L (pH 7.4), embedded in OCT, and frozen on dry ice. All procedures were in accordance with institutional guidelines and followed the American Physiological Society guidelines for animal research.

Plasma total cholesterol was determined with an automatic analyzer (Kodack Ektachem DT System). Plasma LDL-cholesterol was analyzed using the validated methods of the Lipid Research Clinic Program[28] and quantified spectrophotometrically (Kontron Instruments).

LDL Preparation and Determination of the Free Cholesterol and Cholesteryl Esters Content

Human LDLs ($d_{1.019}$-$d_{1.063}$ g/mL) were obtained and modified as previously described.[13,14] LDL preparations were <48 hours old, nonoxidized (<1.2 mmol malondialdehyde/mg protein LDL), and without detectable levels of endotoxin (Limulus Amebocyte Lysate test, Bio Whittaker).

Arrested cells were incubated with nLDL or agLDL (100 μg/mL) for 6, 12, 24, and 48 hours. In some experiments, cells were pretreated for 24 hours with ALLN (25 μmol/L) before adding LDL (50, 100 μg/mL). Cells were then exhaustively washed and harvested into 1 mL of 0.10 N NaOH. Lipid extraction and TLC were performed as previously described.[13,14]

Semiquantitative and Real-Time PCR

Arrested cells were incubated with nLDL or agLDL for 6, 12, 24, and 48 hours. RNA and protein were isolated by using the Tripure isolation Reagent (Roche Molecular Biochemicals) according to the manufacturer. LRP and LDL receptor mRNA levels were analyzed by semiquantitative RT-PCR as previously described.[14] The specific oligonucleotides for SREBPs were SREBP-1 forward primer: 5′-atgtagtcgatggccttgcg-3′; SREBP-1 reverse primer: 5′tgtgaccctgcagatccagc-3′; SREBP-2 forward primer: 5′-tgggaccattctgaccacca-3′; and SREBP-2 reverse primer: 5′-tgtgacctcgcagatccagc-3′. Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalize results. Amplification was carried out by 20 (GAPDH), 20 (LRP), 25 (LDL receptor), 20 (SREBP-1), and 22 (SREBP-2) cycles. PCR products (10 μL) were resolved by electrophoresis in 1.5% to 2% agarose gels and transferred onto nylon membranes by the standard capillary technique. Blots were UV cross-linked. Detection of digoxigenine (DIG)-labeled nucleic acids was performed with an anti-DIG antibody linked to alkaline phosphatase, and CSPD (Disodium 3-(4-methoxispiro (1,2-dioxetane-3,2′-15′-chloro) tricyclo [3.3.1.1^{3,7}] decan)-4-yl) phenylphosphate (Roche Molecular Biochemicals) was used as substrate.

TaqMan fluorescent Real-Time PCR primers and probes (6′FAM-MGB) for LRP and LDL receptor were designed by use of Primer Express software from PE biosystems and were as follows: LRP forward: 5′-gagctgaaccacgccttg-3′; LRP reverse: 5′-ggtagacactgccactccgatac-3′; LRP probe: 5′-ttgccatggtgacacag-3′; LDL receptor forward: 5′- tgacaatgctcaccaagctctg-3′; LDL receptor reverse: 5′-ctcacgctactgggcttcttct-3′; and LDL receptor probe: 5′-ctgccagcaacgtcg-3′. Human *gapdh* (4326317E) was used as endogenous control. The specificity and the optimal primer and probe concentrations were tested. Taqman real-time PCR was performed with 2-μL/well of RT products (1 μg total RNA) in 25 μL of TaqMan PCR Master Mix (PE Biosystem) with the primers at 300 nmol/L and the probe at 200 nmol/L. PCR was performed at 95°C for 10 minutes (for AmpliTaq Gold activation) and then run for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute on the ABIPRISM 7000 Detection System. The threshold cycle (Ct) values were determined and normalized to the housekeeping gene *gapdh*. Because the targets have similar amplification efficiency as endogenous controls, we have used the comparative Ct method (delta delta Ct) to perform relative quantification of LRP and LDL receptor.

Western Blot Analysis

SDS/PAGE was run as previously described.[14] Blots were incubated with monoclonal antibodies against human LRP (β-chain; Research Diagnostics, clone 8B8 RDI 61067, dilution 1:40). To test equal protein loading for the different samples, blots were also incubated with monoclonal antibodies against human α-actinin (Chemicon International, MAB 1682, dilution 1:10000).

LRP In Situ Hybridization

Abdominal aorta sections (n=3) from the same segment of normocholesterolemic and hypercholesterolemic animals were analyzed. Conventional histology (Masson Trichrome) was performed to identify the lesion type.

For riboprobe synthesis, human LRP cDNA corresponding to nucleotides 2221 to 2764 (544 mer) was cloned in plasmid vectors using standard techniques. Antisense and sense riboprobes were synthesized using T3 and T7 RNA polymerase (Promega), respectively, and DIG-labeled nucleotides (Roche Molecular Biochemi-

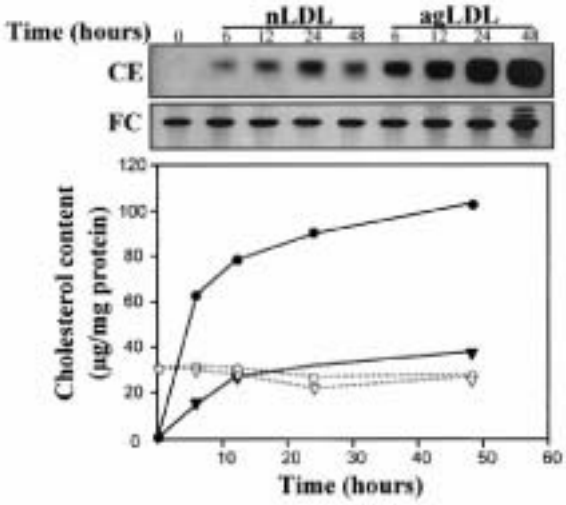

Figure 1. Thin layer chromatography of the VSMC intracellular cholesterol accumulation induced by nLDL and agLDL. VSMCs were incubated with nLDL (triangles) or agLDL (circles) (100 µg/mL) for increasing times. Filled symbols indicate Cholesteryl esters (CE); open symbols, free cholesterol (FC). Results are expressed as microgram of cholesterol per milligram of protein and are shown as mean±SEM (n=2).

cals). In situ hybridization studies were performed on a set of 20-µm-thin sections. Sections were fixed before being permeabilized by proteinase K. Proteinase K was then deactivated and the sections were treated with TEA, postfixed again, and blocked with 2 mg/mL glycine in PBS. After wash with SSC×2, sections were prehybridized with a solution of 50% formamide, SSC×5, 50 µg/mL heparin, 5% dextran sulfate, 0.01%SDS, Denhardt's solution ×5, 100 µg/mL polyAmRNA, 25 µg/mL tRNA, 25 µg/mL salmon sperm DNA. Sections were then incubated with the probe at 1 µg/mL in the prehybridization solution overnight at 53°C. Digoxigenin labeled nucleotides were detected by an alkaline phosphatase conjugated anti-digoxigenin antibody provided by the Dig-labeled immunodetection kit, using NBT/BCIP as substrate (Vector Laboratory). Results were evaluated with an Olympus Vanox fluorescence microscope. Images were digitalized using a Sony 3CCD camera. Controls were performed by the incubation of the section with the sense riboprobe and with the empty-plasmid riboprobes.

Data Analysis

Data were expressed as mean±SEM. A statview (Abacus Concepts) statistical package for the Macintosh computer system was used for all analysis. Multiple groups were compared by nonparametric tests, Wilcoxon, or Mann-Whitney U, as needed. Statistical significance was considered when $P<0.05$.

Results

nLDL and agLDL Downregulate LDL Receptor and Upregulate LRP mRNA Expression in a Time- and Dose-Dependent Manner

Differences in cholesteryl ester (CE) levels between VSMCs incubated with nLDL or agLDL were highly significant ($P<0.05$) (Figure 1). VSMC-CE accumulation derived from agLDL increased from undetectable levels to 103±5.2 µg/mg protein at 48 hours. On the contrary, VSMC-CE accumulation from nLDL was slightly increased (from undetectable levels to 32.2±3.16 µg/mg protein at 48 hours), reaching a plateau after 12 hours of incubation. Free cholesterol content was not changed by either agLDL or nLDL.

LRP was slightly upregulated by nLDL and strongly by agLDL in a time-dependent (Figure 2A) and dose-dependent (Figure 2C) manner. AgLDL was able to upregulate LRP expression at the lowest concentration tested (50 µg/mL). Maximal LRP induction by both nLDL and agLDL (100 µg/mL) was observed after 24 hours; however, agLDL induced a much higher LRP expression than nLDL (agLDL, 3±0.12 versus nLDL, 1.76±0.32; $P<0.05$). In absence of LDL (control), LRP expression remained unaltered along the tested times.

Both nLDL and agLDL downregulated LDL receptor mRNA expression in a time-dependent (Figure 2B) and

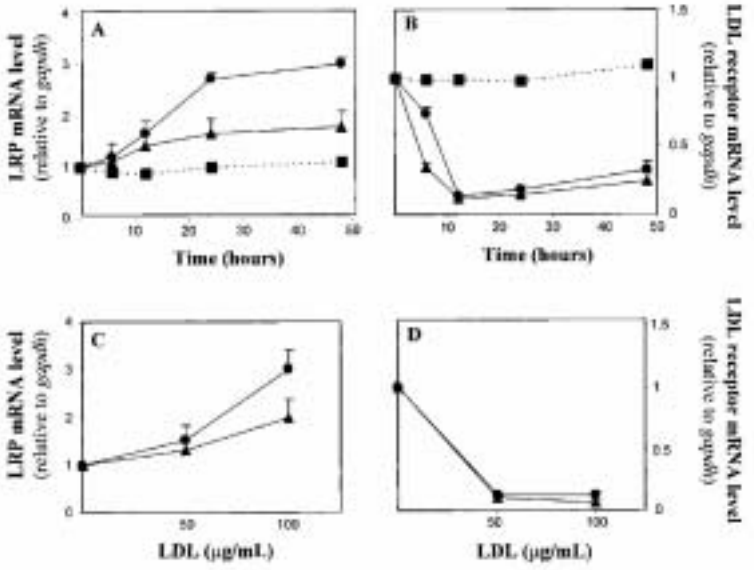

Figure 2. Time and dose response of LRP and LDL receptor to nLDL and agLDL. VSMCs were incubated in the absence (squares) or presence of nLDL (triangles) or agLDL (circles) (100 µg/mL) for increasing times (A and B) or incubated with increasing concentrations of nLDL (triangles) or agLDL (circles) for 24 hours (C, D). A and C, Real-time PCR quantification of LRP mRNA. B and D, Real-time PCR quantification of LDL receptor mRNA. Data were processed with a specially designed software program based on Ct values of each sample and normalized to *gapdh* mRNA (n=3).

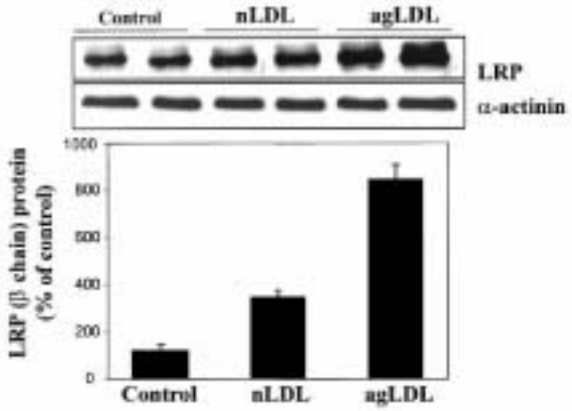

Figure 3. Western blot showing LRP (β-chain) and α-actinin protein levels in VSMCs incubated with nLDL or agLDL (100 μg/mL). Results are expressed as a percentage of control cells (incubated in absence of LDL) and are shown as mean±SEM (n=3).

dose-dependent (Figure 2C) manner. The lowest concentration tested of both nLDL and agLDL (50 μg/mL) completely reduced LDL receptor expression. The time-course assays performed with the highest LDL concentration (100 μg/mL) showed a complete LDL receptor downregulation after 6 hours with nLDL and after 12 hours with agLDL. A slight reversion of the LDL downregulation was observed after 24 and 48 hours of LDL incubation. In absence of LDL (control), LDL receptor expression remained unaltered along the tested times.

Western blot analysis showed that both nLDL and agLDL (100 μg/mL, 24 hours) were able to induce LRP protein synthesis. However, agLDL induced higher LRP protein expression than nLDL (agLDL: 8.3±1.2 versus nLDL: 3.3±0.5; $P<0.05$) (Figure 3).

nLDL and agLDL Downregulated SREBP-2 mRNA Levels in a Time- and Dose-Dependent Manner

We evaluated SREBPs mRNA expression levels in VSMCs exposed to increasing concentrations of nLDL and agLDL, and although SREBP-1 remained unaltered, SREBP-2 levels were downregulated in a dose-dependent (Figure 4A) and time-dependent (Figure 4B) manner. The lowest concentration tested of both nLDL and agLDL (50 μg/mL) decreased SREBP-2 mRNA expression by ≈50%, and a complete SREBP-2 downregulation was observed at 100 μg/mL. The time-course assays performed with the highest LDL concentration (100 μg/mL) showed that, as observed with the LDL receptor mRNA expression, there was a delay on the SREBP-2 downregulation induced by agLDL (12 hours) compared with that induced by nLDL (6 hours). In addition, similarly to the LDL receptor expression, a slight reversion of the SREBP-2 downregulation was observed after 24 and 48 hours of incubation with nLDL or agLDL.

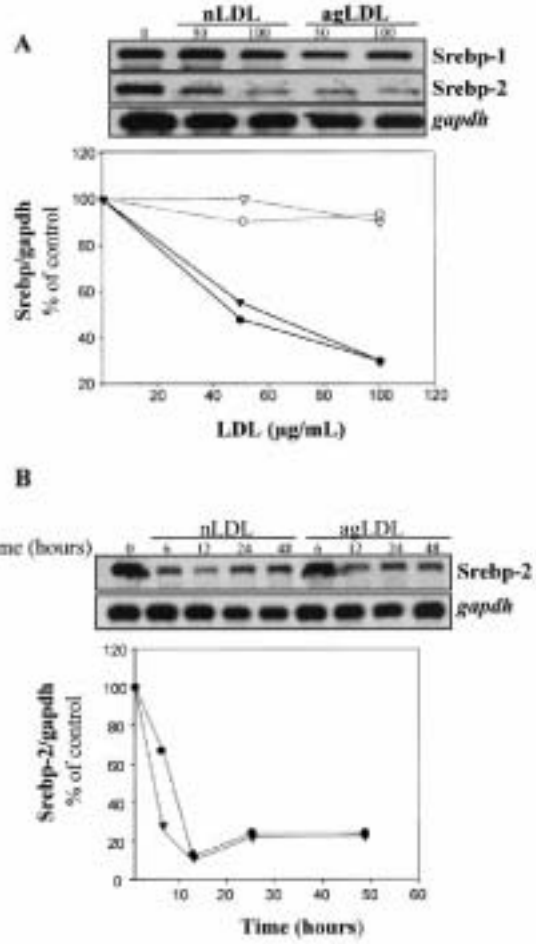

Figure 4. Effect of nLDL and agLDL on SREBP mRNA levels. A, VSMCs were incubated with increased concentrations of nLDL (triangles) or agLDL (circles) for 24 hours, and SREBP-1 (open symbols) or SREBP-2 (filled symbols) mRNA levels were determined by RT-PCR. B, Time-course regulation of SREBP-2 by nLDL and agLDL (100 μg/mL). Results were normalized by *gapdh* mRNA level and are expressed as percentage of controls (n=2).

ALLN Prevents the LDL Receptor Downregulation and the LRP Upregulation Caused by nLDL and agLDL

To analyze the possible involvement of SREBPs in LDL-mediated upregulation of LRP expression, we analyzed the effect of ALLN (25 μmol/L), an inhibitor of SREBP catabolism, on LRP mRNA levels. ALLN blocked LRP upregulation induced by LDL not only at mRNA (Figure 5B) but also at the protein level (Figure 5D). As expected, ALLN almost completely prevented the downregulation on LDL receptor mRNA expression induced by nLDL and agLDL (100 μg/mL, 24 hours) (Figure 5C). ALLN also decreased the CE accumulation derived from agLDL by 36±4% at 50 μg/mL agLDL and by 46±6.8% at 100 μg/mL agLDL (Figure 5E), indicating that LRP function is affected by ALLN. Taken together, these results suggest the involvement of SREBP-2 on the LRP upregulation

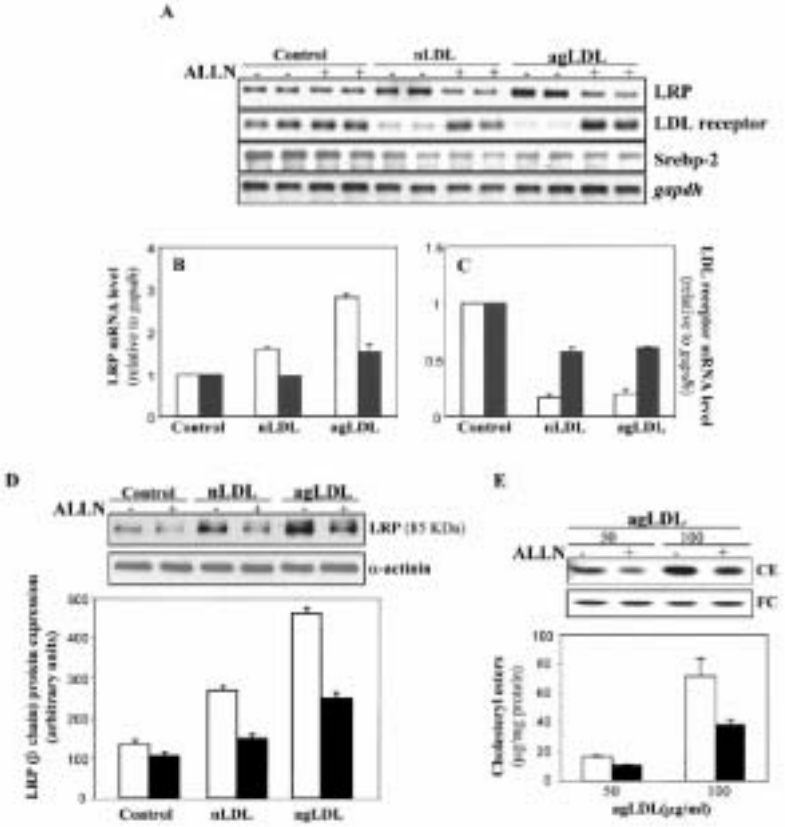

Figure 5. Effect of ALLN on LRP upregulation induced by nLDL and agLDL. VSMCs were incubated with nLDL or agLDL (100 μg/mL) and ALLN (25 μmol/L) during 24 hours. A, Autoradiography showing LRP and LDL receptor mRNA expression levels obtained by semiquantitative RT-PCR. B, Real-time PCR quantification of LRP mRNA in ALLN absence (open bars) or presence (closed bars). C, Real-time PCR quantification of LDL receptor mRNA in ALLN absence (open bars) or presence (closed bars). Data were processed with a specially designed software program based on Ct values of each sample and normalized to gapdh mRNA (n=2). D, Western blot showing LRP (β-chain) and α-actinin protein levels. E, Thin-layer chromatography showing the FC and CE bands of VSMC. Bar graphs showing the quantification of CE bands in absence (open bars) or presence (closed bars) of ALLN. Results are expressed as microgram of cholesterol per milligram of protein and are shown as the mean of two experiments performed in duplicate (deviations <5% of the mean do not appear in the computer-originated graphs).

caused by nLDL and agLDL. ALLN did not show any effect on SREBP-2 mRNA expression in control or LDL-incubated VSMCs (Figure 5A).

LRP mRNA Levels Were Higher in VSMCs of Hypercholesterolemic Pigs

To determine whether hypercholesterolemia influences LRP expression in VSMCs in vivo, we performed in situ hybridization analysis. As shown in the Table, animals fed the hypercholesterolemic diet showed higher cholesterol plasma levels. Masson Trichromic staining shows very incipient lesions in hypercholesterolemic pigs (Figure 6B). In situ hybridization analysis revealed that there is an increase in LRP expression (arrows) in the intima-media layer (just below developing plaques) of hypercholesterolemic (Figure 6D) compared with normocholesterolemic pigs (Figure 6C). Controls with sense riboprobes were negative for normocholesterolemic (Figure 6E) and hypercholesterolemic (Figure 6F) pigs.

Plasma Lipid Profile in Normolipemic and Hyperlipemic Pigs

	Normolipemic	Hyperlipemic
Total cholesterol, mg/dL	65.1±16	463±202*
LDL cholesterol, mg/dL	34.0±10.9	333±120*

Results are mean±SD.
*$P<0.01$ vs normolipemic animals.

Discussion

In human VSMCs, LRP is the lipoprotein receptor that internalizes agLDL obtained by vortexing[13] or incubation with versican.[14] Because vortexing generates agLDL similar to that isolated from the arterial wall[15] and versican is one of the main proteoglycans interacting with the LDL in the arterial intima,[16] the LRP-mediated agLDL uptake could have a crucial role in VSMC-lipid deposition in atherosclerotic plaques.

In this work, we demonstrate that agLDL strongly upregulates LRP at transcriptional level. The increase on mRNA LRP transcription leads to a large increase in LRP protein expression. In these cells, both nLDL and agLDL completely downregulate LDL receptor expression. Consequently, by inducing LRP expression in human VSMC, agLDL could lead to a progressive intracellular accumulation of cholesterol in these cells. The equal capacity of nLDL and agLDL to downregulate LDL receptor expression can be explained by their identical capacity to downregulate SREBP-2 mRNA levels. LDL receptor downregulation is prevented by ALLN, an inhibitor of SREBP-2 catabolism, confirming the expected response of cells to cholesterol loading. Interestingly, nLDL and agLDL were unable to upregulate LRP expression in ALLN-treated VSMCs. Furthermore, LRP upregulation, like LDL receptor downregulation, seems to be dependent on SREBP-2 downregulation. However, other factors besides SREBP-2 must be involved on LRP upregulation, because

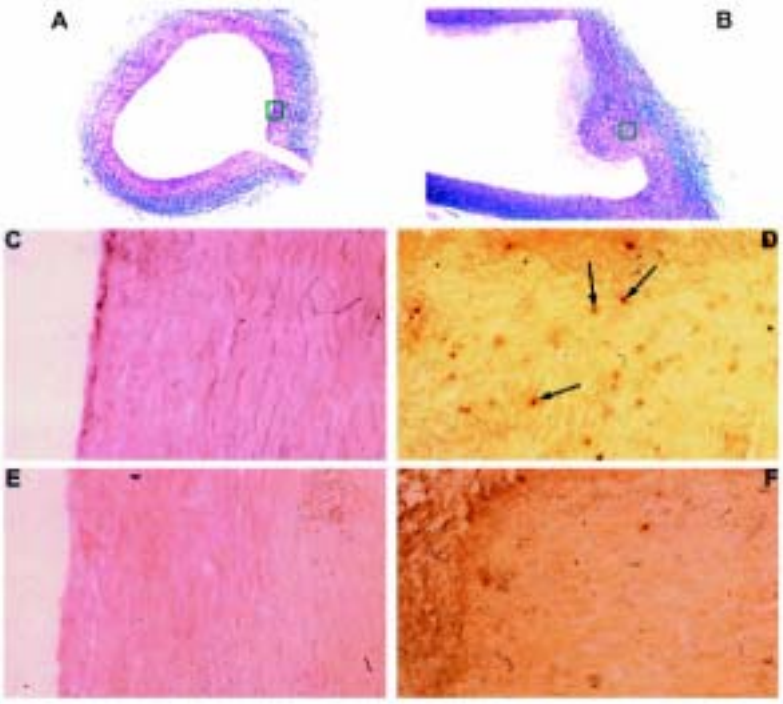

Figure 6. In situ hybridization analysis of LRP expression in normocholesterolemic and hypercholesterolemic pigs. Masson trichromic stain of samples from abdominal aorta of normocholesterolemic (A) and hypercholesterolemic (B) pigs. In situ hybridization with LRP antisense riboprobe in normocholesterolemic (C) and hypercholesterolemic (D) pigs. Controls with LRP sense riboprobe in normocholesterolemic (E) and hypercholesterolemic (F) pigs. Arrows indicate LRP mRNA expression. Magnification ×100.

agLDL has much more capacity than nLDL to increase LRP expression levels, whereas both lipoproteins have equal capacity to completely downregulate SREBP-2 levels. These results suggest that the large amount of cholesterol internalized from agLDL into cells could modulate other transcription factors likely involved in the LRP upregulation. Although the LRP gene does not have SRE-1 sequences in its promoter, an SRE-1 site in the unusually long 5′-untranslated region has been described.[26] Other genes such as microsomal triglyceride transfer protein[27] or 7α-hydroxylase[28] have been described to be upregulated through SREBPs downregulation. Our results obtained in human VSMCs are in agreement with those obtained in macrophages, because LRP upregulation has been observed in cells incubated with cholesterol and 25-hydroxycholesterol.[23] In addition, the LRP upregulation observed in vitro has been corroborated in vivo; in situ hybridization analysis revealed that LRP expression is upregulated in the vessel wall of hypercholesterolemic animals. LRP upregulation in hypercholesterolemic aortas is concomitant with the SREBP-2 downregulation previously described by our group.[11] Our results in vivo are in agreement with those obtained in blood mononuclear cells, in which dietary cholesterol has been shown to increase LRP mRNA levels,[32] and in aortas of Watanabe rabbits, although the main cellular component at both early and late stages of atherosclerosis in this animal model are infiltrated macrophages.[33]

To our knowledge, this is the first demonstration that exposure to high LDL concentration and cellular accumulation of CE increases LRP expression in VSMCs. Our results suggest that hypercholesterolemia might increase the capacity of VSMCs to take up LDL from the intima by regulating cellular LRP levels. In addition, LRP upregulation may influence other pathways involved in atherothrombosis, because LRP mediates the degradation of molecular complexes involved in thrombogenesis and fibrinolysis.[4,5,34]

Acknowledgments

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References

1. Krieger M, Herz J. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Ann Rev Biochem*. 1994;63:601–637.
2. Willnow TE. The low density lipoprotein receptor gene family: multiple roles in lipid metabolism. *J Mol Med*. 1999;77:306–315.
3. Neels JG, Horn IR, Van den Berg BMM, et al. Ligand-receptor interactions of the low density lipoprotein receptor-related protein, a multiligand endocytic receptor: fibrinolysis 38. *Proteolysis*. 1998;12:219–240.
4. Mikhailenko I, Kounnas MZ, Strickland DK. Low density lipoprotein receptor-related protein/α$_2$-macroglobulin receptor mediates the cellular internalization and degradation of thrombospondin. *J Biol Chem*. 1995; 270:9503–9540.
5. Willnow TE, Goldstein JL, Orth K, et al. Low density lipoprotein receptor-related protein and gp330 bind similar ligands, including plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance. *J Biol Chem*. 1992;267:26172–26180.
6. Warshawsky I, Herz J, Bruce GI, et al. The low density lipoprotein receptor-related protein can function independently from heparan sulfate proteoglycans in tissue factor pathway inhibitor endocytosis. *J Biol Chem*. 1996;271:25873–25879.
7. Kowal RC, Herz J, Goldstein JL, et al. Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoliprotein E-enriched lipoproteins. *Proc Natl Acad Sci U S A*. 1989;86: 5810–5814.
8. Beisiegel U, Weber W, Ihrke G, et al. The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. *Nature*. 1989;341:162–164.

9. Chappel DA, Fry GL, Waknitz LE, et al. Lipoprotein lipase induces catabolism of normal triglyceride-rich lipoproteins via the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor in vitro. *J Biol Chem*. 1992;268:14168–14175.
10. Kounnas MZ, Chappell DA, Wong H, et al. The cellular internalization and degradation of hepatic lipase is mediated by the low density lipoprotein receptor-related protein and requires cell surface proteoglycans. *J Biol Chem*. 1995;270:9307–9312.
11. Fujioka Y, Cooper AD, Fong LG. Multiple processes are involved in the uptake of chylomicron remnant by mouse peritoneal macrophages. *J Lipid Res*. 1998;39:2339–2349.
12. ReblinT, Niemeier A, Meyer N, et al. Cellular uptake of lipoprotein [a] by mouse embryonic fibroblasts via the LDL receptor and the LDL receptor-related protein. *J Lipid Res*. 1997;38:2103–2110.
13. Llorente-Cortés V, Martínez-González J, Badimon L. LDL receptor-related protein mediates uptake of aggregated LDL in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2000;20:1572–1579.
14. Llorente-Cortés V, Otero-Viñas M, Hurt-Camejo E, et al. Human coronary smooth muscle cells internalize versican-modified LDL through both the low density lipoprotein receptor-related protein and the LDL receptor. *Arterioscler Thromb Vasc Biol*. 2002;22:387–393.
15. Tirziu D, Jinga VV, Serban G, et al. The effects of low density lipoprotein modified by incubation with chondroitin 6-sulfate on human aortic smooth muscle cells. *Atherosclerosis*. 1999;147:155–166.
16. Laatsa J, Hiltunen T, Särkioja T, et al. Expression of α_2-macroglobulin receptor/low density lipoprotein receptor-related protein and scavenger receptor in human atherosclerotic lesions. *J Clin Invest*. 1994;93:2014–2021.
17. Handschig K, Schulz S, Schnurer C, et al. Low density lipoprotein receptor-related protein in atherosclerotic development: up-regulation of gene expression in patients with coronary obstruction. *J Mol Med*. 1998;76:596–600.
18. Hussaini IM, Srikumar K, Quesenberry PJ, et al. Colony stimulating factor-1 modulates α-macroglobulin receptor expression in murine bone marrow macrophages. *J Biol Chem*. 1990;265:19441–19446.
19. Misra UK, Gawdi G, González-Gronow M, et al. Coordinate regulation of the α_2-macroglobulin signaling receptor and the low density lipoprotein receptor-related protein/α_2 macroglobulin receptor by insulin. *J Biol Chem*. 1999;274:25785–25791.
20. Hussaini IM, LaMarre J, Lysiak JJ, et al. Transcriptional regulation of LDL receptor–related protein by IFN-γ and the antagonistic activity of TGF-β_1 in the RAW 264.7 macrophage-like cell line. *J Leukocyte Biol*. 1998;59:733–739.
21. LaMarre J, Wolf BB, Kittler El W, et al. Regulation of macrophage α_2-macroglobulin/low density lipoprotein receptor-related protein by lipopolysaccharide and interferon-γ. *J Clin Invest*. 1993;91:1219–1224.
22. Herz J, Kowal RC, Goldstein JL, et al. Proteolytic processing of the 600 KD low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. *EMBO J*. 1988;9:1769–1776.
23. Kött H, Herz J, Stanley KK. Structure of the low-density lipoprotein receptor-related protein (LRP) promoter. *Biochim Biophys Acta*. 1989;1009:229–236.
24. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*. 1997;89:331–340.
25. Sakai J, Nohturffi A, Goldstein JL, et al. Cleavage of sterol regulatory element-binding proteins (SREBPs) at site-1 requires interaction with SREBP cleavage-activating protein: evidence from in vivo competition studies. *J Biol Chem*. 1998;273:5785–5793.
26. Gaeta BA, Borthwick I, Stanley KK. The 5′-flanking region of the α_2 MR/LRP genes contains and enhancer-like cluster of Sp1 binding sites. *Biochim Biophys Acta*. 1994;1219:307–313.
27. Alfon J, Royo T, Garcia-Moll X, et al. Platelet deposition on eroded vessel wall at stenotic shear rate is inhibited by lipid-lowering treatment with atorvastatin. *Arterioscler Thromb Vasc Biol*. 1999;19:1812–1817.
28. Lipid Research Clinic Program. Manual of Laboratory Operation. Washington, DC: US Government Printing Office; 1974. DHEW publication No. NIH75-628.
29. Sato R, Miyamoto W, Inoue J, et al. Sterol regulatory element-binding protein negatively regulates microsomal triglyceride transfer protein gene transcription. *J Biol Chem*. 1999;274:24714–24720.
30. Peet DJ, Turley SD, Ma W, et al. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR α. *Cell*. 1998;93:693–704.
31. Rodriguez C, Martínez-González J, Sánchez-Gómez S, et al. LDL down-regulates CYP51 in porcine vascular endothelial cells and in the arterial wall through a sterol regulatory element binding protein-2-dependent mechanism. *Circ Res*. 2001;88:268–274.
32. Boucher P, Lorgeril M, Salen P, et al. Effect of dietary cholesterol on low density lipoprotein receptor, 3-hydroxy-3-methylglutaryl-CoA reductase and low density lipoprotein receptor-related protein mRNA expression in healthy humans. *Lipids*. 1998;33:1177–1186.
33. Watanabe Y, Inaba T, Shimano H, et al. Induction of LDL receptor-related protein during the differentiation of monocyte-macrophages: possible involvement in the atherosclerosis process. *Arterioscler Thromb*. 1994;14:1000–1006.
34. Biessen EA, van Teijlingen M, Vietsch H, et al. Antagonists of the mannose receptor and the LDL receptor-related protein dramatically delay the clearance of tissue plasminogen activator. *Circulation*. 1997;95:46–52.

III.6. ARTICLE IV

Llorente-Cortés V, Otero-Viñas M*, Berrozpe M, Badimon L. Intracellular lipid accumulation, Low density lipoprotein receptor-related protein expression, and cell survival in vascular smooth muscle cells derived from normal and atherosclerotic human coronaries.*

**Ambdós autors han contribuït per igual a l'article.*

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Intracellular lipid accumulation, Low density lipoprotein receptor-related protein expression, and cell survival in vascular smooth muscle cells derived from normal and atherosclerotic human coronaries.

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Abstract

Background Vascular smooth muscle cells (VSMC) regulation during atherosclerotic plaque progression is determinant for plaque stability.

Aims To study lipid accumulation, Low density lipoprotein receptor-related protein (LRP) expression, and cell survival in VSMC isolated from non-atherosclerotic areas (normal VSMC) and advanced atherosclerotic plaques (plaque-VSMC) of human coronaries.

Design Normal or plaque-VSMCs were obtained from the intima by a modification of the explant technique.

Results. **Aggregated LDL** (agLDL) (100 μg/mL) internalization induced higher intracellular cholesteryl ester (CE) accumulation in plaque-VSMC compared to normal VSMC (89.28±6.1 *vs* 60.34±4.1 μg CE/mg protein; $P<0.05$). This internalization was associated to LRP expression since plaque-VSMC show higher levels of LRP mRNA (6.06±0.55 *vs* 3.87±0.28; $P<0.05$) and LRP protein expression than normal VSMC. However, plaque-VSMC showed lower proliferative response than normal VSMC (6536±636 *vs* 11151±815 cpm [^{3}H]thymidine; $P<0.05$) and did not respond to platelet derived growth factor BB (PDGF-BB) stimulus. In agreement, Bcl_2/BAX ratio was significantly lower in plaque-VSMC compared to normal VSMC (0.14±0.05 *vs* 0.51±0.07; $P<0.05$) and it was independent of lipid loading.

Conclusions These results indicate that higher intracellular lipid deposition in plaque-VSMC is related to higher LRP expression levels. However, LRP-mediated agLDL internalization is not directly related to the reduced survival of plaque-VSMC.

Keywords Human vascular smooth muscle cells, aggregated LDL, Low density lipoprotein receptor-related protein, DNA synthesis, cholesteryl esters.

There are several receptors that may contribute to intracellular cholesterol accumulation in the vascular wall, mainly scavenger receptors (SRs), VLDL receptor, LDL receptor and Low density lipoprotein receptor-related protein (LRP) [1,2]. With the exception of LDL receptor, the other receptors are highly expressed in atherosclerotic lesions [3-5]. SRs mediate the uptake of many negatively charged ligands including oxidized LDL [6,7]. In contrast, LRP has been shown to act as an endocytosis-mediated receptor for different plasma lipoproteins such as apolipoproteinE-enriched VLDL [8,9], lipoprotein lipase, hepatic lipase and lipoprotein lipase-trygliceride-rich lipoprotein complexes [10,11], chylomicron remnant

[12], lipoprotein (a) [13]. We recently demonstrated that aggregated LDL (agLDL), one of the main modifications of LDL in the intima arterial [14,15], is bound and internalized through LRP, receptor that is upregulated by agLDL uptake in human VSMC and by hypercholesterolemia in the vascular wall [16-19]. Furthermore, LRP-mediated agLDL internalization could be considered as one of the main mechanisms for VSMC intracellular lipid deposition. VSMC are the main cellular component in the healthy vessell wall and the lost of VSMC content is a key factor determining vascular wall stability during atherosclerotic lesion progression. VSMC number has been described to be regulated by proliferative [20,21] and apoptotic mechanisms [22]. In advanced atherosclerotic plaques, apoptotic VSMC have been detected [23] and there is higher apoptotic rate in VSMC isolated from advanced lesions [24]. Besides a wide variety of growth factors identified in atherosclerotic plaques that induce apoptosis [25], oxidized LDL also seems to induce VSMC apoptosis [26]. In contrast, lipid accumulation derived from agLDL, highly present in the arterial intima, prevent apoptosis of macrophages [27]. It is unknown whether agLDL uptake regulate VSMC survival. This study has analyzed agLDL internalization and LRP expression in VSMC derived from healthy and atherosclerotic coronary vessels. Our results indicate higher LRP expression levels and higher intracellular cholesterol accumulation derived from agLDL uptake in plaque-VSMC. However, LRP-mediated agLDL internalization was not associated to the lower DNA synthesis and the lower Bcl_2/BAX survival ratio of plaque-VSMC.

Methods

Coronary artery sampling

Human coronary arteries were obtained from explanted hearts immediately after surgical excision at transplant operations performed at the Hospital de la Santa Creu i Sant Pau.All procedures were approved by the Institutional Review Committee. Coronary arteries were dissected, immersed in cell maintenance media, examined under low magnification with a zoom stereo microscope and classified in two categories: areas with or without atherosclerotic as deduced from absence of fibro-fatty tissue or visible plaques [28,29]. At this point, samples were used either for immunohistochemistry or cell culture.

Immunohistochemical analysis

Immunohistochemistry specimens were immersed in fixative solution (4% paraformaldehyde in 0.1 mol/L PBS, pH 7.4 overnight at 4ºC), sectioned into blocks, cryoprotected and frozen [29]. Vessels were cross-sectionally cut and consecutive sections (5 μm thick) were collected on chromopotassium-gelatin coated slides for immunohistochemistry or conventional staining and stored at –20ºC until tested. Masson Trichromic staining (performed in representative cross-sectional preparations from each block), allowed to classify the lesions into group I (initial lesions, arterial intimal thickening (AIT), n=8), group II (mild growing lesions: type I-II and III, n=15), group III (advanced high risk lesions: type IV-V, n=8), group IV (severe lesions: type VII-VIII, n=10).

Sections were thawed and blocked with PBS-1% bovin serum albumin (BSA) for 1 hour at room temperature. After washing in PBS, sections were incubated with primary antibodies diluted 1/50-1/10 (depending on the antibody) during 3 hours at RT or diluted 1/100-1/400 overnight at 4ºC. As secondary antibodies, horseradish peroxidase-conjugated rabbit antimouse IgG alkaline phosphatase-conjugated rabbit antimouse Ig diluted 1/1000 were used. The antibodies, their specificity and source are detailed in Table I. Several sections from independent blocks were processed. Incubations with only primary or secondary antibodies were simultaneously performed as a control of the immunostaining procedure. Controls using nonimmune sera from mouse and rabbit were also carried out. Nuclei were counterstained with Hoechst 33258 colorant.

Table I. Characteristics of the antibodies used in immunohistochemical studies

Antibodies	Specificity	Source	Type	Acquisition
Anti LRP α2	LRP α2	Mouse	Monoclonal	Research Diagnostica
Anti LDL receptor	LDL receptor	Rabbit	Policlonal	Amersham
Anti α-actin	Smooth muscle cells	Mouse	Monoclonal	Dako

Human coronary VSMC isolation

Primary cultures of human VSMC were obtained from human coronary arteries of explanted hearts at transplant operations performed at the Hospital de la Santa Creu i Sant Pau. Normal or plaque-VSMC were obtained from the intima of non-atherosclerotic areas (n=5) or advanced atherosclerotic lesions (group III) (n=4) respectively, by means of a

modification of the explant technique [28,29]. In three cases,VSMC were obtained from normal and atherosclerotic segments of the same coronary artery (Table II). Cultures from individual patients were maintained as separate cultures and cells were not pooled. Subconfluent cells were passaged by trypsinization. Cells derived from 2nd passage were used in order to preserve most of their *in vivo* properties. VSMC were growth in medium 199 containing 20% foetal calf serum (FCS) plus 2% human serum, were arrested in medium with 0.4% FCS for 48 hours and then incubated with LDL in absence of FCS or lipoprotein deficient serum (LPDS).

Table II. Patient characteristics

	Age	**Sex**	**Clinical status**
		Normal VSMC	
H92	25	M	Motor vehicle accident
H95	59	M	Ischemic cardiopathy
H108	57	M	Ischemic cardiopathy
H110	14	F	Nonischemic cardiopathy
H112	25	F	Nonischemic cardiopathy
		Plaque VSMC	
H95	59	M	Ischemic cardiopathy
H108	57	M	Ischemic cardiopathy
H79	61	M	Nonischemic cardiopathy
H112	25	F	Nonischemic cardiopathy

LDL preparation and determination of the free and cholesteryl ester content

Human LDLs ($d_{1.019}$-$d_{1.063}$ g/mL) were obtained from pooled sera of normocholesterolemic volunteers and modified by vortexing as previously described [30]. LDL preparations were less than 48 hours old, non-oxidized (less than 1.2 mmol malonaldehyde/mg protein LDL) and without detectable levels of endotoxin (Limulus Amebocyte Lysate test, Bio Whittaker). Quiescent normal or plaque-VSMC from H95, H108 and H112 patients were incubated with increasing concentrations of native LDL (nLDL) or agLDL (50,100,200 μg/mL) for 24 hours. Then exhaustively washed and harvested into 0.10 mol/L NaOH. Lipid extraction and thin layer chromatography (TLC) was performed as previously described [30].

Real-time PCR

Quiescent VSMC were incubated with nLDL or agLDL (100 μg/mL) for 24 hours. Cells were washed with PBS and total RNA and protein were isolated by using the Tripure^{TM} isolation Reagent (Roche Molecular Biochemicals) according to the manufacturer. LRP and LDL receptor mRNA were analyzed by real-time PCR as previously described [19]. TaqMan fluorescent Real-Time PCR primers and probes (6'FAMMGB) for LRP and LDL receptor were designed by use of Primer Express software from PE biosystems. The specificity and the optimal primer and probe concentrations were tested. Bcl_2 (Hs00153350) BAX (Hs00180269) and CPP32 (Hs00263337) were used as "assay on demand" (PE biosystems). Human 18S rRNA (4 319413E) and *gapdh* (4326317E) were used as endogenous control (PE Biosystems). Taqman real-time PCR was performed with 2 μL/well of RT products (1 μg total RNA) in 25 μL of TaqMan PCR Master Mix (PE Biosystems) with the primers at 300 nmol/L and the probe at 200 nmol/L. PCR was performed at 95ºC for 10 min (for AmpliTaq Gold activation) and then run for 40 cycles at 95ºC for 15 seconds and 60ºC for 1 minute on the ABIPRISM 7000 Detection System (PE biosystems). The threshold cycle (Ct) values were determined and normalized to the housekeeping gene gapdh or 18S rRNA as needed. Since the targets have similar amplification efficiency than endogenous controls, we have used the comparative Ct method (delta delta Ct) to perform relative quantification of LRP, LDL receptor, Bcl_2 and BAX.

Western blot analysis

SDS-PAGE was run as previously described [17]. Blots were incubated with monoclonal antibodies against human LRP (β-chain, Research Diagnostics, clone 8B8 RDI 61067, dilution 1:40) or against human CPP32 (Transduction Laboratories, clone 19, dilution 1:1000). To test equal protein loading for the different samples, blots were also incubated with monoclonal antibodies against human α-actin (Dako, clone 1A4, 1:10000).

DNA synthesis

Proliferation assays were performed in VSMC (2^{nd} passage) obtained from normal and atherosclerotic plaque areas of the patient H95 and H108. VSMC DNA synthesis was measured by [^{3}H]thymidine incorporation. VSMC were seeded in 24-well plates at 20.000 cells/cm^2 and grown to confluence in the current medium. After 48 hours, the growth medium

was replaced by an arresting medium containing 0.2% FCS and maintained for a further 24 hours. At this time, nLDL and agLDL (100 μg/mL) were added to the culture medium and maintained for 24 hours. Then, cells were exhaustively washed and cultured in a medium containing 1 μCi/mL [^{3}H]thymidine in the absence or presence of 10^{-9} mol/L PDGF-BB for 24 hours. At the end of this period, the cells were washed with PBS, fixed with cold methanol 95% for 5 min and incubated with 10% trichloroacetic acid (TCA) for 10 min in an ice-bath. Then, the cells were dissolved in 0.10 mol/L NaOH and the amount of [^{3}H]thymidine incorporated was determined by scintillation using Microbeta 1450 (Trilux).

Image analysis

Results were evaluated with an Olympus Vanox AHBT3 microscope and digitalized by a Sony 3CCD camera. The expression of LRP and α-actin proteins were semi-quantitatively assessed by scoring the proportion of staining [31,32]. First, the entire section was systematically scanned at 200x magnification and five non-overlaping fields were selected to perform the analysis. The percentage of positive staining with each specific antibody was determined, by using the Visilog 4.1.2 Image Analysis System, dividing the area of the target protein by the total area of the field. In some cases, results were normalized by the total nuclei number in each field. The results were expressed as percentage of positive areas and are shown as the mean±SEM of semi-quantifications from a minimum of five sections each, from coronary lesions processed in independent immunostainings.

Statistical analysis

Comparisons among groups were performed by parametric (one factor ANOVA) or non-parametric (Kruskal-Wallis, Mann-Whitney U test) analysis as needed. Statistical significance was considered when $p<0.05$.

Results

Comparison of cholesteryl ester accumulation from LDL in normal and plaque-VSMC.

In order to test whether VSMC-agLDL internalization plays a role in the lipid accumulation associated to atherosclerotic lesion progression, normal and plaque-VSMC, obtained as described in Methods, were incubated with increasing concentrations of agLDL

(50,100, 200 μg/mL) for 24 hours. In three cases VSMC were obtained from normal and atherosclerotic segments of the same coronary artery (Table II). Both normal and plaque-VSMC show undetectable levels of CE (<0.5±0.2 μg CE/mg protein) when cultured in absence of LDL. Since the CE content of control VSMC was negligible and LDL reduced endogenous cholesterol synthesis to undetectable levels, the increase in CE content observed in VSMC reflects the cholesterol that enter through LDL uptake [30]. No significant differences were found in CE increase induced by nLDL in plaque *versus* normal VSMC (1.5±0.42 *vs* 1.35±0.31 μgCE/mg protein at 100 μg/mL). However, agLDL induced higher intracellular CE accumulation in plaque-VSMC compared to normal VSMC (89.28±6.1 *vs* 60.34±4.1 at 100 μg/mL agLDL, $P<0.05$; 97.86±6 *vs* 63.2±3.5 at 200 μg/mL agLDL, $P<0.05$) (Figure 1). nLDL or agLDL did not alter the free cholesterol (FC) content of normal or plaque-VSMC (data not shown) [16,17,30].

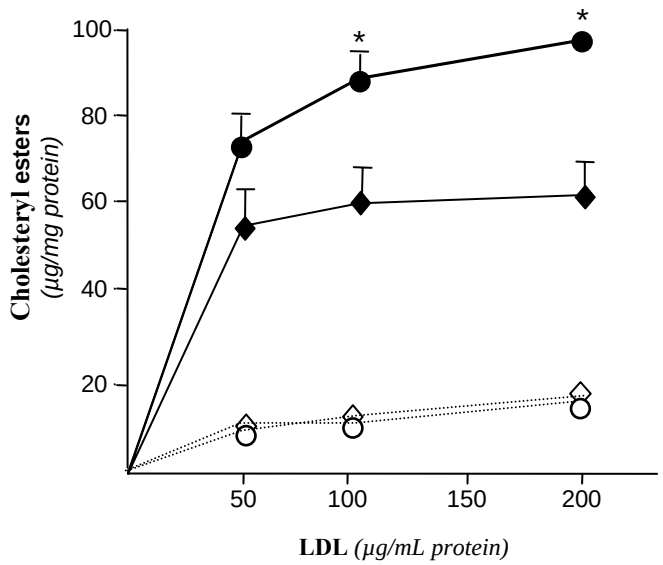

Figure 1. Thin layer chromatography analysis of intracellular cholesterol accumulation induced by nLDL and agLDL in normal and plaque-VSMC. Line graphs showing quantification of CE bands incubated with agLDL (closed symbols) and nLDL (open symbols) from TLC in normal (romboid) and plaque-VSMC (circles). Results are expressed as μg CE per milligram protein and are shown as mean±SEM of two independent experiments performed with VSMC from H95, H108 and H112 patients. **P*<0.05, *versus* normal VSMC.

Comparison of LRP expression in normal and plaque-VSMC.

LRP, receptor that binds and internalizes agLDL [16-18], and LDL receptor mRNA expression were compared in normal and plaque-VSMC obtained as described in Methods. In three cases VSMC were obtained from normal and atherosclerotic segments of the same

coronary artery (Table II). As shown in Figure 2, the levels of LRP mRNA expression were much higher than those of LDL receptor mRNA either in normal VSMC (LRP: 3.87±0.28 *vs* LDLr: 0.57±0.16; $P<0.05$) or plaque-VSMC (LRP: 6.06±0.55 vs LDLr: 0.39±0.15; $P<0.05$). LRP mRNA expression was significantly higher ($P<0.05$) in plaque-VSMC than in normal VSMC (Figure 2A). In contrast, LDL receptor mRNA expression was significantly lower in plaque-VSMC ($P<0.05$) (Figure 2B). VSMC from normal and advanced plaques obtained from the same patient followed the pattern described for the rest of samples, there was higher LRP expression in VSMC from atherosclerotic lesions. Western blot analysis (Figure 2C) showed significantly higher LRP protein expression in plaque-VSMC than in normal VSMC.

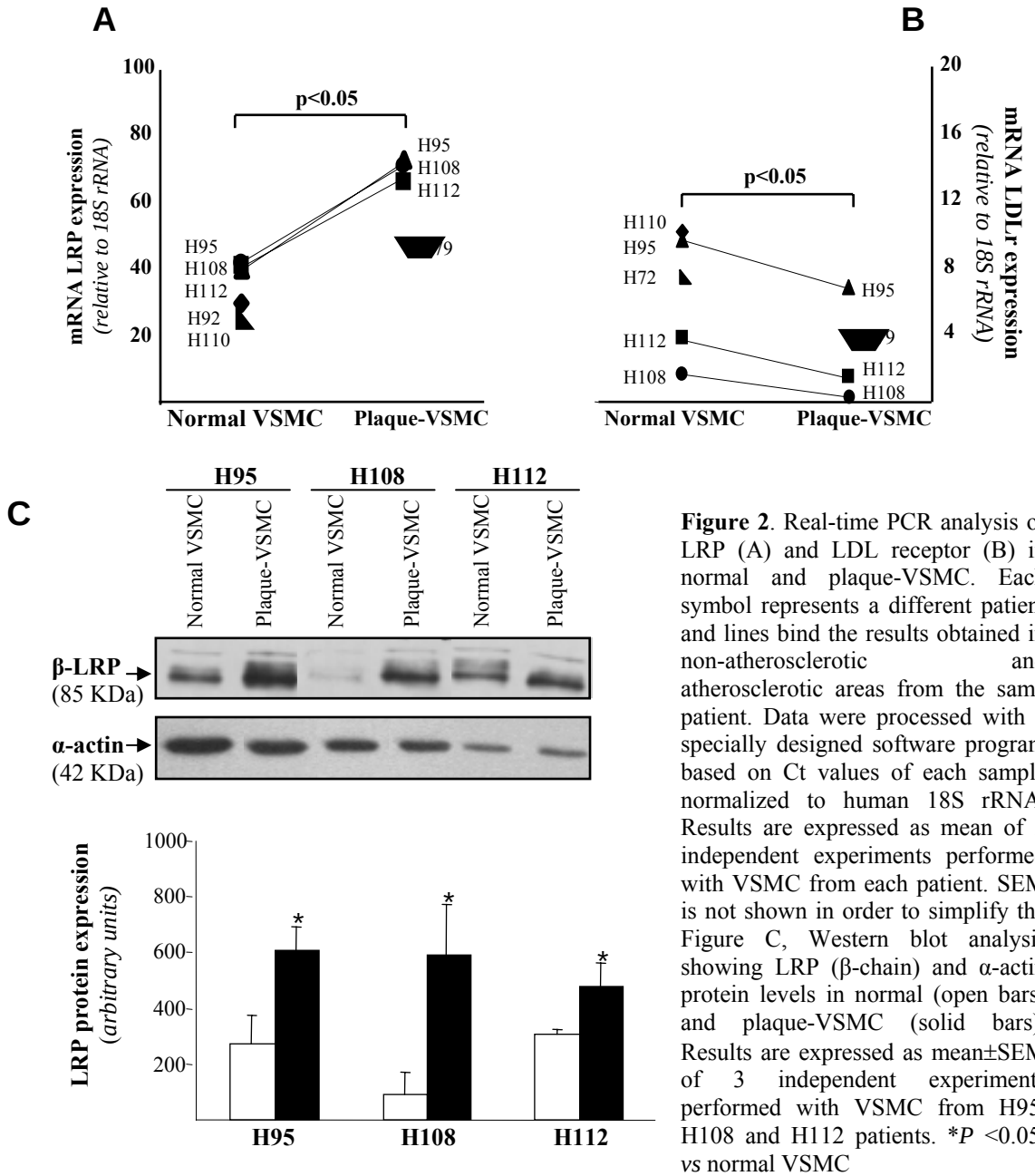

Figure 2. Real-time PCR analysis of LRP (A) and LDL receptor (B) in normal and plaque-VSMC. Each symbol represents a different patient and lines bind the results obtained in non-atherosclerotic and atherosclerotic areas from the same patient. Data were processed with a specially designed software program based on Ct values of each sample normalized to human 18S rRNA. Results are expressed as mean of 3 independent experiments performed with VSMC from each patient. SEM is not shown in order to simplify the Figure C, Western blot analysis showing LRP (β-chain) and α-actin protein levels in normal (open bars) and plaque-VSMC (solid bars). Results are expressed as mean±SEM of 3 independent experiments performed with VSMC from H95, H108 and H112 patients. *P <0.05, *vs* normal VSMC

Evolution of lipid deposition, α-actin positive cells, and LRP expression during atherosclerotic lesion progression in the human coronary arteries.

Immunohistochemical analysis of lipid (measured as apoB100 and ORO staining), VSMC (measured as α-actin) and LRP was performed in the 4 groups of plaques previously described in Methods. Image analysis quantification showed a significant increase in intimal lipid deposition in group II and group III *versus* group I (Figure 3A). Figure 3B shows the results of the percentage of intimal surface occupied by α-actin. A marked decrease in the VSMC α-actin positive cells was observed with atherosclerotic lesion progression.

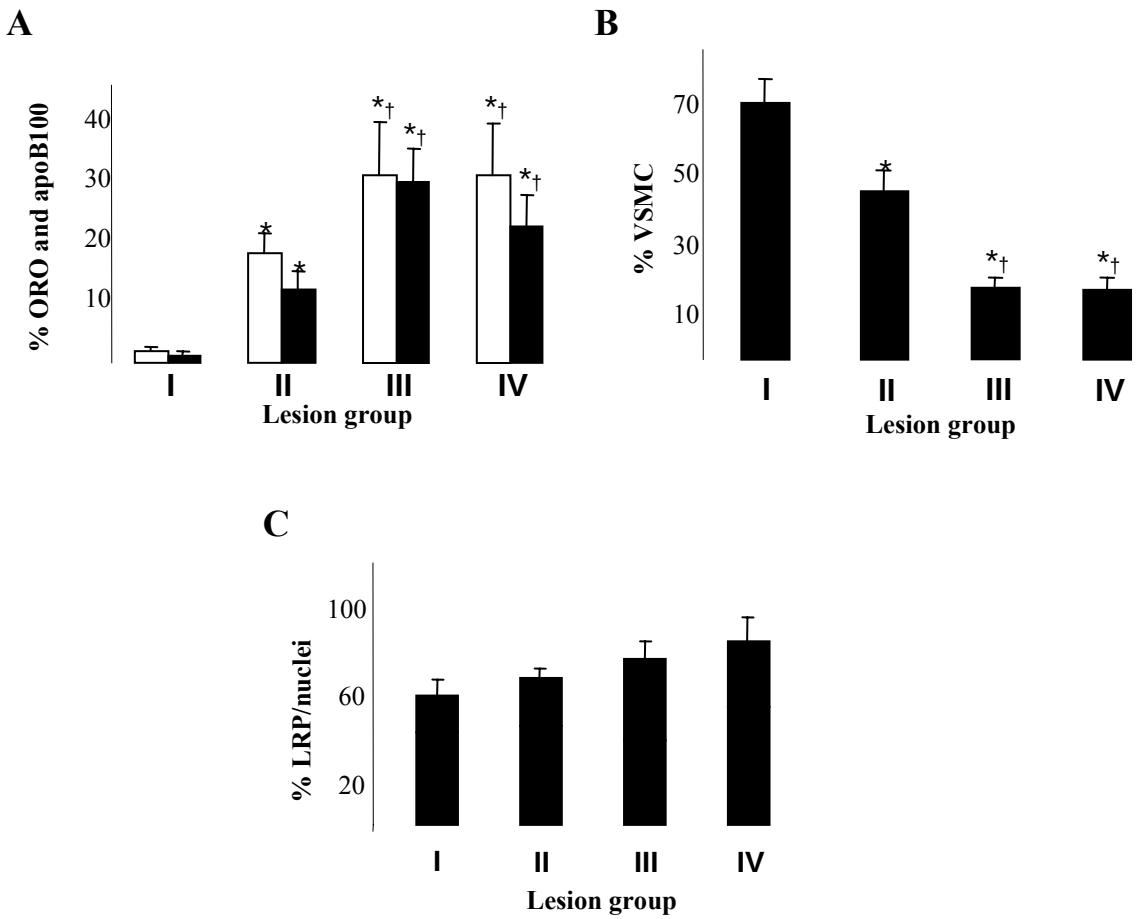

Figure 3. Image analysis quantification of lipid, VSMC content and LRP staining in group I, group II, group III and group IV lesions. A, Bar graphs showing the percentage of apoB-100 (open bars) and ORO (closed bars) B, Bar graphs showing the percentage of intimal area occupied by VSMC. C, Bar graphs showing the quantification of LRP positive cells in the vessel wall. Results are expressed as the percentage of marker positive areas and shown as the mean±SEM of semi-quantifications of five sections each, from coronary lesions processed in independent immunostainings. **P* <0.05, *versus* group I; †*P*<0.05, *versus* group II.

Concerning LRP expression, there was a moderate increase on LRP expression in the vessel wall with lesion progression (Figure 3C). Figure 4 shows a representative group I

(panel A) and group III (advanced high risk lesions) (panel B). In group I lesion, LRP was highly expressed in the intima and adventicia with minor expression in the media (panel C). However, in high risk lesions, LRP expression was highly detected both in the plaque and the internal tunica media (panel D). LDL receptor expression was low and homogeneous both in group I (panel E) or group III lesion (panel F).

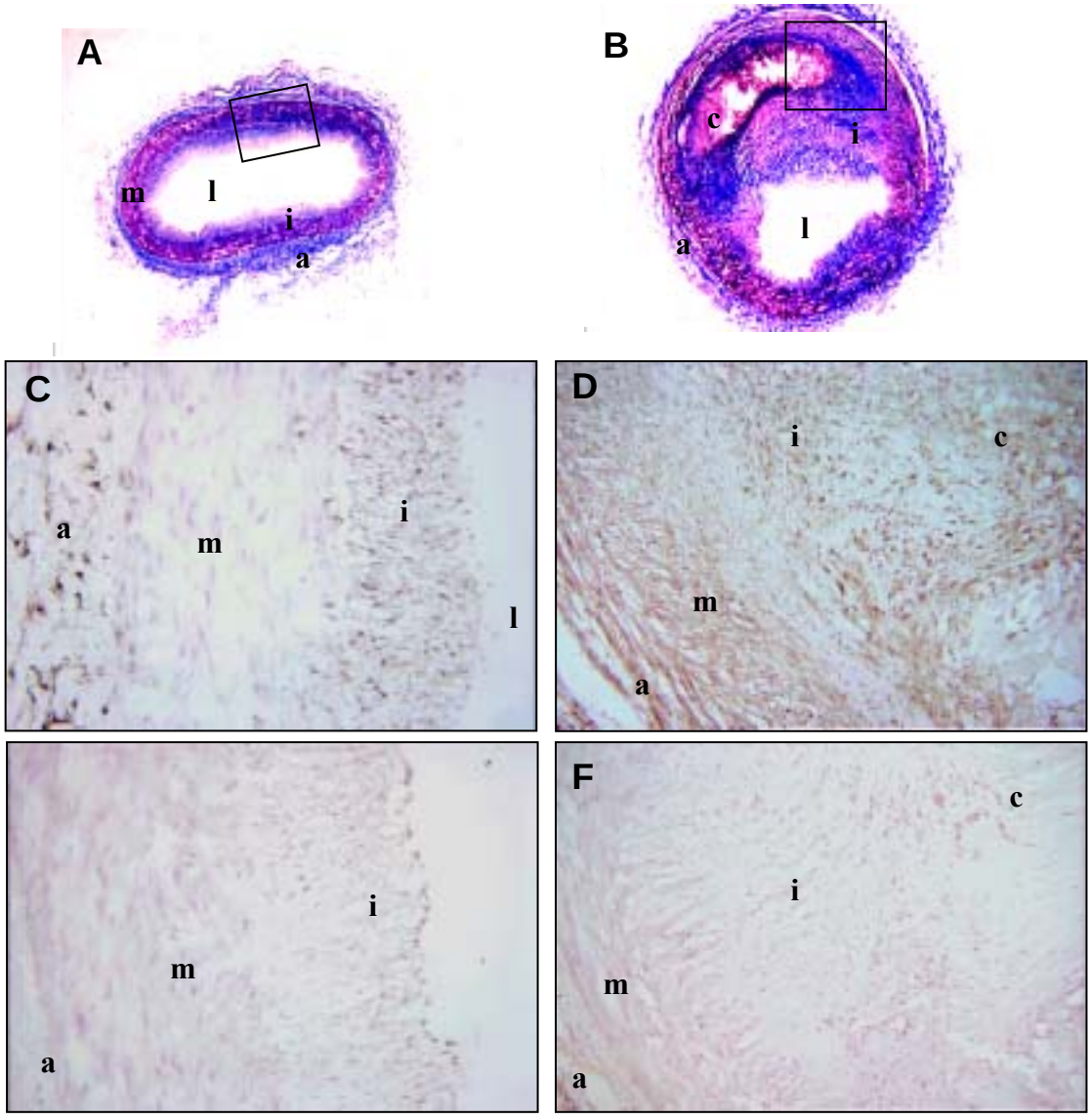

Figure 4. Representative Masson Trichromic staining in group I (A) and group III (B) lesions. Representative LRP immunostaining in group I (C) and group III (D) lesions. Representative LDL receptor immunostaining in group I (E) and group III (F) lesions. l, lumen; i, intima; m, media; c, lipid core. Bar: 100 μm.

Effects of agLDL loading in normal and plaque-VSMC survival .

In order to know whether LRP-mediated agLDL internalization was responsible for the decrease in VSMC number during atherosclerotic lesion progression, we analyzed DNA synthesis and Bcl_2/BAX ratio, index of VSMC survival, in normal and plaque-VSMC. As shown in Figure 5, plaque-VSMC showed lower DNA duplication rate than normal VSMC in absence of LDL (6536±636 *vs* 11151±815 cpm [^{3}H]thymidine; $P<0.05$). Neither nLDL nor agLDL (100 μg/mL) exerted any significant effect on the proliferative rate of normal VSMC. However, both nLDL and agLDL significantly increased [^{3}H]thymidine incorporation of plaque-VSMC ($P<0.05$).

PDGF-BB efficiently increased the proliferative rate of normal VSMC ($P<0.05$). However, PDGF-BB did not significantly alter [^{3}H]thymidine incorporation of plaque-VSMC independently of the absence or presence of nLDL or agLDL.

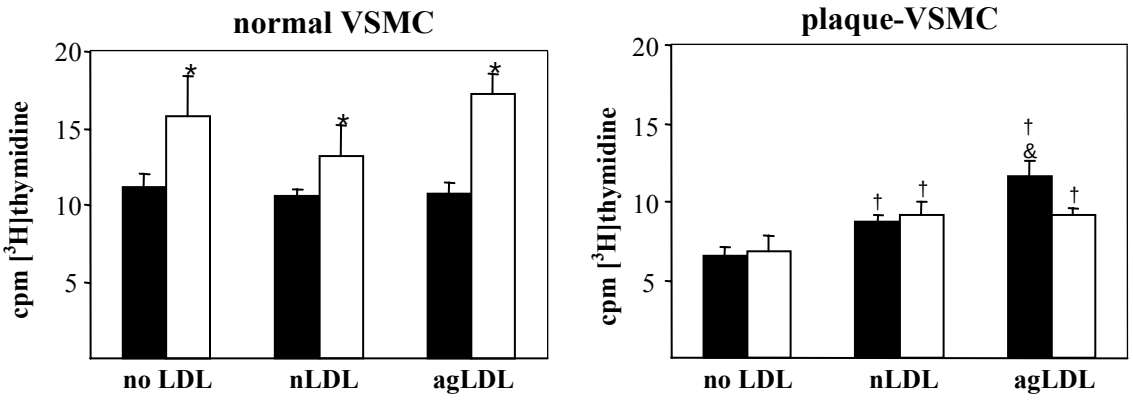

Figure 5. [^{3}H]thymidine incorporation analysis of VSMC DNA synthesis. Normal and plaque-VSMC were incubated by nLDL or agLDL (100 μg/mL). Then, VSMC were exhaustively washed and incubated with PDGF-BB (10^{-9} mol/L) and [^{3}H]thymidine (1 μCi/mL) for 24 hours. Bar graphs showing quantification [^{3}H]thymidine in absence (closed bars) or presence (open bars) of PDGF-BB stimulus. Results are expressed as cpm [^{3}H]thymidine x10^3 incorporated and are shown as men±SEM of 2 independent experiments performed with VSMC from H95 and H108 patients. **P* <0.05, PDGF-BB *versus* no PDGF-BB; †*P*<0.05, LDL *versus* no LDL; &*P*<0.05, agLDL *versus* nLDL.

As shown in Figure 6A, Bcl_2/BAX ratio was significanlty lower in plaque-VSMC compared to normal VSMC (plaque-VSMC:0.14±0.05 *vs* normal VSMC: 0.51±0.07; $P<0.05$) independent of the presence of nLDL or agLDL. No statistically significant differences were found in CPP32 expression levels at mRNA (Figure 6B) or protein levels (Figure 6C) between plaque and normal VSMC.

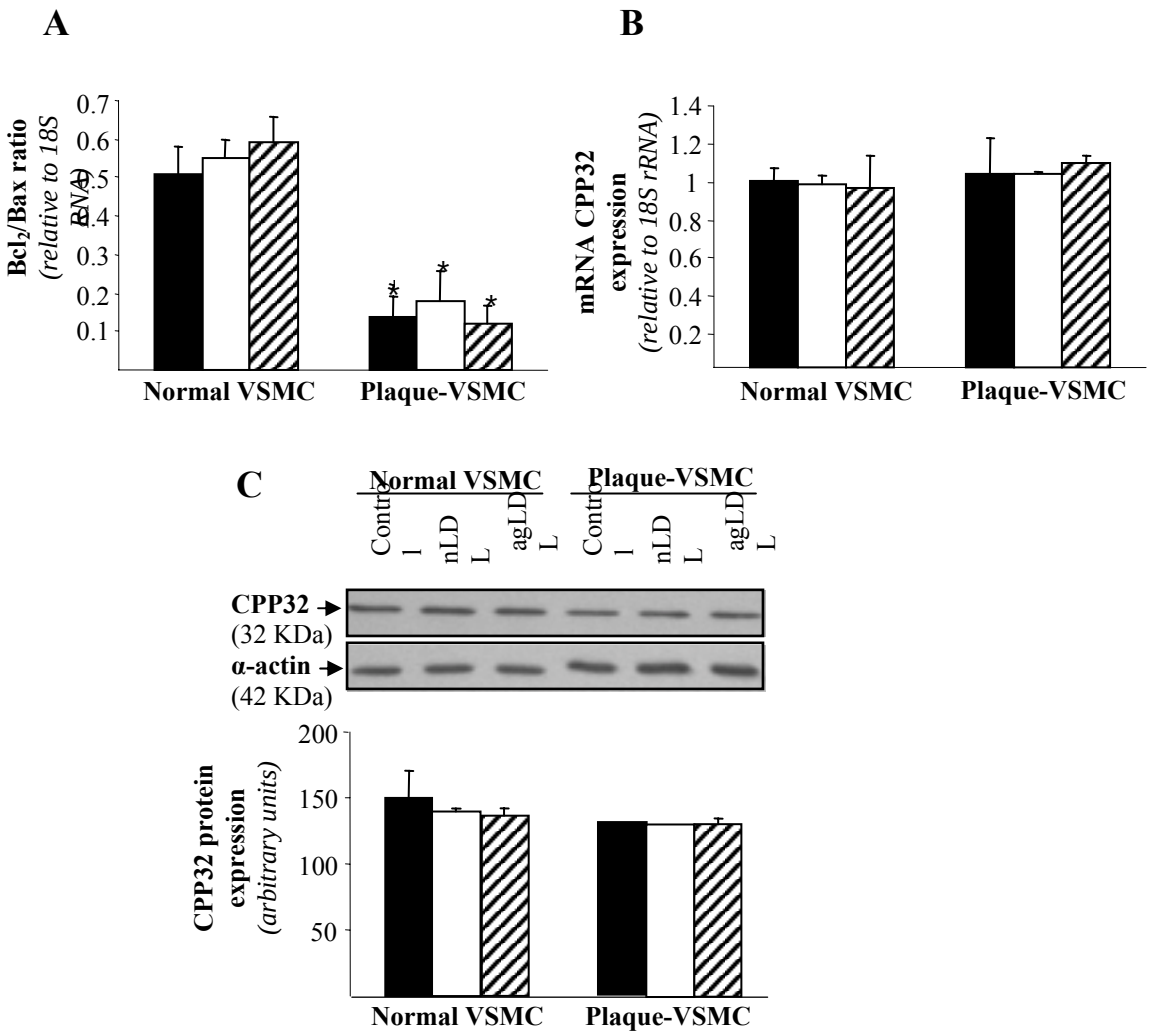

Figure 6. Real time PCR quantification of Bcl_2/BAX mRNA ratio (A) and CPP32 mRNA (B) expression levels in normal and plaque-VSMC incubated in the absence (solid bars) or presence of nLDL (open bars) or agLDL (hatched bars) (100 μg/mL) for 24 hours. Data were processed with a specially designed software program based on Ct values of each sample. Results are shown as men±SEM of 3 independent experiments performed with VSMC from H95,H108 and H112 patients. B. Western blot analysis showing CPP32 protein and α-actin levels in normal and plaque-VSMC incubated in the absence (solid bars) or presence of nLDL (open bars) or agLDL (hatched bars) (100 μg/mL) for 24 hours. Results are expressed as mean±SEM of 3 independent experiments performed with VSMC from H95, H108 and H112 patients. * P<0.05, plaque-VSMC *versus* normal VSMC.

Discussion

In agreement with the role of LRP on VSMC-agLDL internalization previously described in our group [16-19], plaque-VSMC LRP over-expression was associated to increased intracellular CE accumulation from agLDL, one of the most important LDL modifications in the arterial intima [14,15]. The results obtained *in vitro* are in agreement with the increase in lipid accumulation and LRP expression during coronary atherosclerotic lesion progression observed by us and others [3-5]. These results are also in agreement with the effect of hypercholesterolemia upregulating LRP expression in the porcine vascular wall [19] and in blood mononuclear cells [33]. Taken together, these results indicate that LRP mediated agLDL internalization likely contributes to lipid accumulation recruitment in the arterial wall.

According to the described reduction of the intimal VSMC number with plaque progression, we found that plaque-VSMC proliferate less, have no response to PDGF-BB stimulus and have lower Bcl_2/BAX survival ratio. Although these results indicate that plaque-VSMC have higher tendency to apoptosis, they are not apoptotic in cell culture since they do not show CPP32 overexpression. The higher tendency to apoptosis and limited proliferation of plaque-VSMC are phenotypic characteristics that seem not to be lost by subculture and they are an intrinsec property of these cells [22,24,25]. In fact, some of the genetic differences underlying these properties have been elucidated such as an inability to phosphorylate RB (blocking proliferation) [34] and defective IGF-1 signaling in plaque-VSMC [35]. Additionally, a role for the LRP receptor limiting cell proliferation has been described in different cell types [36-38]. In fact, LRP1 cytoplasmic tail contain residues that can be phosphorylated by both protein kinase A and protein kinase C (PKC). Phosphorylation by PKC seems to be a key mechanism for LRP mediated signal transduction [39]. Considering that PKC is a key regulator of proliferation and apoptosis, the regulation of LRP cytoplasmic tail phosphorylation might be a key event determining VSMC fate.

However, the lower proliferation and survival rate associated to plaque-VSMC were not due to higher capacity of plaque-VSMC to internalize agLDL through LRP since agLDL increased the proliferation rate of plaque-VSMC in absence of PDGF-BB and agLDL did not alter the survival rate Bcl_2/BAX or CPP32 expression levels. The lack of pro-apoptotic effects of agLDL on VSMC survival is in agreement with the results demonstrating the inhibitory effect of agLDL on macrophage activation-induced apoptosis [27] and those obtained by Knockx *et al* that demonstrate how agLDL induces death associated protein (DAP) kinase in

human VSMC but not apoptosis [40]. Our results indicate that LRP mediated agLDL uptake could be one of the main pro-atherogenic mechanisms increasing intracellular lipid deposition in VSMC. However, LRP mediated agLDL uptake seems not to be responsible for the reduced survival of plaque-VSMC.

References

[1] Ylä-Herttuala S. Expression of lipoprotein receptors and related molecules in atherosclerotic lesions. *Curr Opin Lipidol* 1996; 7: 292-297.

[2] Krieger M, Herz J. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu Rev Biochem* 1994; 63: 601-637.

[3] Hiltunen TP, Luoma JS, Nikkari T, Ylä-Herttuala. Expression of LDL receptor, VLDL receptor, LDL receptor-related protein, and scavenger receptor in rabbit atherosclerotic lesions. *Circulation* 1998; 97: 1079-1086.

[4] Moestrup SK, Gliemann J, Pallesen G. Distribution of α_2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res* 1992; 269: 375-382.

[5] Luoma J, Hiltunen T, Särkioja T, Moestrup SK, Gliemann J, Kodama T *et al*. Expression of α_2-macroglobulin receptor/low density lipoprotein receptor-related protein and scavenger receptor in human atherosclerotic lesions. *J Clin Invest* 1994; 93: 2014-2021.

[6] Boullier A, Bird DA, Chang M, Dennis EA, Friedman P, Gillotte-Taylor K *et al*. Scavenger receptors, oxidized LDL and atherosclerosis. *Ann N Y Acad Sci* 2001; 947: 214-22.

[7] Steinberg D & Witztum JL. Lipoproteins, lipoprotein oxidation, and atherogenesis. *In Molecular Basis of Cardiovascular Disease*. K.E Chien, ed. 1998: 458-475. W.B. Saunders. Philadelphia.

[8] Kowal RC, Herz J, Goldstein JL, Esser V, Brown MS. Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apolipoprotein E-enriched lipoproteins. *Proc Natl Acad Sci USA* 1989; 86: 5810-5814.

[9] Beisiegel U, Weber W, Ihrke G, Herz J, Stanley KK. The LDL-receptor related protein, LRP, is an apoprotein E-binding protein. *Nature* 1989; 341: 162-164.

[10] Chappel DA, Fry GL, Waknitz MA, Iverius PH, Williams SE, Strickland DK. The low density lipoprotein receptor-related protein/α2 macroglobulin receptor binds and mediates catabolism of bovine milk lipoprotein lipase. *J Biol Chem* 1992; 268: 14168-14175.

[11] Chappel DA, Fry GL, Waknitz LE, Muhonen LE, Pladet MW, Iverius PH *et al*. Lipoprotein lipase induces catabolism of normal triglyceride-rich lipoproteins via the low density lipoprotein receptor related protein/α_2-macroglobulin receptor in vitro. *J Biol Chem* 1992; 268: 14168-14175.

[12] Krapp A, Ahle S, Kersting S, Hua Y, Kneser K, Nielsen M *et al*. Hepatic lipase mediates the uptake of chylomicrons and β-VLDL into cells via the LDL receptor-related protein LRP. *J Lipid Res* 1996; 37: 926-936.

[13] Reblin T, Niemeier A, Meyer N, Willnow TE, Kronenberg F, Dieplinger H *et al*. Cellular uptake of lipoprotein [a] by mouse embryonic fibroblasts via the LDL receptor and the LDL-receptor related protein. *J Lipid Res* 1997; 38: 2103-2110.

[14] Hoff HF, O'Neil J. Lesion-derived low density lipoprotein and oxidized low density lipoprotein share a lability for aggregation, leading to enhanced macrophage degradation. *Arterioscler Thromb* 1991; 11: 1209-1222.

[15] Guyton JR, Klemp KF, Mims MP. Altered ultrastructural morphology of self-aggregated low density lipoproteins: coalescence of lipid domains forming droplets and vesicles. *J Lipid Res* 1991; 32: 953-961.

[16] Llorente-Cortés V, Martínez-González J, Badimon L. LDL receptor-related protein mediates uptake of aggregated LDL in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2000; 20: 1572-1579.

[17] Llorente-Cortés V, Otero-Viñas M, Hurt-camejo E, Martínez-González J, Badimon L. Human coronary smooth muscle cells internalize versican-modified LDL through LDL receptor-related protein and LDL receptors. *Arterioscler Thromb Vasc Biol* 2002; 22: 387-393.

[18] Llorente-Cortés V, Otero-Viñas M, Badimon L. Differential role of heparan sulfate proteoglycans on aggregated LDL uptake in human vascular smooth muscle cells and mouse embryonic fibroblasts. *Arterioscler Thromb Vasc Biol* 2002; 22:1905-1911.

[19] Llorente-Cortés V, Otero-Viñas M, Sánchez S, Rodríguez C, Badimon L. LDL up-regulates Low density lipoprotein receptor-related protein expression in vascular smooth

muscle cells. Possible involvement of sterol regulatory element binding protein-2-dependent mechanism. *Circulation* 2002; 106: 3104-3110.

[20] Bicknell KA, Surry EL, Brooks G. Targeting the cell machinery for the treatment of cardiovascular disease. *J Pharm Pharmacol* 2003; 55: 571-591.

[21] Afroze T, Husain M. Cell cycle dependent regulation of intracellular calcium concentration in vascular smooth muscle cells: a potential target for drug therapy. *Curr Drug Targets Cardiovasc Haematol Disord* 2001; 1: 23-40.

[22] Bennett MR. Apoptosis of vascular smooth muscle cells in vascular remodelling and atherosclerotic plaque rupture. *Cardiovasc Res* 1999; 41: 361-368.

[23] Geng Y-J, Libby P. Evidence for apoptosis in advanced human atheroma: colocalization with interleukin-1β converting enzyme. *Am J Pathol* 1995; 147: 251-266.

[24] Bennet MR, Evan GI, Schwartz SM. Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic vessels. *J Clin Invest* 1995; 95: 2266-2274.

[25] Bennett MR, Boyle JJ. Apoptosis of vascular smooth muscle cells in atherosclerosis. *Atherosclerosis* 1998; 138: 3-9.

[26] Hsieh C-C, Yen M-H, Yen C-H, Lau Y-T. Oxidized low density lipoprotein induces apoptosis via generation of reactive oxygen species in vascular smooth muscle cells. *Cardiovasc Res* 2001; 49: 135-145.

[27] Kubo N, Kikuchi J, Furukawa Y, Sakai T, Ohta H, Iwase S *et al*. Regulatory effects of aggregated LDL on apoptosis during foam cell formation of human peripheral blood monocytes. *FEBS letters* 1997; 409: 177-182.

[28] Varela O, Martínez-González J, Badimon L. The response of smooth muscle cells to α-thrombin depends on its arterial origin: comparison among different species. *Eur J Clin Invest* 1998; 28: 313-323.

[29] Martínez-González J, Berrozpe M, Varela O, Badimon L. Heterogeneity of smooth muscle cells in advanced human atherosclerotic plaques: intimal smooth muscle cells expressing a fibroblast surface protein are highly activated by platelet-released products. *Eur J Clin Invest* 2000; 31: 939-949.

[30] Llorente-Cortés V, Martínez-González J, Badimon L. Esterified cholesterol accumulation induced by aggregated LDL uptake in human vascular smooth muscle cells is reduced by HMG-CoA reductase inhibitors. *Arterioscler Thromb Vasc Biol* 1998; 18: 738-746.

[31] Padró T, Bieker R, Ruiz S, Steins M, Retzlaff S, Burger H *et al*. Overexpression of vascular endothelial growth factor (vEGF) and its cellular receptor (vEGFR-2) in the bone marrow of patients with acute myeloid leukemia. *Leukemia* 2002; 16: 1302-1310.

[32] Gobbi H, Dupont WD, Simpson W, Plummer WD Jr, Schuyler PA, Olson SJ *et al*. Transforming growth factor-□ and breast cancer risk in women with mammary epithelial hyperplasia. *J Natl Cancer Inst* 1999; 91: 2096-2101.

[33] Boucher P, Lorgeril M, Salen P, Crozier P, Delaye J, Vallon JJ *et al*. Effect of dietary cholesterol on low density lipoprotein receptor, 3-hydroxy-3-methylglutaryl-CoA reductase and low density lipoprotein receptor-related protein mRNA expression in healthy humans. *Lipids* 1998; 33: 1177-1186.

[34] Bennett MR, macdonald K, Chan SW, Boyle JJ, Weissberg PL. Cooperative interactions between RB and p53 regulate cell proliferation, cell senescence, and apoptosis in human vasuclar smooth muscle cells from atherosclerotic plaques. *Circ Res* 1998; 82: 704-712.

[35] Patel V, Zhang Q-J, Soos M, Siddle K, Weissberg PL, Bennett MR. Defect of insulin-like growth factor 1 signaling underlies increased apoptosis of human atherosclerotic plaque-derived vascular smooty muscle cells. *Circ Res* 2001; 88: 895-902.

[36] Loukinova E, Ranganathan S, Kuznetsov S, Gorlatova N, Migliorini MM, Loukinov D *et al*. Platelet-derived growth factor (PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP). *J Biol Chem* 2002; 277: 15499-15506.

[37] Boucher P, Liu PV, Gotthardt M, Hiesberger T, Anderson RGW, Herz J. Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low Density lipoprotein receptor-related protein in caveolae. *J Biol Chem* 2002; 275: 15507-15513.

[38] Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: role in vascular wall integrity and protection from atherosclerosis. *Science* 2003; 300: 329-32.

[39] Van der Geer P. Phosphorylation of LRP1: regulation of transport and signal transduction. Trends Cardiovascular Med 2002; 12(4): 160-165.

[40] Martinet W, Schrijvers DM, De Meyer GRY, Thielemans J, Knaapen MWN, Herman AG *et al*. Gene expression profiling of apoptosis-related genes in human atherosclerosis. *Arterioscler Thromb Vasc Biol* 2002; 22: 2023-2029.