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Basic Research

Infusions of Large Synthetic HDL Containing Trimeric apoA-I Stabilize Atherosclerotic Plaques in Hypercholesterolemic Rabbits

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ABSTRACT

Background: Among strategies to reduce the remaining risk of cardiovascular disease, interest has focused on using infusions of synthetic high-density lipoprotein (sHDL).

Methods: New Zealand rabbits underwent a perivascular injury at both carotids and were randomly allocated into 2 protocols: (1) a single-dose study, where rabbits were treated with a single infusion of sHDL containing a trimeric form of human apoA-I (TN-sHDL, 200 mg/kg) or with Placebo; (2) a multiple-dose study, where 4 groups of rabbits were treated 5 times with Placebo or TN-sHDL at different doses (8, 40, 100 mg/kg). Plaque changes were analysed *in vivo* by intravascular ultrasound. Blood was drawn from rabbits for biochemical analyses and cholesterol efflux capacity evaluation.

Results: In both protocols, atheroma volume in the Placebo groups increased between the first and the second intravascular ultrasound evaluation. A stabilization or a slight regression was instead observed vs baseline in the TN-sHDL-treated groups ($P < 0.005$ vs Placebo after infusion). TN-sHDL treatment caused a sharp rise of plasma-free cholesterol levels and a significant increase of total cholesterol efflux capacity. Histologic analysis of carotid plaques showed a reduced

RÉSUMÉ

Contexte : Parmi les stratégies visant à réduire le risque résiduel de maladie cardiovasculaire, la perfusion de lipoprotéines de haute densité synthétiques (HDLs) suscite un grand intérêt.

Méthodologie : Des lapins de Nouvelle-Zélande ont subi une lésion périvasculaire aux deux carotides et ont été répartis de façon aléatoire dans deux protocoles : (1) une étude portant sur une seule dose, au cours de laquelle les lapins ont reçu une seule perfusion de HDLs renfermant une forme trimère d'apoA1 humaine (TN-HDLs, 200 mg/kg) ou un placebo; ou (2) une étude portant sur diverses doses, au cours de laquelle 4 groupes de lapins ont été traités à 5 reprises par un placebo ou par des perfusions de TN-HDLs à différentes doses (8, 40, 100 mg/kg). L'évolution des plaques a été analysée in vivo par échographie intravasculaire. Des échantillons de sang ont été prélevés pour les analyses biochimiques et l'évaluation de la capacité d'efflux du cholestérol.

Résultats : Dans les deux protocoles, le volume de l'athérome dans les groupes sous placebo a augmenté entre la première et la seconde évaluation par échographie intravasculaire. Par contre, dans les groupes ayant reçu les perfusions de TN-HDLs, une stabilisation ou une

Drugs affecting lipid metabolism have revolutionized the treatment of atherosclerosis reducing the risk of cardiovascular disease (CVD) by 30% to 40%. There is, however, an urgent need for the further reduction of the unacceptably high remaining risk.[1] Therefore, the development of drugs targeting the atherosclerotic process still represents an important area of contemporary clinical research.[1,2]

It is a well-known fact that the concentration of high-density lipoprotein cholesterol (HDL-C) is inversely related to the risk of cardiovascular events.[3,4] The most popular mechanistic hypothesis underlying this association is the stimulation of the reverse cholesterol transport (RCT).[5] However, recent studies have suggested that the plasma HDL-C concentration may not always reflect HDL function or, more significantly from a clinical perspective, explain the reduced CVD risk.[4,6-10] Indeed, carriers of inherited HDL disorders accumulate small cholesterol-poor HDL particles, which are very efficient in cholesterol efflux capacity (CEC) as

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macrophage accumulation in TN-sHDL-treated rabbits compared with Placebo ($P < 0.05$).

Conclusions: Our results demonstrate that acute and subacute treatments with TN-sHDL are effective in stabilizing atherosclerotic plaques in a rabbit model. This effect appears to be related to a reduced intraplaque accumulation of inflammatory cells. Besides recent failures in proving its efficacy, sHDL treatment remains a fascinating therapeutic option for the reduction of cardiovascular risk.

légère régression des plaques ($p < 0,05$ comparativement au placebo après perfusion) ont été observées par rapport aux valeurs initiales. Le traitement par TN-HDLs a été associé à une élévation marquée du taux plasmatique de cholestérol libre et à une augmentation significative de la capacité d'efflux du cholestérol total. Les analyses histologiques des plaques dans les carotides ont révélé une diminution de l'accumulation de macrophages chez les lapins ayant reçu des perfusions de TN-HDLs comparativement à ceux sous placebo ($p < 0,05$).

Conclusions : Nos résultats montrent que les traitements aigus et subaigus par TN-HDLs stabilisent efficacement les plaques d'athérosclérose dans un modèle de lapin. Cet effet semble lié à une accumulation moindre de cellules inflammatoires dans les plaques. Malgré des résultats récents n'ayant pas corroboré son efficacité, le traitement par les HDLs demeure une option thérapeutique fascinante dans la réduction du risque cardiovasculaire.

a first step of RCT and are not at increased risk for CVD.[11-13] In addition, HDL has been shown to protect the endothelium, inhibit low-density lipoprotein oxidation, play an important role in host defence, and exert anti-inflammatory and antithrombotic effects.[5,14-16]

Taken together, these data justify the rationale of testing infusion therapies with cholesterol-poor HDL (ie, synthetic HDL [sHDL]), as a treatment for patients with atherosclerotic disease. This therapeutic approach showed very promising results in preclinical studies and first clinical trials, where atherosclerosis regression was demonstrated.[17,18] However, in more recent investigations, infusion of sHDL did not show a convincing clinical benefit.[19]

In the context of this therapeutic approach, a recombinant high−molecular mass variant of human apoA-I, named Tetranectin-apoA-I, has been engineered by fusing 3 apoA-I molecules with the trimerization domain of human tetranectin.[20] This trimeric apoA-I is not filtered by glomeruli and hence shows a prolonged half-life as compared with normal apoA-I,[20] thus potentially improving its efficacy. Indeed, sHDL containing the dimeric form of a human apoA-I molecular variant, apoA-I_{Milano}, characterized by a longer half-life, has been formulated and proved effective in both preclinical and clinical studies.[17,21,22] Moreover, sHDL containing trimeric apoA-I (TN-sHDL) maintains the biological functions of monomeric apoA-I by promoting cell cholesterol efflux, stimulating lecithin:cholesterol acyltransferase-mediated cholesterol esterification, and exerting anti-inflammatory effects.[23,24]

The aim of the present study was to evaluate the effect on atherosclerosis of TN-sHDL infusion in rabbits.[17,21] This experimental approach takes advantage of the *in vivo* assessment of plaque volume through intravascular ultrasound (IVUS). In addition, the impact of TN-sHDL on key biomarkers of RCT was evaluated. The results obtained in the present study may guide future developments towards clinical success.

Materials and Methods

Male New Zealand white rabbits, weighing 2.0-2.2 kg, were used for the study. Procedures involving animals and their care were conducted in accordance with institutional guidelines that are in compliance with national (D.L. No. 26, March 4, 2014; G.U. No. 61, March 14, 2014) and international laws and policies (EEC Council Directive 2010/63, September 22, 2010: Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011). The study was approved by the Italian Ministry of Health (Progetto di Ricerca Protocollo 2012/4).

Preparation of TN-sHDL

TN-sHDL was prepared by Roche Diagnostics (Penzberg, Germany). Briefly, recombinant TN was expressed in *Escherichia coli* (StrataGene) and a protein extract was made using the phenol extraction protocol.[20] The crude protein was purified using Zn-chelate, followed by SP-Sepharose chromatography, and lyophilized. To remove endotoxins and *E. coli* lipids, the TN was washed with chloroform:methanol, redissolved in a guanidinium-HCL buffer, gel-filtrated using Sephadex G-25 into 25 mM $(NH_4)_2CO_3$ (pH 8.8), and lyophilized. The TN was finally bound to 1-palmitoyl-2-oleoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine in a 1:45:15 molar ratio, as described.[25]

Experimental protocols

Lipid-rich plaque formation was induced as previously described.[26] Rabbits were anaesthetized, common carotid arteries perivascularly injured by electric current using a bipolar microcoagulator, and all animals fed a 1.5% cholesterol diet throughout the study. Ninety days after surgery, a complete scan of right carotids was recorded by IVUS. Cross-sectional area at the point of maximal stenosis was measured, and only animals with stenosis between 25% and 50% were enrolled for the study.[25] The right jugular vein was cannulated for treatment/blood drawing. Rabbits were then treated following 2 protocols, named single-dose study and multiple-dose study, respectively. For the single-dose study, rabbits were divided into 2 groups of 8 animals each and treated with a single intrajugular infusion, at a constant rate of 1.0 mL/min, of 200 mg/kg of TN-sHDL or with Placebo (5 mM sodium phosphate and 240 mM sucrose, pH 7.3). For the multiple-dose study, selected rabbits were divided into 4 groups of 8 animals each and treated 5 times (once every 3 days) with Placebo or 8, 40, 100 mg/kg body weight of TN-sHDL.

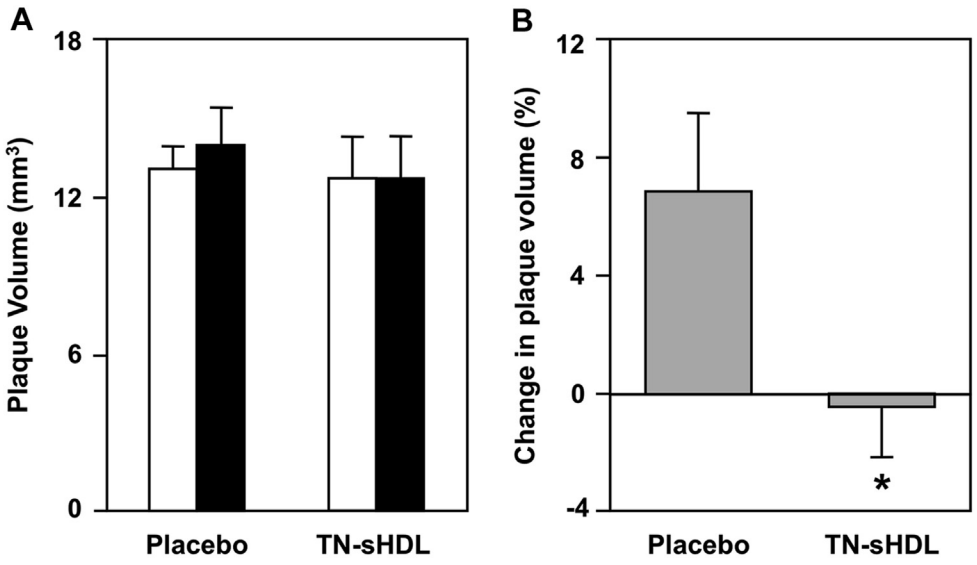

Figure 1. A single infusion of synthetic high-density lipoprotein containing trimeric apoA-I (TN-sHDL) promotes plaque stabilization. (**A**) Absolute values and (**B**) percentage change of atheroma volume evaluated by intravascular ultrasound at the right carotids in rabbits treated with Placebo or 200 mg/kg of TN-sHDL. In (**A**), **open bars** represent pretreatment and **solid bars** represent post-treatment. Data are expressed as mean ± standard deviation, with n = 6 for Placebo and n = 7 for TN-sHDL-treated rabbits. *$P < 0.0001$ vs Placebo by the paired 2-sample *t*-test.

Three days after the single or after the fifth (last) dose, rabbits were anaesthetized and subjected to a second IVUS analysis. Carotids were then excised, embedded in optimal cutting temperature compound, and stored at −80°C. Operators responsible for treatment, animal handling, IVUS analyses, and histologic quantifications were totally blinded with respect to the treatment.

IVUS imaging

IVUS evaluations were performed before the start of the treatments and at sacrifice using a mechanical IVUS system (Galaxy 2; Boston Scientific, Natick, MA), as previously described.[17] Atheroma area was calculated as external elastic membrane area minus luminal area. The sum of areas was multiplied by the slice thickness value (0.5 mm) to obtain plaque volume. The cross-section with the maximal plaque area was referred to as the point of maximal plaque formation (maximal stenosis).

Biochemical evaluations

Biochemical evaluations were performed on rabbits enrolled in the single-dose study. Blood was collected before and 2′, 30′, 60′, 4 hours, 24 hours, 48 hours, and 72 hours after the end of the single infusion, in EDTA-containing tubes. Plasma was separated by centrifugation and stored at −20°C.[27,28] Total and free cholesterol concentrations were measured by using a Roche Diagnostics Cobas auto-analyser.[29,30] Trimeric apoA-I plasma levels were measured using an anti-human apoA-I antibody (F. Hoffmann-La Roche, Basel, Switzerland), which does not recognize rabbit apoA-I.

Histologic evaluation

Cryosections were stained with hematoxylin (Mayer's Haemalum; Bio-Optica, Milano, Italy) and eosin (Bio-Optica) for plaque area measurement. In the single-dose study, neutral lipid accumulation was identified staining selected sections with Oil Red O (Sigma-Aldrich, St. Louis, MO),[31] whereas macrophages were detected incubating the selected sections with the specific antibody RAM-11 (Dako Italia S.p.A, Cernusco sul Naviglio, Italy).[25]

The Aperio ScanScope GL Slide Scanner (Aperio Technologies, Vista, CA) was used to acquire digital images.[22,32] Plaque volume was evaluated by measuring cross-sectional areas of the intima every 0.5 mm within the area of plaque accumulation. The lipid or macrophage content was measured as plaque area occupied by lipids or macrophages/total plaque area × 100.[33,34]

Efflux experiments

CEC of rabbit plasma samples was quantified in J774 murine macrophages.[35] Cells were labelled with [1,2-^{3}H]-cholesterol (PerkinElmer, Milano, Italy) for 24 hours in the presence of an acetyl-coenzyme A acetyltransferases inhibitor (Sandoz S.p.A., Origgio, Italy) used at 2 μg/mL. After 18 hours in bovine serum albumin-containing medium, cholesterol efflux was promoted for 4 hours using 0.5% (v/v) of rabbit plasma samples collected before infusion, and at 4 hours and 72 hours after the end of the single infusion. Aqueous diffusion (AD)-mediated cholesterol efflux was evaluated in J774 murine macrophages under basal conditions, that is, in the absence of 8-(4-Chlorophenylthio) adenosine 3′,5′-cyclic monophosphate (c-CAMP) (Sigma-Aldrich, Milano, Italy). In this condition, specific lipid transporters are expressed at undetectable levels.[36] Total release of cholesterol, that is, AD-dependent plus ATP binding cassette transporter A1 (ABCA1) mediated, was measured by adding cAMP to the J774 cells. ABCA1-mediated CEC was then calculated as the difference between total and AD-dependent CEC.[36]

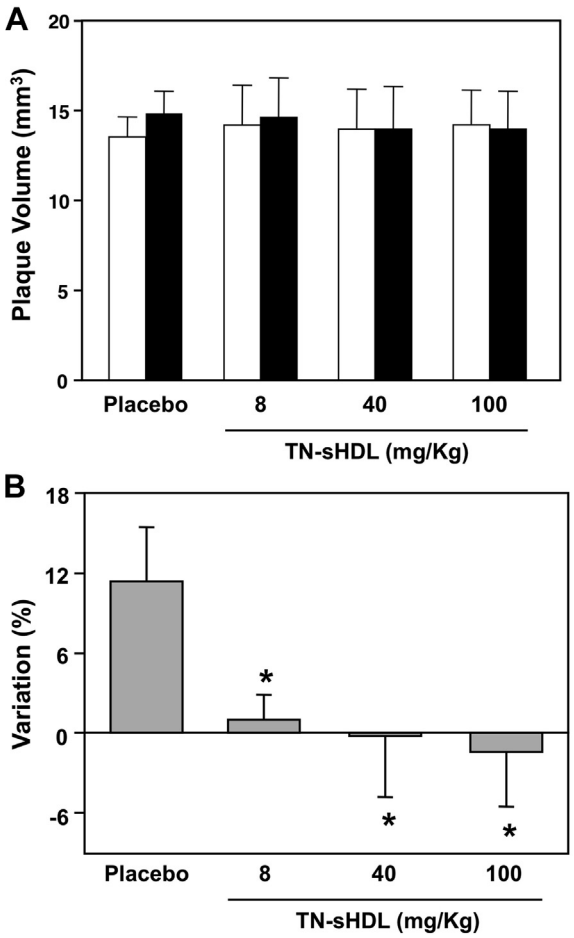

Figure 2. Five infusions of 8, 40, and 100 mg/kg of synthetic high-density lipoprotein containing trimeric apoA-I (TN-sHDL) are effective in stabilizing or moderately regressing rabbit carotid plaques. (**A**) Absolute values and (**B**) percentage change of atheroma volume evaluated by intravascular ultrasound at the right carotids in rabbits treated with Placebo or different doses of TN-sHDL. In (**A**), **open bars** represent pretreatment and **solid bars** represent post-treatment. Data are expressed as mean ± standard deviation, with n = 7 for Placebo, n = 5 for TN-sHDL 8 mg/kg, n = 7 for TN-sHDL 40 mg/kg, and n = 8 for TN-sHDL 100 mg/kg treated rabbits. *$P < 0.005$ vs Placebo by 1-way analysis of variance.

Statistical analysis

Group differences in IVUS absolute plaque volume, free cholesterol levels, and CEC were tested for statistical significance by analysis of variance (ANOVA) for repeated measurements. IVUS percentage variations and histology data were evaluated by the paired 2-sample *t*-test or by 1-way ANOVA. All ANOVA analyses were followed by the Tukey *post hoc* test. The Pearson correlation coefficient was calculated for inter- and intraobserver variability of IVUS measurements and for the association between plasma free cholesterol changes and total-CEC changes. A value of $P < 0.05$ was considered statistically significant. The statistical analyses were performed using the SYSTAT software (Version 13; Systat Software, Inc, Chicago, IL) or Prism (version 6.0) (GraphPad Inc, San Diego, CA).

Results

IVUS scans were analysed for plaque volume measurements. The interobserver and the intraobserver variability in plaque volume measurements were in line with those calculated in previous studies performed by our group (0.853, $P < 0.001$, and 0.915, $P < 0.005$, respectively).[17,26]

Single-dose study

In 3 of 16 animals, the quality of IVUS images did not allow reliable measurements of the plaque area; therefore, the results described below refer to 13 rabbits (6 Placebo and 7 TN-sHDL-treated rabbits). Figure 1 shows absolute plaque volumes and percentage variations that occurred during the treatment period. Pretreatment plaque volumes were not different between the 2 groups ($P > 0.05$). By looking at plaque changes vs baseline, atheroma volume in the Placebo group increased in the time between the first and the second IVUS evaluation (+0.94 ± 0.33 mm^3, Fig. 1A). In contrast, a stabilization was observed vs baseline in TN-sHDL-treated rabbits (−0.05 ± 0.26 mm^3). Comparison between post-treatment plaque volumes of the 2 groups showed a difference that was very close to statistical significance ($P = 0.06$). Nevertheless, absolute and percentage changes of atheroma volume in the Placebo group were significantly different from those found in TN-sHDL-treated rabbits (+0.94 ± 0.33 mm^3 vs −0.05 ± 0.26 mm^3, respectively; $P < 0.0001$, and Fig. 1B). Examples of IVUS images of atherosclerotic plaques recorded before and after treatment with Placebo or TN-sHDL are shown in Supplemental Figure S1.

Multiple-dose study

A total of 32 rabbits were treated, but only 27 animals (7 Placebo, 5 TN-sHDL 8 mg/kg, 7 TN-sHDL 40 mg/kg, and 8 TN-sHDL 100 mg/kg) allowed reliable plaque measurements. Pretreatment plaque volumes were not statistically different among the 4 experimental groups ($P > 0.05$, Fig. 2A). Atheroma volume in the Placebo group increased during the time between the first and the second IVUS evaluation (+1.55 ± 0.58 mm^3). A slight progression or regression was instead observed in TN-sHDL-treated rabbits vs baseline (+0.18 ± 0.25 mm^3, −0.01 ± 0.63 mm^3 and −0.21 ± 0.57 mm^3 in the TN-sHDL 8, 40, and 100 mg/kg groups, respectively, Fig. 2A). As a consequence, absolute and percentage changes of total atheroma volume vs baseline in each TN-sHDL-treated group were significantly different from those measured in the Placebo group ($P < 0.005$, Fig. 2B). No statistical differences were observed among the 3 TN-sHDL-treated groups.

Effect of TN-sHDL infusion on plaque macrophage content and plasma-free cholesterol

Plaque volume, evaluated by histology, did not differ between Placebo and TN-sHDL-treated rabbits ($P > 0.05$). Moreover, TN-sHDL treatment did not affect plaque lipid accumulation, measured by Oil Red O staining (65.7% ± 18.0% vs 70.3% ± 13.0% in Placebo, $P > 0.05$). On the contrary, TN-sHDL-treated rabbits displayed a significantly lower plaque macrophage content compared with that measured in Placebo (69.5% ± 13.4% vs 84.3% ± 9.3%, $P < 0.05$, Fig. 3).

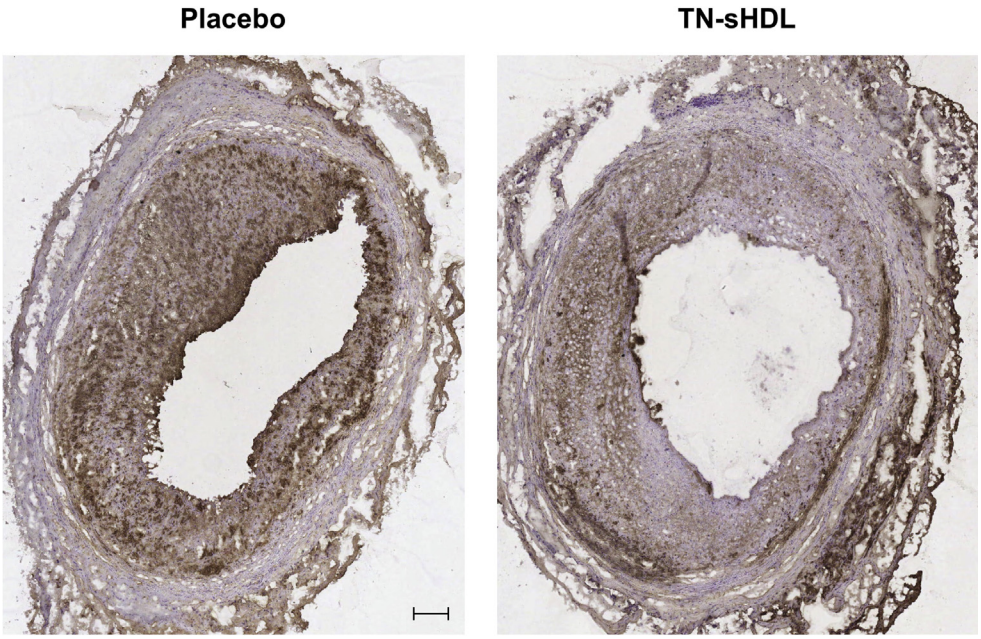

Figure 3. Synthetic high-density lipoprotein containing trimeric apoA-I (TN-sHDL) infusion reduces plaque macrophage content. Representative photomicrographs of immunostaining for macrophages in rabbit carotid plaques infused with Placebo or 200 mg/kg of TN-sHDL. A decreased macrophage content is visible in the TN-sHDL-treated rabbit. **Scale bar** = 100 μm.

Total cholesterol concentration did not change throughout the treatment period, and between the 2 groups of animals (Fig. 4). However, as shown in Figure 4, starting from 2 minutes after the end of the infusion and up to 24 hours, a significant increase in plasma-free cholesterol levels was observed in rabbits treated with TN-sHDL ($P < 0.05$ vs Placebo).

Total cholesterol (mM, mean±SD)

	0h	2min	0.5h	1h	4h	24h	48h	72h
Placebo	24.7±2.3	24.5±7.2	23.2±4.3	24.3±6.1	23.6±4.5	25.5±9.1	25.9±7.0	23.7±8.5
TN-sHDL	23.4±7.3	24.8±5.4	25.4±6.1	23.8±8.6	24.2±8.8	24.6±4.6	25.1±5.9	25.2±8.4

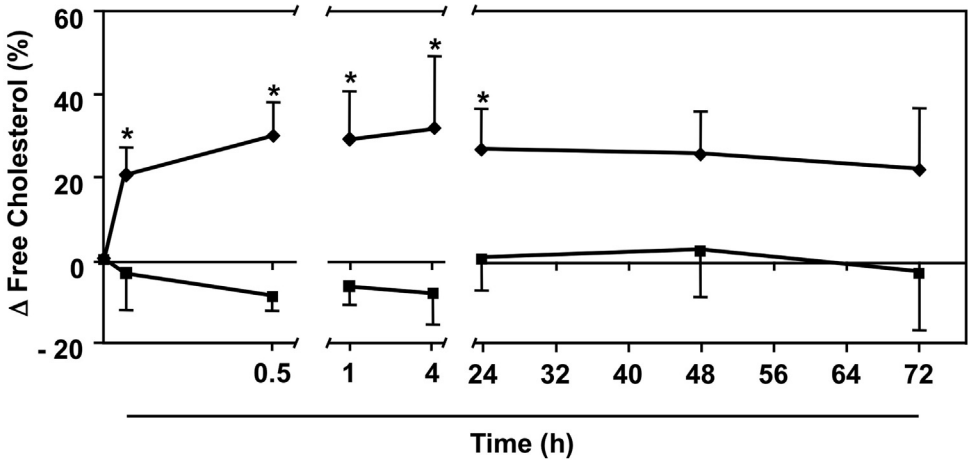

Figure 4. (**Top**) Total cholesterol plasma levels measured before (0) and 2′, 30′, 60′, 4 hours, 24 hours, 48 hours, and 72 hours after the end of the infusion in Placebo and synthetic high-density lipoprotein containing trimeric apoA-I (TN-sHDL)-treated rabbits. (**Bottom**) A single TN-sHDL infusion causes a rapid increase of free cholesterol levels. Percentage change of free cholesterol levels measured in plasma of rabbits treated with Placebo (**squares**) or 200 mg/kg of TN-sHDL (**diamonds**). Blood was collected before (0′) and 2′, 30′, 60′, 4 hours, 24 hours, 48 hours, and 72 hours after the end of the infusion. Data are expressed as mean ± standard deviation (SD), with n = 6 for Placebo and n = 7 for TN-sHDL-treated rabbits. *$P < 0.05$ vs Placebo by analysis of variance for repeated measurements.

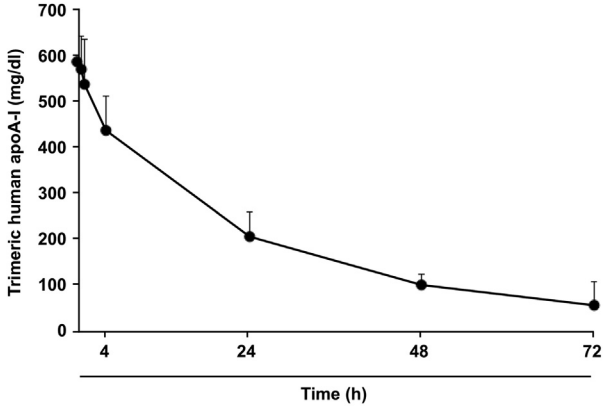

Figure 5. Plasma clearance of trimeric human apoA-I in hypercholesterolemic rabbits infused with 200 mg/kg of synthetic high-density lipoprotein containing trimeric apoA-I. Blood was collected before and 2′, 30′, 60′, 4 hours, 24 hours, 48 hours, and 72 hours after the end of the infusion. Data are expressed as mean ± standard deviation, with n = 7.

Plasma concentration of apoA-I was also measured at each time point. Based on these data, a half-life of 22 hours was calculated (Fig. 5).

Effect of TN-sHDL infusion on CEC

TN-sHDL infusion elicited a marked increase of total, AD-dependent and ABCA1-mediated CEC (Fig. 6). As shown in Figure 6A, the total CEC of rabbit plasma, collected 4 hours after the end of TN-sHDL infusion, was significantly increased compared with the total CEC measured in the Placebo group ($P < 0.0001$). No differences between the 2 treatments were observed instead in plasma samples collected before infusion and after 72 hours. Moreover, for TN-sHDL-treated rabbits, the plasma total CEC at 4 hours was significantly different from that measured before infusion or 72 hours after the end of the infusion ($P < 0.0001$).

Four hours after TN-sHDL infusion, AD-dependent CEC was increased compared with Placebo ($P < 0.005$), and it was significantly higher than that measured before and 72 hours after infusion ($P < 0.05$; Fig. 6B). The ABCA1-mediated CEC was significantly increased at 4 hours after TN-sHDL treatment compared with pretreatment values and returned to baseline at 72 hours after the end of the single infusion (Fig. 6C). No significant differences were observed vs Placebo at each time point analysed.

In the Placebo group, no variations were detected in total, AD-dependent as well as ABCA1-mediated CEC at each time point (Fig. 6, A-C).

Delta plasma free cholesterol at 4 hours after infusion correlated strongly and positively with the increase of total efflux capacity at the same time point ($R^2 = 0.868$, $P < 0.0001$).

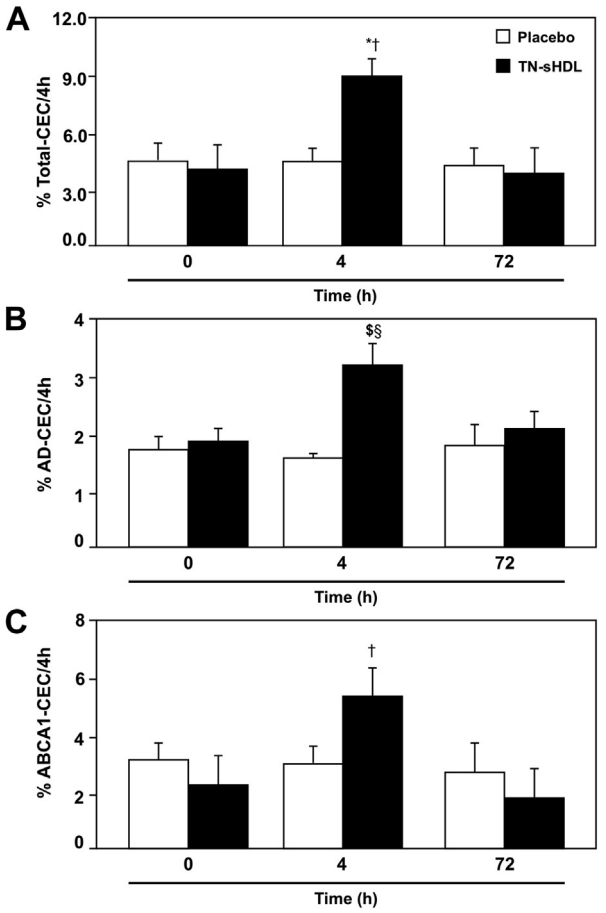

Figure 6. Synthetic high-density lipoprotein containing trimeric apoA-I (TN-sHDL) infusion increases cholesterol efflux capacity (CEC). CEC was measured on rabbit plasma collected before infusion (0 hour), at 4 hours, and 72 hours after the end of infusion with Placebo (**open bars**) or 200 mg/kg of TN-sHDL (**solid bars**). (**A**) Total, (**B**) aqueous diffusion (AD)-dependent, and (**C**) ATP binding cassette transporter A1 (ABCA1)-mediated CEC of rabbit plasma are shown. All efflux values are reported as the average of 3 determinations in different wells. Data are expressed as mean ± standard deviation, with n = 5. (**A**) *$P < 0.0001$ vs 4 hours Placebo; †$P < 0.0001$ vs 0 hour and 72 hours TN-sHDL. (**B**) \$$P < 0.005$ vs 4 hours Placebo; §$P < 0.05$ vs 0 hour and 72 hours TN-sHDL. (**C**) †$P < 0.0001$ vs 0 hour and 72 hours TN-sHDL. Data were analysed by analysis of variance for repeated measurements.

Discussion

The main result of the present study is that a single intravenous infusion of TN-sHDL promoted plaque stabilization in a rabbit model of atherosclerosis.[26] This stabilization occurred without plaque lipid removal, but was paralleled by a significant reduction in plaque macrophage content. Similar effects were achieved by treating the rabbits with 5 infusions of TN-sHDL at different doses (8, 40, and 100 mg/kg), where stabilization or a moderate regression of atherosclerotic lesions was observed. These results were obtained in a rabbit model, already used to test the efficacy of sHDL infusion on atherosclerotic lesions,[37,17] that allows the *in vivo* assessment of plaque volume through IVUS, one of the clinical imaging modalities to evaluate the impact of therapies on plaque progression/regression.[38-40] The efficacy of TN-sHDL treatment must be evaluated considering that these results were obtained within a short time and after 1 or few administrations. Of note, first choice pharmacologic treatments, that is, statins, minimally affect plaque size, and this effect occurs only when these drugs are administered at high doses and for 18-24 months.[41]

The trimeric human apoA-I has been synthesized with the aim of increasing the half-life as compared with normal apoA-I,[20] based on the hypothesis of a size-dependent rate of catabolism for apoA-I.[42] Indeed, in the present experimental conditions, a half-life of 22 hours was found, a much higher value than that measured for normal apoA-I.[43] This observation seems to exclude the formation of immune complexes with trimeric apoA-I that were shown to cause an accelerated catabolism of TN-sHDL in a previous study in monkeys.[44]

When TN-sHDL was infused at 3 different doses, only a trend towards a dose-related effect on plaque volume was detected, because no significant differences were observed among treatments. This result is in line with those obtained by infusion of other sHDL preparations, that is, ETC-216 and CER-001. Specifically, ETC-216 administered at 45 mg/kg dose did not show significantly higher efficacy than the 15 mg/kg dose and CER-001 showed its best efficacy at the lowest dose tested.[38,45] These results may be explained by recent epidemiologic observations indicating that HDL-C levels do not correlate linearly with CVD risk, but they follow a U-shaped association, thus suggesting that HDL functionality is not reflected by HDL concentration.[46] As a consequence, the highest sHDL dose tested may not necessarily determine a greater effect on plaque burden when compared with a lower dose used. Although speculative, 8, 40, and 100 mg/kg of TN-sHDL may be close to the bottom of the U-curve, thus showing a comparable efficacy in their atheroprotection.

Animal[47] and human[48] studies have shown that the cholesterol efflux potential of HDL is a better inverse predictor of CVD than plasma HDL-C levels per se. For this reason, CEC was evaluated in rabbit plasma after Placebo or TN-sHDL infusion. TN-sHDL caused a marked increase of total-CEC in rabbit plasma collected 4 hours after the end of the infusion. In agreement with these data, a rapid increase of plasma-free cholesterol was detected after TN-sHDL infusion that positively correlated with the delta total-CEC at 4 hours. A similar sharp rise in free cholesterol concentration was also observed after the infusion of MDCO-216, CSL112, and CER-001, both in animal and human studies.[49,50] Efflux data revealed that TN-sHDL infusion increased the ABCA1-mediated route, but also importantly affected AD-dependent CEC. A physical explanation for the use of the AD pathway by TN-sHDL may be related to its size. It has been shown that reconstituted HDL with diameter greater than 9 nm is a good acceptor in the AD pathway, whereas smaller particles are efficient acceptors of cholesterol via ABCA1.[51] Coherently, TN-sHDL particles, which have a mean diameter of approximately 9.5 nm (unpublished results), efficiently use the AD pathway. The relevant use of this efflux modality differentiates TN-sHDL from other sHDL tested for atherosclerosis regression, such as MDCO-216, CSL-112, that, being constituted by smaller particles, mainly promote cholesterol efflux via the ABCA1 transporter.[49,50] In addition, the observed increase in AD-dependent CEC could also be partially related to the phospholipid component of the sHDL particles, because phospholipids promote AD efflux.[52]

Although it is clear that the ABCA1 pathway plays a major role in cholesterol removal from cell components of atherosclerotic plaque, pharmacologic interventions increasing non−ABCA1-mediated cholesterol efflux[53,54] did not provide cardiovascular benefit in patients with atherosclerotic CVD.[55] It is thus interesting to note that no significant removal of neutral lipids from plaques was observed in TN-sHDL-infused rabbits vs Placebo. This result differs from that obtained after infusion of A-I_{Milano}-containing sHDL where a dose-dependent lipid removal from plaques was detected.[21] Interestingly, TN-sHDL-treated rabbits displayed a significantly lower macrophage content in carotid plaques compared with the Placebo group, implying that the stabilization of the stenosis process mediated by this sHDL treatment may be a consequence of reduced monocyte migration into the atherosclerotic lesions. Indeed, in previous studies, sHDL infusion was shown to significantly lower endothelial expression of VCAM-1, ICAM-1, and MCP-1.[56,57] Alternatively, the lower macrophage content of plaques in TN-sHDL-treated rabbits could be the result of macrophage cholesterol removal occurring through the ABCA1 pathway. Cholesterol unloading has in fact been shown to restore the migration ability of macrophages,[58] possibly inducing their transmigration out from plaques.[59]

Conclusions

We showed that infusion of sHDL containing trimeric human apoA-I led to a stabilization of atherosclerotic lesions. This result might be the consequence of different HDL-mediated mechanisms, including anti-inflammatory effects related to the increased cholesterol efflux. The long-term efficacy of this kind of therapeutic strategies has still to be proven in the clinic, but an experimental study clearly demonstrated that acute regression of atherosclerotic plaques by sHDL infusion was maintained up to 6 months.[57]

In conclusion, our results may provide the scientific rationale to further develop lipidated TN-ApoA-I and support the evidence of health benefits by sHDL infusion in the treatment of atherosclerosis.

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Disclosures

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Supplementary Material

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