High-Density Lipoproteins Reduce Endothelial-to-Mesenchymal Transition

Frank Spillmann, Kapka Miteva, Burkert Pieske, Carsten Tschöpe, Sophie Van Linthout

Objective—Endothelial-to-mesenchymal transition is an inflammation-induced process by which endothelial cells transdifferentiate into fibroblasts. Based on the endothelial-protective and antifibrotic effects of high-density lipoproteins (HDL), we aimed to investigate whether HDL can reduce endothelial-to-mesenchymal transition.

Approach and Results—Therefore, human aortic endothelial cells were stimulated with the profibrotic factor transforming growth factor (TGF)-β1 in the presence or absence of HDL. Their impact on the transition of endothelial cells to mesenchymal-like cells was analyzed. Phase contrast microscopy demonstrated that HDL abrogated the TGF-β1–induced spindle-shape morphology in human aortic endothelial cells. Furthermore, HDL decreased the TGF-β1–mediated induction of a-smooth muscle actin expression and concomitant loss in endothelial cadherin expression, as shown by immunofluorescence staining and flow cytometry. In addition, HDL decreased the TGF-β1–induced collagen deposition in human aortic endothelial cells involving the scavenger receptor class B, type 1 and downstream phosphatidylinositol-3-kinase following the findings that the HDL-mediated reduction was abrogated by scavenger receptor class B, type 1 siRNA knockdown and phosphatidylinositol-3-kinase inhibition, respectively. The HDL-mediated reduction in endothelial-to-mesenchymal transition was associated with an induction of the inhibitory Smad, Smad 7.

Conclusions—We provide the first in vitro evidence that the endothelial-protective and antifibrotic effects of HDL include the reduction in endothelial-to-mesenchymal transition. (Arterioscler Thromb Vasc Biol. 2015;35:1774-1777. DOI: 10.1161/ATVBAHA.115.305887.)

Key Words: aortic endothelial cells • endothelial-to-mesenchymal transition • fibrosis • HDL • transforming growth factor

Endothelial-to-mesenchymal transition (EndMT) is an inflammation-induced process by which endothelial cells transdifferentiate into mesenchymal-like cells, characterized by induced a-smooth muscle actin (α-SMA) expression, loss of endothelial cell markers, and increased collagen deposition. This phenomena contributes to different cardiovascular pathologies, including cardiac fibrosis and vascular remodeling, and is among others triggered by the lack of nitric oxide (NO).

Dyslipidemia, characterized by low high-density lipoproteins (HDL)-cholesterol, high low-density lipoprotein-cholesterol, and high triglyceride levels, underlies endothelial dysfunction and atherosclerosis and is also strongly associated with the progression of cardiac fibrosis. The endothelial-protective effects of HDL, including their antioxidative, anti-inflammatory, antiapoptotic, and proangiogenic features, are well recognized. HDL also have the potential to regenerate/repair the endothelium: they induce the mobilization of endothelial progenitor cells/circulating angiogenic cells from the bone marrow, promote their subsequent incorporation at the site of endothelial damage, and induce the migration of mesenchymal stromal cells. The latter may contribute to endothelial repair because mesenchymal stromal cells are able to render support to endothelial cells as pericyte-like cells and to induce angiogenesis in a paracrine manner. HDL exert besides these abovementioned pleiotropic endothelial-protective effects also antifibrotic effects. The endothelial-protective and antifibrotic characteristics of HDL, on the one hand, and the characteristics of EndMT, on the other hand, triggered us to investigate whether HDL reduce EndMT.

Materials and Methods

Materials and methods related to cultured human aortic endothelial cells (HAEC), including HDL dose determination (Figure I in the online-only Data Supplement), immunostainings, flow cytometry, mRNA quantification, collagen content, nitric oxide analysis, knockdown via siRNA, western blot, and statistics appear in the online-only Data Supplement.

Results

HDL decreased the transforming growth factor (TGF)-β1–induced morphological transition of an endothelial to spindle-shaped mesenchymal-like cell phenotype, as shown
by phase contrast pictures (Figure II in the online-only Data Supplement). In accordance with this observation, immuno-fluorescence staining demonstrated less α-SMA–positive and VE-cadherin–negative HAEC, when HAEC were stimulated with TGF-β1 in the presence of HDL compared with TGF-β1 alone (Figure 1A). Flow cytometry further assessed that HDL downregulated the percentage of TGF-β1–induced α-SMA–positive and VE-cadherin–negative HAEC by 1.6-fold (P<0.0005; Figure 1B). In addition, TGF-β1 supplementation induced collagen I and III mRNA expression and collagen accumulation in HAEC by 5.5-fold (P<0.0005), 1.7-fold (P<0.05), and 1.3-fold (P<0.001), respectively. In contrast, HDL abrogated the TGF-β1–mediated rise in collagen I and III mRNA expression and content, leading to levels not different from unstimulated basal conditions (Figure 2A–2C). The HDL-mediated reduction in collagen content was abrogated in TGF-β1–supplemented scavenger receptor class B, type 1 (SR-BI) siRNA compared with scrambled siRNA-transfected HAEC, indicating that the HDL-mediated decrease in collagen in TGF-β1–supplemented HAEC occurred in a SR-BI–dependent manner (Figure 2D). Furthermore, HDL induced the phosphorylation state of SR-BI downstream targets Akt and eNOS (Figure IIIA and IIIB in the online-only Data Supplement) and raised the TGF-β1–downregulated NO levels by 1.2-fold (P<0.05) (control, 6800±570; HDL, 6200±430; TGF-β1, 4800±330; TGF-β1+HDL, 6000±310). The HDL-mediated reduction of TGF-β1–induced collagen deposition in HAEC could be abrogated via the use of the phosphatidylinositol-3-kinase inhibitor Ly294002, whereas no significant inhibition could be reached with the NO-inhibitor L-NAME (P=0.0898; Figure IIIIC in the online-only Data Supplement). With respect to TGF-β1 downstream signaling involving Smads and the EndMT-inducing transcription factors Snail 1, Slug, and ZEB1, HDL induced Smad 7 expression by 1.2-fold (P<0.05), whereas it increased Snail 1 by 1.7-fold (P<0.005) and reduced Slug and ZEB1 mRNA expression by 2.0-fold (P<0.05) and 1.4-fold (P<0.05) under TGF-β1 conditions (Figure IV in the online-only Data Supplement), respectively.

Finally, HDL supplementation to HAEC stressed with TGF-β1 for 48 h declined the TGF-β1–induced collagen deposition in HAEC, providing the first evidence that HDL not only can reduce the development of EndMT, but might also reverse EndMT or induce mesenchymal-to-endothelial transition (Figure V in the online-only Data Supplement).17

**Discussion**

The present study provides the first in vitro evidence that HDL reduce EndMT as documented by their capacity (1) to reduce the TGF-β1–induced transition to a spindle-shaped morphology; (2) to decrease the TGF-β1–induced α-SMA expression and collagen accumulation; and (3) to abrogate the TGF-β1–mediated loss of the endothelial cell marker VE-cadherin.

The endothelial-protective effects of HDL are well established. Low plasma HDL levels are an independent predictor of endothelial dysfunction in healthy individuals and diabetic patients.18 In contrast, elevation of plasma HDL by niacin, infusion of synthetic HDL,19 gene transfer with apolipoprotein (apo) A-I,6 the main apolipoprotein of HDL, or the use of an apo A-I mimetic peptide20 improves impaired endothelial...
function. The HDL-mediated improvement in endothelial function involve their induction in endothelial NO production.\(^6,21\) Lack of NO, the main hallmark of endothelial dysfunction,\(^22\) results in EndMT,\(^4\) a process which relevance in the induction of cardiac fibrosis\(^1,2\) and in the development of heart failure with preserved ejection fraction\(^2,23\) follows from recent publications. TGF-β1, the primary cytokine driving fibrosis in various organs,\(^24–26\) is a potent inducer of EndMT. In this study, we demonstrate for the first time that HDL reduce TGF-β1–induced EndMT in HAEC, as shown by the HDL-mediated reduction in the transition toward spindle-shaped cells, collagen accumulation, α-SMA expression, and loss of the endothelial cell marker VE-cadherin, induced on TGF-β1 stimulation. We showed that HDL decreased the TGF-β1–induced collagen production in HAEC in a SR-BI–dependent manner, which further corroborates the findings that HDL protect the endothelium involving the SR-BI receptor.\(^11\) In agreement, HDL induced the phosphorylation state of SR-BI downstream targets Akt and eNOS. Furthermore, the HDL-mediated decline in TGF-β1–induced collagen deposition could be abrogated by phosphatidyl inositol-3-kinase blockage. However, no significant abrogation of the HDL-mediated reduction in collagen deposition could be reached by NO inhibition, despite the observation that HDL upregulated the TGF-β1–impaired NO release in HAEC. These findings suggest that the HDL-mediated induction of phosphorylated eNOS and NO production under TGF-β1 conditions is an epiphenomenon and therefore not per se linked to EndMT. We further demonstrated that HDL induced inhibitory Smad7 expression and decreased the expression of the EndMT transcription factors Slug and ZEB1,\(^27\) indicative for blocking TGF-β1-Smad-EndMT signaling. Though, interestingly, an induction of the EndMT transcription factor Snail was found on supplementation with HDL, suggesting a fine tuned balance between those transcription factors and their impact on EndMT.

In brief, this study supports the antibiotic effects of HDL,\(^7,28\) which are less established compared with their endothelial-protective characteristics.

In conclusion, we demonstrate a novel endothelial-protective and antibiotic effect of HDL: the reduction in EndMT. However, further in-depth investigations are needed to elucidate the exact underlying mechanisms.

Acknowledgments

This study was supported by the DZHK to C. Tschöpe and S. Van Linhout, by MEDIA to C. Tschöpe, and by the European Foundation for the study of diabetes (EFSD) to S. Van Linhout. We acknowledge the assistance of the BCRT Flow Cytometry Laboratory. We thank Annika Koschel, Gwendolin Matz, and Marzena Sosnowski (in alphabetic order) for excellent technical support.

Disclosures

None.

References


### Significance

Endothelial-to-mesenchymal transition, an inflammation-induced process by which endothelial cells transdifferentiate into mesenchymal-like cells, contributes to different cardiovascular pathologies, including cardiac fibrosis and vascular remodeling. We show here for the first time that high-density lipoprotein (HDL) can reduce this process in human aortic endothelial cells. This finding further corroborates the well-recognized endothelial-protective effects of HDL, as well as their antifibrotic features. Furthermore, it suggests that patients with low HDL or dysfunctional HDL are more prone to endothelial-to-mesenchymal transition and potential subsequent cardiac fibrosis or vascular remodeling. Therefore, therapies directed at increasing HDL concentrations and HDL quality could overcome endothelial-to-mesenchymal transition and the early onset of cardiovascular remodeling processes.
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Arterioscler Thromb Vasc Biol. 2015;35:1774-1777; originally published online June 18, 2015; doi: 10.1161/ATVBAHA.115.305887
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/35/8/1774

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2015/06/18/ATVBAHA.115.305887.DC1

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Supplemental Figure I. Dose response of HDL on TGF-β1-induced collagen deposition in HAEC. Bar graphs represent the mean ± SEM of the ratio of the absorbance at 540 nm of Sirius Red-stained HAEC towards the absorbance at 495 nm of crystal violet-stained HAEC stimulated with TGF-β1 in the presence or absence of 5, 50, or 100 µg of HDL protein/ml versus unstimulated HAEC, as indicated, n=12/group.
Supplemental Figure II. HDL decrease the TGF-β1-induced transition of HAEC towards cells with a spindle shaped morphology. Representative phase contrast pictures of HAEC cultured under basal, TGF-β1, HDL, or TGF-β1+HDL conditions, as illustrated. Bar depicts 200 µm. Arrows indicate HAEC with a spindle shape.
Supplemental Figure III. HDL induce the phosphorylation state of Akt and eNOS in TGF-β1 supplemented HAEC and decrease the TGF-β1-induced collagen deposition in a phosphatidyl inositol 3 kinase-dependent manner. A. and B. Upper panels: representative Western blots of phosphorylated (p)-Akt and total (tot.) Akt and p-eNOS and tot. eNOS, respectively; lower panels: bar graphs represent the mean ± SEM of the ratio of p-Akt / tot. Akt and p-eNOS / tot. eNOS, respectively, with the basal group set as 100% and n=3/without TGF-β1 and n=4/with TGF-β1 groups. C. Bar graphs depict the absorbance at 540 nm of Sirius Red-stained HAEC towards the absorbance at 495 nm of crystal violet-stained HAEC stimulated with TGF-β1 in the presence of 50 µg HDL protein/ml with or without 1 µM of Ly294002 or 0.1 mM L-NAME, as indicated, n=6-8/group.
Supplemental Figure IV. HDL modulate the expression of Smad 7 and of EndMT-inducing transcription factors. A. Upper panel: representative Western blot of Smad 7 and GAPDH: bar graphs represent the mean ± SEM of the ratio of Smad 7 to GAPDH, respectively, with the basal group set as 100% and n=3/without TGF-β and n=4/ with TGF-β groups. Bar graphs represent the mean ± SEM of B. Snail 1, C. Slug and D. ZEB1 mRNA expression, depicted as the relative expression towards CDKN1b and the basal condition set as 1 with n=4-6/group.
**Supplemental Figure V. HDL reverse TGF-β1-induced collagen deposition.** Bar graphs depict the absorbance at 540 nm of Sirius Red-stained HAEC towards the absorbance at 495 nm of crystal violet-stained HAEC stimulated without or with TGF-β1, or with TGF-β1 to which HDL together with TGF-β1 is added 48h after plating for 24h, as indicated, with n=9-10/groups without HDL and n=12/HDL treatment group.

| TGF-β | - | + | + |
| HDL   | - | - | + |

Supplemental Figure V. HDL reverse TGF-β1-induced collagen deposition. Bar graphs depict the absorbance at 540 nm of Sirius Red-stained HAEC towards the absorbance at 495 nm of crystal violet-stained HAEC stimulated without or with TGF-β1, or with TGF-β1 to which HDL together with TGF-β1 is added 48h after plating for 24h, as indicated, with n=9-10/groups without HDL and n=12/HDL treatment group.
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**MATERIALS AND METHODS**

**Cell culture**

Human aortic endothelial cells (HAEC) (Lonza Walkersville, Walkersville, MD, USA) in passage 4 to 6 were cultured in EBM-2 basal medium supplemented with EGM-2 Single Quots (Lonza, USA). Twenty-four hours (h) later, the medium was daily exchanged by EBM-2 basal medium containing ascorbic acid, gentamicinsulfate/ampicillin B and hydrocortisone of the supplier, in the presence or absence of 10 ng/ml transforming growth factor-ß1 (TGF-ß1) (R&D Systems), with or without 50 µg/ml of HDL (MP Biomedicals, Solon, Ohio, USA). The concentration of 50 µg of HDL protein/ml for HAEC was chosen since we and others previously demonstrated protective effects of HDL in endothelial cells at this concentration. Furthermore, a dose response experiment evaluating the effect of 5, 50, or 100 µg protein HDL/ml on TGF-ß1-induced collagen deposition in HAEC indicated that 50 as well as 100 µg protein HDL/ml reduced the TGF-ß1-induced collagen deposition in HAEC, an effect which was not found by 5 µg protein HDL/ml (Supplemental Figure 1). To assess the underlying mechanisms of HDL, HDL was added to TGF-ß1-supplemented HAEC in the presence or absence of 1 µM of the PI3K inhibitor Ly 294002 or 0.1 mM of the NO-inhibitor L-NAME and its impact on collagen deposition determined 48h after plating and daily refreshment of the respective medium. The potential therapeutic effect of HDL was assessed via addition of HDL combined with TGF-ß1 for 24h to HAEC which were previously 48h stimulated with daily refreshed TGF-ß1 followed by evaluation of its impact on collagen deposition. Phase contrast pictures were taken using the 10x objective of a Zeiss Axio Observer Z1 microscope (Carl Zeiss, Oberkochen, Germany).

**Anti-human VE Cadherin and α-Smooth Muscle Actin Staining**

HAEC (10,000 cells/well) were plated in a CellCarrier black 96-well plate (PerkinElmer, Waltham, Massachusetts, USA). After 48h, with daily refreshment of TGF-ß1 and/or HDL supplementation, the cells were fixed with Fixation/Permeabilization solution (BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit) and stored in PBS until staining. HAEC were stained with anti-human α-smooth muscle actin-phycocerythrin diluted (1:20) in 1x Perm/Wash Buffer (BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit) for 30 min at room temperature, followed by staining with anti-human VE cadherin-allophycocyanin (Biolegend) diluted (1:20) in PBS overnight at 4°C, and DAPI (Sigma) staining diluted (1:1000) in PBS for 3 min at room temperature (RT). Fluorescent images were taken by Operetta high content screening system (PerkinElmer, Inc. USA) using the 40x LWD objective.

**Flow cytometry**

Cells were collected 6 days after daily refreshed TGF-ß1 and/or HDL supplementation. Flow cytometry analysis was performed on a MACSQuant Miltenyi Biotec flow cytometer after cell labeling with a PE-conjugated α-SMA antibody (R&D Systems, Minneapolis, MN, USA) or APC- conjugated VE-cadherin antibody (Biolegend, San Diego, California, USA). Cadherin and α-SMA positive cells were analyzed with FlowJo 8.7. software (Tree Star).

**Gene expression analysis**

RNA was isolated using the RNeasy Mini Kit according to the manufacturer’s protocol (Qiagen GmbH, Hilden, Germany), followed by cDNA synthesis. To assess the mRNA expression of the target genes collagen I, III, Snail, Slug, ZEB1 and CDKN1b, real-time PCR (Eppendorf Mastercycler egradient realplex, Hamburg, Germany) was performed using gene expression assays for Col1a1 Hs00164004_m1, Col3a1 Hs00943809_m1, Snail Hs00195591_m1 Slug Hs00950344_m1 ZEB1 Hs00232783_m1 and CDKN1b Hs00153277_m1 from Applied Biosystems, respectively. mRNA expression was normalized to the housekeeping gene CDKN1b and relatively expressed with the control group set as 1.
Collagen content
HAEC were 48h after plating and daily refreshment of TGF-ß1 and/or HDL supplementation, fixed in methanol overnight at -20ºC, washed once with PBS and incubated in 0.1% Direct Red 80 (Sirius red) staining solution at RT for 60 min. By the siRNA experiment, fixation of the cells took place 72h post plating and daily refreshment of TGF-ß1 and/or HDL supplementation. By the HDL therapy experiment, cells were fixated 72h after plating, by which in the HDL treatment group, HDL combined with TGF-ß1 was supplemented to HAEC, 48h after plating and daily refreshment of TGF-ß1. After second washing with PBS, the Sirius red staining of the HAEC was eluted in 0.1N sodium hydroxide at RT for 60 min on a rocking platform. The optical density representative for the accumulation of collagen I and III was measured at 540 nm. For normalization to cell amount, HAEC were stained with crystal violet and the absorbance was measured at 495 nm. Data are represented as the ratio of the absorbance at 540 nm (Sirius Red) towards the absorbance at 495 nm (crystal violet).

Nitric oxide measurement
Intracellular NO was measured with DAF-FM diacetate (4-amino-5-methylamino-2’,7’-difluorofluorescein diacetate, Invitrogen) as described previously. After daily supplementation with TGF-ß1 with or without HDL for 2 days, HAEC were incubated at 37°C for 30 min in PBS containing 1 µM of DAF-FM diacetate. After loading, cells were rinsed twice with PBS and incubated with fresh PBS at 37°C for 30 min. NO fluorescence intensity was read in a Berthold Mithras LB 940 reader at 495 nm excitation and 515 nm emission wavelength. For normalization to cell amount, HAEC were stained with crystal violet and the absorbance was measured at 495 nm. Data express the ratio of NO fluorescence intensity divided towards the average of the absorbance at 495 nm (crystal violet), per condition.

siRNA knockdown
HAEC were transfected with Lipofectamine RNAiMAX (Life Technologies) with SR-BI or scrambled siRNA (Life Technologies) following the manufacturer’s protocol.

Western Blot
After daily supplementation with TGF-ß1 with or without HDL for 2 days, HAEC were lysed in lysis buffer (Invitrogen) containing proteinase inhibitors (Roche). An equal amount of protein was loaded into SDS-polyacrylamide gels. Phosphorylated Akt and total Akt (New England Biolabs, Cell signalling, Ipswich, MA, USA), phosphorylated eNOS (New England Biolabs), total eNOS (BD Biosciences, San Diego, CA, USA), Smad 7 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (Bidesign, Memphis, TN, USA) were detected with each specific antibody, followed by incubation with an IR dye secondary antibody (LI-COR Biosciences, Lincoln/Nebraska, USA). All blots were visualized with Odyssey (LI-COR Biosciences). Quantitative analysis of the intensity of the bands was performed with Odyssey V3.0 software.

Statistical analysis
Data are presented as mean ± SEM. Assumption of Gaussian distribution was consistently tested by the method of Kolmogorov and Smirnov. Statistical differences between groups were assessed with the unpaired t-test. When no Gaussian distribution was reached, a non-parametrical test was used. Differences were considered to be significant when the two-sided P-value was lower than 0.05.
References