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Effect of myeloperoxidase oxidation and *N*-homocysteinylation of high-density lipoprotein on endothelial repair function

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Abstract: Endothelial cell (EC) migration is essential for healing vascular injuries. Previous studies suggest that high-density lipoprotein (HDL) and apolipoprotein A-I (apoA-I), the major protein constituent of HDL, have endothelial healing functions. In cardiovascular disease, HDL is modified by myeloperoxidase (MPO) and *N*-homocysteine, resulting in apoA-I/apoA-II heterodimer and *N*-homocysteinylation (*N*-Hcy) apoA-I formation. This study investigated whether these modifications attenuate HDL-mediated endothelial healing. Wound healing assays were performed to analyze the effect of MPO-oxidized HDL and *N*-Hcy HDL *in vitro*. HDL obtained from patients

with varying troponin I levels were also examined. MPO-oxidized HDL reduces EC migration compared to normal HDL *in vitro*, and *N*-Hcy HDL showed a decreasing trend toward EC migration. EC migration after treatment with HDL from patients was decreased compared to HDL isolated from healthy controls. Increased apoA-I/apoA-II heterodimer and *N*-Hcy apoA-I levels were also detected in HDL from patients. Wound healing cell migration was significantly negatively correlated with the ratio of apoA-I/apoA-II heterodimer to total apoA-II and *N*-Hcy apoA-I to total apoA-I. MPO-oxidized HDL containing apoA-I/apoA-II heterodimers had a weaker endothelial healing function than did normal HDL. These results indicate that MPO-oxidized HDL and *N*-Hcy HDL play a key role in the pathogenesis of cardiovascular disease.

Keywords: acute myocardial infarction (AMI); high-density lipoprotein (HDL); myeloperoxidase (MPO); *N*-homocysteinylation; wound repair.

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Introduction

High-density lipoprotein (HDL) and its major protein, apolipoprotein A-I (apoA-I), are well established as negative risk factors for the development of atherosclerosis (Boden 2000). HDL reduces the atherosclerosis risk by multiple physiologic functions, including anti-oxidant and anti-inflammatory properties, and reverse cholesterol transport (Barter et al. 2004; Garner et al. 1998; Rubin et al. 1991; Zhang et al. 2003). In addition, HDL can inhibit LDL oxidation, resulting in suppressed inflammation and endothelial cell (EC) protection from apoptosis (Barter et al. 2004; de Souza et al. 2010; Navab et al. 2000a,b; Suc et al. 1997). These properties could promote endothelial healing after vascular injury. The failure of endothelial integrity causes arteriosclerotic progress and plaque destabilization (Silvestre-Roig et al. 2014). Although the multiple functions of HDL protect against endothelial failure, these protective functions might be affected when HDL is modified during clinical conditions such as inflammation and oxidative stress.

ApoA-I, which plays a key anti-atherosclerotic role, is modified by various reactions such as oxidation, glycation, and homocysteinylation (Brown et al. 2013; Hoang et al. 2007; Miyazaki et al. 2014; Shao et al. 2010a,b). In this study, we focused on myeloperoxidase (MPO) oxidation and *N*-homocysteinylation, which produce MPO-oxidized HDL and *N*-homocysteinylation (*N*-Hcy) HDL during atherosclerotic conditions. MPO potentially participates in atherosclerosis promotion and propagation. For example, MPO is released from macrophages in atherosclerotic lesions, and HDL isolated from the atherosclerotic lesions contains numerous MPO-modified proteins such as chlorinated, nitrated, and sulfoxidated apoA-I (Pankhurst et al. 2003; Shao et al. 2005). Similarly, oxidation of LDL by MPO is also an important event in the development of atherosclerosis (Malle et al. 2006; Podrez et al. 2000). In addition, apoA-I/apoA-II heterodimers are produced via a tyrosine-tyrosine bond formed by MPO-oxidation. In a previous study, we demonstrated that apoA-I/apoA-II heterodimer levels were higher in plasma from subjects with acute myocardial infarction (AMI), compared to normal subjects (Kameda et al. 2012). We further showed that MPO-oxidized HDL has lower anti-inflammatory ability than normal HDL (Kameda et al. 2015). Therefore, this study focused on the MPO-oxidized HDL which might be further losing its other anti-atherosclerotic functions. Homocysteine (Hcy) is another protein modulator and is also a risk factor for atherosclerosis. Indeed, high Hcy levels are strongly associated with AMI (Alam et al. 2012; Al-Obaidi et al. 2000). Hcy can be converted to homocysteine thiolactone (HcyT), which has high reactivity and binds several proteins such as albumin, hemoglobin, fibrinogen, and apoA-I (Jakubowski 2002). We previously reported that 1.0–7.4% (of total apoA-I) *N*-Hcy apoA-I is present in normal human serum (Ishimine et al. 2010). Further, elevated *N*-Hcy apoA-I indicates a lower cholesterol efflux capacity (Yano et al. 2016). Thus, MPO-oxidized HDL and *N*-Hcy HDL dysfunction have been partly unveiled, however, few studies have examined the association of endothelial healing and HDL modification.

This study investigated whether MPO-oxidation and *N*-homocysteinylation can attenuate HDL-induced endothelial healing, in an effort to elucidate the factors that contribute to atherosclerotic disease. Using wound healing assays, the effect of MPO-oxidized and *N*-Hcy HDL on endothelial cell migration was assessed *in vitro*. Moreover, with respect to altered HDL protein composition induced by MPO and HcyT, HDL-induced endothelial healing in patients with varying troponin I levels was compared to HDL levels in healthy subjects.

Results

MPO-oxidized HDL reduces the capacity to promote cell migration during wound healing

To confirm the modification of HDL by MPO, the MPO-treated HDL protein components were separated by SDS-PAGE under reducing conditions and immunoblotted with anti-apoA-I and anti-apoA-II antibodies. Prominent bands were detected at apparent molecular masses of 8.5 and 28 kDa, corresponding to the apoA-II and apoA-I monomers, respectively (Figure 1(A)). In addition, a band was observed with both antibodies at an apparent molecular mass of 37 kDa, which represented apoA-I/apoA-II heterodimers. With respect to CBB staining, the apoA-I/apoA-II heterodimer (37 kDa) level increased in staining intensity after MPO treatment (Figure 1(B)).

Next, wound healing assays were performed using MPO-oxidized HDL. The cells that migrated past the wound edge were photographed (Figure 2(A)). Normal HDL increased HUVEC migration into the wound area, compared to control. MPO-oxidized HDL did not increase migration above the control level. When the migrated cells were quantified, HDL increased HUVEC migration to $123.4 \pm 15.4\%$ of the control value ($p = 0.007$), while MPO-oxidized HDL decreased migration to $97.8 \pm 17.5\%$ of the control value ($p = 0.003$) (Figure 2(B)). Thus, MPO treatment significantly impaired the HDL-induced endothelial healing compared to untreated HDL. In addition, MPO alone did not affect EC migration, while MPO-oxidized HDL decreased migration to $78.9 \pm 12.3\%$ of normal HDL (Figure 2(C)). Since the main apolipoprotein of HDL, apoA-I, is modulated by MPO oxidation resulting in the production of apoA-I/apoA-II heterodimer, we checked a change of HDL in size after the MPO treatment. Consequently, larger size of HDL was observed in the HDL sample treated with MPO oxidation compared to the untreated HDL sample (Figure 1(D)). In particular, we identified an increase in MPO-oxidized HDL larger than 9.2 nm. Moreover, apoA-II was observed in the enlarged HDL as well as apoA-I (Figure 1(E)).

N-homocysteinylation HDL has a downward trend in promoting cell migration during wound healing

In these experiments, we first verified the extent of apoA-I *N*-homocysteinylation by isoelectric focusing and

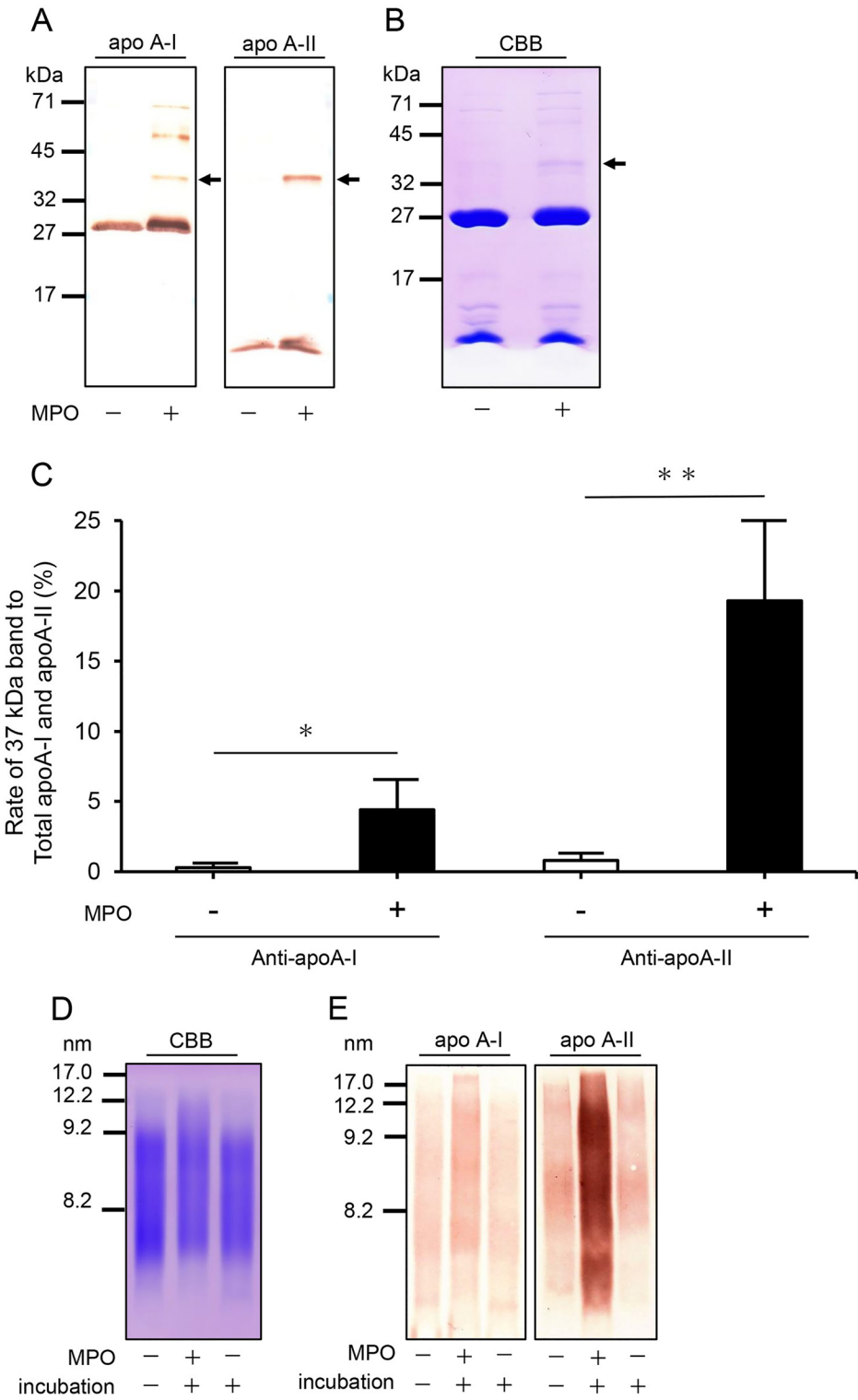


Figure 1: ApoA-I/apoA-II heterodimer formation is induced by MPO catalysis. HDL treated with (+) or without (–) MPO was analyzed by SDS-PAGE and immunoblotting (0.3 µg protein/lane) for apoA-I and apoA-II (A) or by staining with Coomassie Brilliant Blue (CBB) (15 µg protein/lane) (B). Arrows indicate the apoA-I/apoA-II heterodimer. The molecular masses of the standards are listed on the left. (C) The relative amount of apoA-I/apoA-II heterodimer to total apoA-I and total apoA-II were analyzed with CS Analyzer4. Effects of MPO treatment on HDL particle size. HDL treated with MPO was subjected to nondenaturing PAGE followed by CBB staining (8 µg protein/lane) (D) and immunoblotting (6 µg protein/lane) (E). The particle sizes (nm) of the standards are listed on the left. The values indicate the mean + SDs ($n = 3$). * $p < 0.05$, ** $p < 0.01$. The statistical tests were used unpaired t -test.

immunoblotting using anti-apoA-I antibody (Figure 3(A)). *N*-Hcy apoA-I had a higher isoelectric point (pI) depending on the number Hcy residues on the molecule. *N*-Hcy-apoA-I reacts with 2-aminoethanethiol due to the presence of the -SH group derived from Hcy. *N*-Hcy apoA-I levels increased during HcyT treatment in a concentration-dependent manner (0, 1, and 10 mM) (Figure 3(B)).

Using these *N*-Hcy HDLs, the effect on wound healing capacity was evaluated. Treatment with *N*-Hcy HDL significantly increased HUVEC migration relative to control (Figure 4(A)). Both 1 and 10 mM *N*-Hcy HDL induced HUVEC migration by $144.8 \pm 38.8\%$ and $129.1 \pm 25.5\%$, while normal HDL induced $162.0 \pm 76.9\%$ migration, compared to control, and no significant difference was observed in cell migration during wound healing.

HDL from the patients with varying troponin I levels has reduced capacity to promote cell migration during wound healing

Wound healing capacity was determined using HDL isolated from seven blood samples with varying troponin I concentrations (5.92–852.70 ng/ml) (Table 1). To assess the HDL MPO-oxidation and *N*-homocysteinylation from the patient plasmas, we performed immunoblotting analysis (Figures 5(A), (B) and 6(A)). First, increased apoA-I/apoA-II heterodimers were confirmed in several patient samples (Figure 5(A) and (B)). The ratio of apoA-I/apoA-II heterodimer to total apoA-I and total apoA-II were higher in the patient group than in healthy subjects ($5.5 \pm 5.3\%$ vs. $0.5 \pm 0.4\%$, $p = 0.028$ and $36.6 \pm 11.7\%$ vs. $17.5 \pm 6.7\%$, $p = 0.003$, respectively) (Figure 5(D)). Second, *N*-homocysteinylation was also detected in all patient samples. HDL *N*-homocysteinylation was significantly higher in the patient group than in healthy subjects ($19.1 \pm 3.8\%$ vs. $6.9 \pm 4.2\%$, $p < 0.001$) (Figure 6(B)). Moreover, a prominent 12 kDa band, which corresponds to SAA, was observed in two patient samples by SDS-PAGE followed by CBB staining (Figure 5(C)).

HDL from healthy subjects increased HUVEC migration into the wound area compared to control (Figure 7(A)). However, HDL from patients did not increase migration. HDL from healthy subjects increased HUVEC migration to $120.9 \pm 9.6\%$ of the control value, while HDL from patients increased migration to $103.1 \pm 12.1\%$ of the control value ($p = 0.052$ vs. healthy subjects). These results suggest that HDL from healthy subjects promotes HUVEC migration, whereas HDL of patients inhibited HDL-induced endothelial healing (Figure 7(A)).

Wound healing cell migration was significantly correlated with the ratio of apoA-I/apoA-II heterodimer to total apoA-II ($r = -0.603$, $p = 0.022$) and the ratio of *N*-Hcy apoA-I

to total apoA-I ($r = -0.696$, $p = 0.006$). However, there was no significant correlation between wound healing cell migration and the ratio of apoA-I/apoA-II heterodimer to total apoA-I ($r = -0.333$, $p = 0.246$) (Figure 7(B)–(D)). There was also no correlation between SAA levels and repair capacity ($r = -0.414$, $p = 0.142$) (Supplementary Figure 1).

Discussion

Dysfunctional ECs are pleiotropically and profoundly implicated in atherosclerosis progression, including increased expression of cellular adhesion molecules (Albelda et al. 1994; Hwang et al. 1997), cytokine release involved in atherogenesis (Torzewski et al. 1997), and reduced nitric oxide (NO) production (Oemar et al. 1998). In contrast, HDL exerts atheroprotective effects multilaterally on ECs. HDL inhibits endothelial adhesion molecules (Cockerill et al. 1995), stimulates endothelial NO synthase (Gong et al. 2003), and even promotes EC proliferation (Tauber et al. 1980, 1981). However, the protective functions of this rescue lipoprotein can be attenuated by some modifications. Therefore, thorough investigation of the intimate crosstalk between modified HDL and ECs will lead to the discovery of biomarkers and novel therapeutic targets. In this study, we investigated two factors, MPO and Hcy, because previous studies demonstrated that plasma MPO protein levels and Hcy levels are associated with cardiovascular disease risk (Liu et al. 2019; Refsum et al. 1998; Wilcken and Wilcken 1976; Zhang et al. 2001). Moreover, those factors modulate HDL function. However, the effect of these modulations on HDL function has not been fully elucidated. Interestingly, we found that both MPO oxidation and possible *N*-homocysteinylation attenuate the endothelial healing function of HDL *in vitro*.

Regarding MPO oxidation, the intensity of the apoA-I/apoA-II heterodimer band observed by immunoblotting was clearly increased after treating HDL with MPO. ApoA-I/apoA-II heterodimers have an apparent molecular mass of 37 kDa (Kameda et al. 2012). In addition, we hypothesize that the two 50 and 80 kDa bands we observed represented apoA-I homodimers and homotrimers (Jayaraman et al. 2008). MPO induces tyrosine-tyrosine bond formation between apoA-I and apoA-II, and also between apoA-I monomers. Hence, apoA-I homodimers and homotrimers were also produced by MPO oxidation. According to the results of Native-PAGE analysis, it seems these changes in MPO oxidation also lead HDL particles to be larger. Although MPO oxidation enhanced the apoA-I and apoA-II bands in particles larger than 9.2 nm, it was unclear whether apoA-I/apoA-II heterodimers were formed in these particles. Higher apoA-I/apoA-II heterodimer levels were also observed in Troponin I-positive patient plasmas, consistent

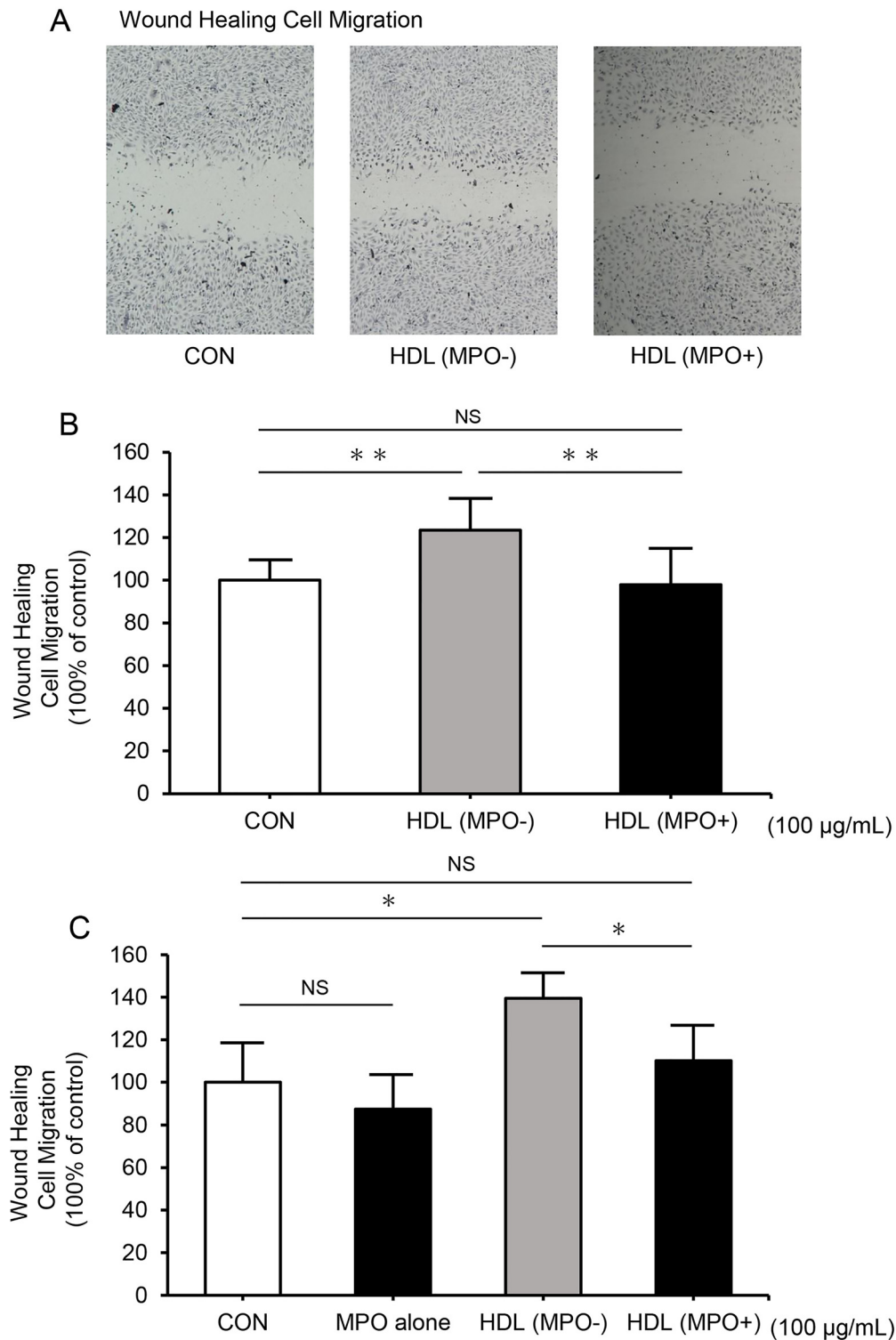


Figure 2: HDL oxidation by MPO reduces HUVEC migration.

(A) Monolayer HUVECs were wounded by manual scraping and incubated with 100 µg/ml normal HDL (MPO-) or MPO-oxidized HDL (MPO+) for 12 h. Migrated cells were photographed in low-power fields and quantified in 12 random wound widths per well ($n = 3$ wells). The percentage of migrated cells treated with HDLs compared to cells with PBS treatment (control; CON, 100%). (B) Quantification values are expressed as the mean + SDs ($n = 3$). (C) The wounded monolayer HUVECs were also incubated with 100 µg/ml normal HDL (MPO-) or MPO-oxidized HDL (MPO+) and MPO alone for 12 h to confirm that the MPO itself does not affect the wound healing cell migration. Cells migrated into the gaps were also expressed as percentage in comparison to control. NS, not significant. * $p < 0.05$. ** $p < 0.01$. The statistical tests were used ANOVA followed by post hoc Tukey's multiple comparison test.

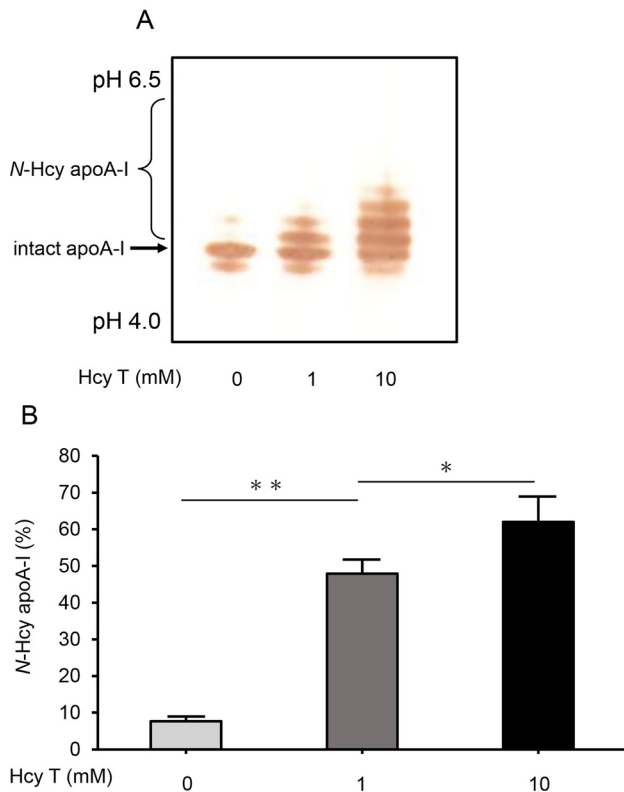


Figure 3: Isoelectric focusing (IEF) for HDL and HDL treated with HcyT.

Representative IEF patterns of apoA-I and N-Hcy apoA-I are shown. HDL was incubated with or without 1 or 10 mM HcyT at 37 °C for 24 h. (A) The samples were analyzed by IEF followed by immunoblotting for apoA-I (0.6 µg protein/lane). (B) The relative amounts of N-Hcy apoA-I were analyzed with CS Analyzer4. The values indicate the mean + SDs ($n = 3$). * $p < 0.05$, ** $p < 0.01$. The statistical tests were used ANOVA followed by post hoc Tukey's multiple comparison test.

with our previous report showing that apoA-I/apoA-II heterodimer levels were significantly higher in plasma from AMI patients compared to controls (Kameda et al. 2012). Analyzing apoA-I oxidation products should provide more specific information about cardiovascular disease than analyzing MPO protein levels because MPO is activated by cardiovascular disease, in addition to numerous infectious and inflammatory diseases (Lu et al. 2015; Nussbaum et al. 2012; Shao et al. 2010c).

Regarding the effect of MPO on HDL function, MPO-oxidized HDL containing apoA-I/apoA-II heterodimers induce weak endothelial migration function *in vitro*. We confirmed that these effects are not directly mediated by MPO, but are specific to MPO-oxidized HDL (Figure 1(C)). A previous study shows that MPO catalyzes HDL oxidation, resulting in apoA-I tyrosine nitration and chlorination. Indeed, MPO-oxidized HDL containing Cl-tyrosine was ineffective at preventing HUVEC apoptosis

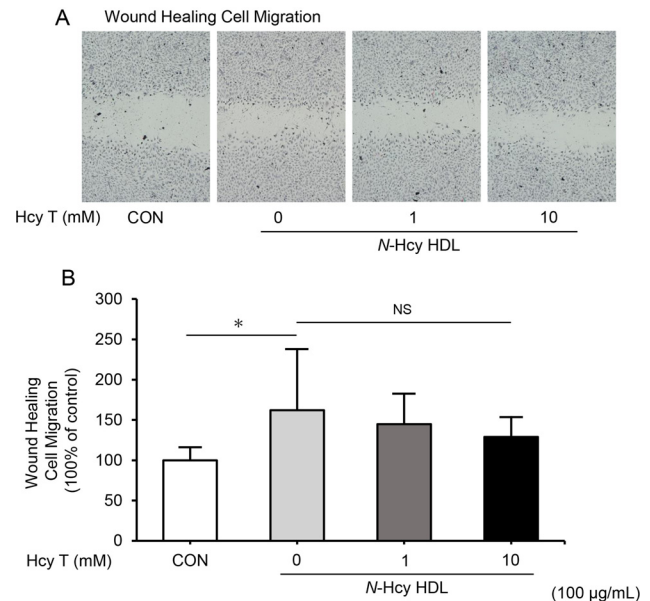


Figure 4: Evaluation of endothelial cells repair capacity of HDLs treated with HcyT (1 and 10 mM).

(A) Monolayer HUVECs were wounded by manual scraping and incubated with 100 µg/ml normal HDL or N-homocysteinylated HDL for 12 h. Migrated cells were photographed in low-power fields and quantified in 12 random wound widths per well ($n = 3$ wells). The percentage of migrated cells treated with HDLs compared to cells with PBS treatment (control; CON, 100%). (B) Quantification values are expressed as the mean + SDs ($n = 3$), NS, not significant. * $p < 0.05$. The statistical tests were used ANOVA followed by post hoc Tukey's multiple comparison test.

(Undurti et al. 2009). Moreover, MPO-catalyzed HDL nitration and chlorination impaired EC repair (Pan et al. 2013). In addition to the apoA-I modifications shown in previous studies, we observed a high negative correlation between wound healing cell migration and the ratio of apoA-I/apoA-II heterodimer to total apoA-II. Tyrosine nitration and chlorination were identified in MPO-oxidized apoA-I (Shao et al. 2005), but the dimerization binding site was unclear. It is possible that the repair capacity is reduced by masking the tyrosine residues of apoA-1 by apoA-II. Although further experiments are needed, there might be a direct effect between tyrosine modification in HDL and EC repair dysfunction. In addition, considering the high negative correlation between EC repair function and the ratio of apoA-I/apoA-II heterodimer to total apoA-II, it is possible that apoA-II plays a role in HDL function to promote EC proliferation. Aside from apoA-I/apoA-II heterodimers, a prominent 12 kDa band, which corresponds to SAA, was observed in two patient samples. SAA can displace apoA-I from HDL particles (Cabana et al. 2004; Coetzee et al. 1986; Sato et al. 2016), suggesting a relationship between SAA and EC repair capacity disorder.

Table 1: Profiles of the subjects in this study.

Number	Wound Healing Cell Migration	Heterodimer*	Heterodimer*	N-Hcy apoA-I*	Troponin I
	(% of control) (\pm SD)	(% of total apoA-I)	(% of total apoA-II)	(%)	(ng/ml)
P1	89 \pm 29	9.4	54.8	18.8	8.53
P2	102 \pm 19	11.4	45.7	23.0	8.22
P3	94 \pm 8	0.2	28.7	15.0	5.92
P4	106 \pm 13	2.1	30.2	15.6	9.21
P5	118 \pm 12	0.4	22.4	16.3	11.54
P6	121 \pm 19	12.3	44.4	20.5	852.70
P7	94 \pm 17	2.8	29.8	24.7	578.43
H1	135 \pm 5	0.2	15.7	0.0	–
H2	115 \pm 20	0.5	29.5	4.9	–
H3	118 \pm 16	1.3	16.8	11.0	–
H4	111 \pm 25	0.2	22.0	11.1	–
H5	133 \pm 56	0.3	16.4	8.7	–
H6	121 \pm 15	0.2	13.8	3.4	–
H7	113 \pm 21	0.6	8.0	9.4	–

Wound healing capacity was measured using HDL isolated from patients with varying troponin I concentrations (P1–P7) and healthy subjects (H1–H7). Wound healing cell migration was performed in three different wells for each HDL sample. The data were expressed as mean \pm standard deviation. *The relative amounts of apoA-I/apoA-II heterodimer and N-Hcy apoA-I were analyzed with western blot analysis by using CS Analyzer4. These relative amounts were obtained from single measurements. Troponin I values were obtained in the clinical laboratory for clinical purpose. These data were linked in Figures 5–7.

However, contrary to expectations, the presence of SAA was not always necessary to impair EC repair capacity, and no correlation between SAA levels and repair capacity was observed in this experiment. Since the number of patients with high SAA was small in this study, further evaluation is required.

Next, we investigated another candidate, Hcy, which can disrupt HDL-mediated wound-healing capacity. HcyT, a cyclic compound derived from Hcy, reacts with proteins to form an amide bond with the ϵ -amino group in lysine residues (Jakubowski 1997, 1999). Indeed, we successfully observed N-Hcy apoA-I in HDL treated with HcyT. Previous studies showed that hyperhomocysteinemia increases cardiovascular disease risk (Liu et al. 2019; Refsum et al. 1998; Wilcken and Wilcken 1976). Thus, Hcy toxicity is likely involved in various proatherogenic properties, including EC dysfunction, smooth muscle cell proliferation activation, thrombogenesis, and reduction of plasma HDL level (Cattaneo 2001; Handy et al. 2005; Lentz 2001; Liao et al. 2006; Mikael et al. 2006; Tsai et al. 1994; Wang et al. 2000). In this study, we detected higher N-Hcy apoA-I levels in patients than in the control group. Moreover, we showed a negative correlation between the N-Hcy apoA-I to total apoA-I ratio and EC migration. Some researchers showed that patients with hyperhomocysteinemia had increased malondialdehyde (MDA) levels (Moselhy and Demerdash 2003) and oxidative radicals generated by Hcy (Feng et al. 2018; Hu et al. 2019). HDL undergoes N-homocysteinylolation *in vivo*, in addition to multiple

oxidative modifications, as described above. In light of this result, it is necessary to confirm the effect on EC proliferation under oxidation associated with N-Hcy HDL production in future studies.

Several limitations of this study should be acknowledged. First, patient blood samples were selected by only troponin I levels. It may include the effects of diseases associated with troponin elevation other than cardiovascular disease. The results showed troponin I levels might not directly correlate with increased HDL modification. There are many other effects that are widely recognized as critical to the development of atherosclerosis, such as LDL levels and LDL modification (Malle et al. 2006; Podrez et al. 2000). The interactive role of these factors also remained to be considered and analyzed. This study indicates a key role for MPO-oxidized HDL containing apoA-I/apoA-II heterodimer in the endothelial healing function of HDL, but there may be interactions between the risk factors (troponin I, levels of MPO-oxidation, N-homocysteinylolation and SAA) in certain individuals. Thus, monitoring risk factors in individuals may provide a more useful estimate of the endothelial healing function of HDL. Furthermore, it is likely that there are many other modifications (e.g., chlorination, nitration, oxidation of methionine and tryptophan) on MPO-treated HDL (Lu et al. 2015; Pan et al. 2013; Shao et al. 2005, 2010b; Undurti et al. 2009). It is possible that dimerization and these modifications are simultaneously acting to reduce HDL function. As another possible protein, apoM containing sphingosine 1-phosphate

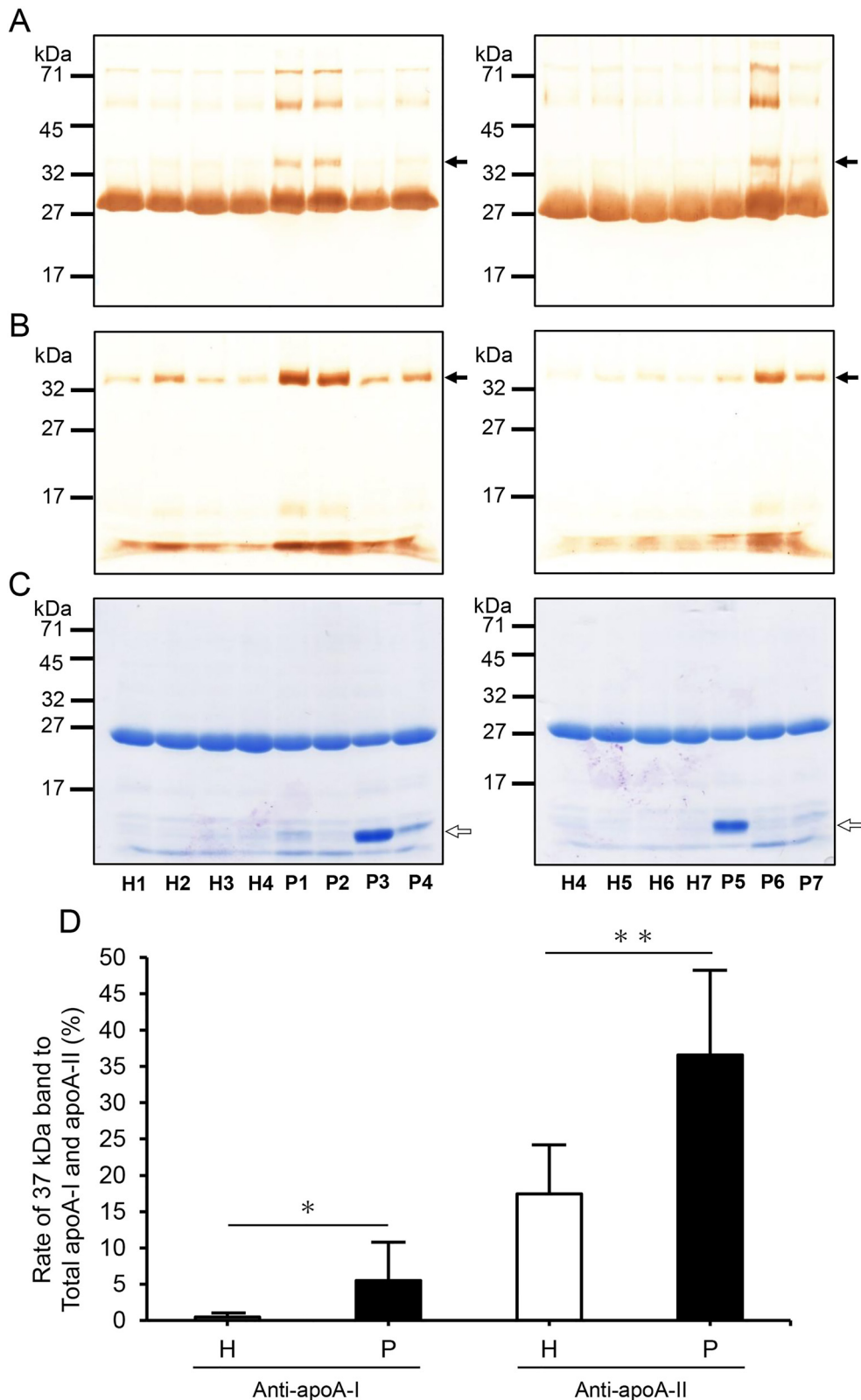


Figure 5: Identification of apoA-I/apoA-II heterodimers in HDL plasma samples from seven patients with varying troponin I levels (P1–P7) and healthy human plasmas (H1–H7).

The samples were analyzed by SDS-PAGE. ApoA-I (A) and apoA-II (B) -containing bands were visualized by immunoblotting (0.6 μ g protein/lane) or by staining with CBB (15 μ g protein/lane). (C) The black arrows indicate the apoA-I/apoA-II heterodimer. The white arrows indicate serum amyloid A (SAA). The molecular masses of the standards are listed on the left. (D) The relative amounts of apoA-I/apoA-II heterodimer to total apoA-I and total apoA-II were analyzed with CS Analyzer4. The values indicate the mean + SDs. * p < 0.05, ** p < 0.01. The statistical tests were used unpaired t -test.

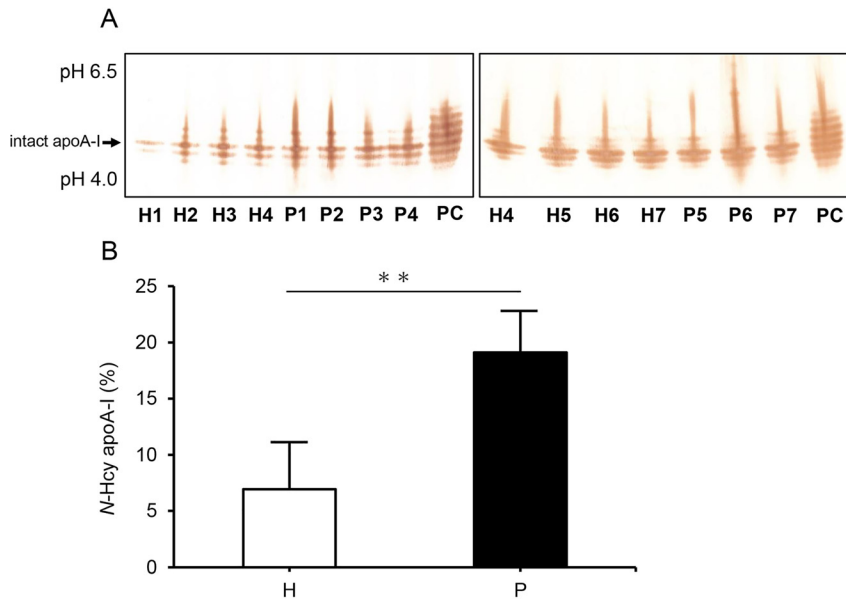


Figure 6: Identification of *N*-homocysteinylation in HDL plasma samples from seven patients with varying troponin I levels (P1–P7) and healthy human plasma (H1–H7).

(A) The samples were analyzed by IEF followed by immunoblotting for apoA-I (0.6 µg protein/lane). (PC: positive control; 10 mM *N*-homocysteinylation HDL), (B) The relative amounts of *N*-Hcy apoA-I were analyzed with CS Analyzer4. The values indicate the mean + SDs. *** $p < 0.01$. The statistical tests were used unpaired *t*-test.

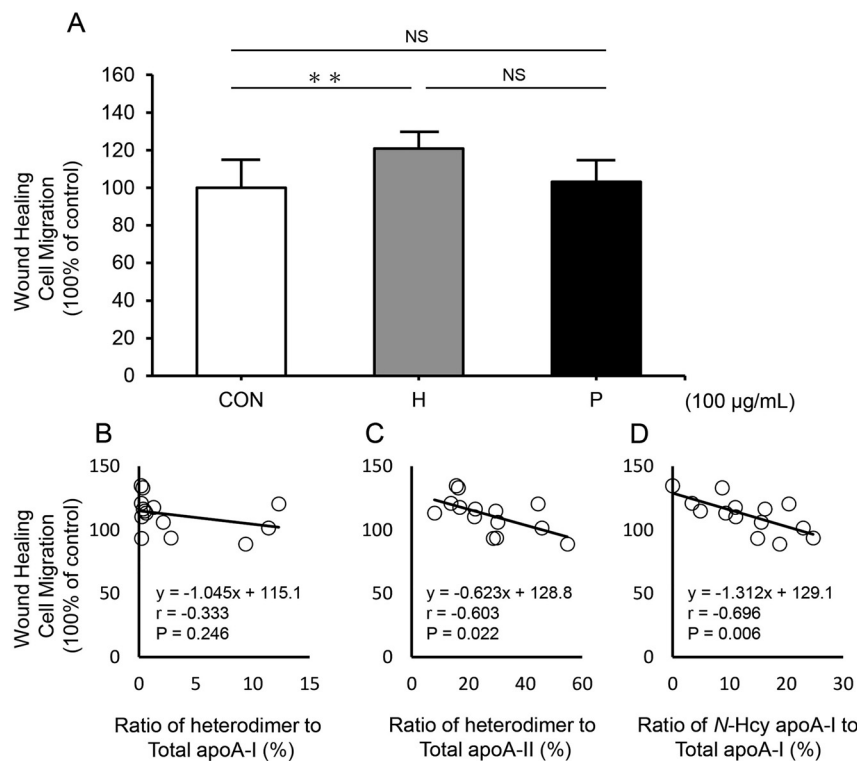


Figure 7: HDL from patients with varying troponin I levels has a reduced capacity to promote HUVEC migration.

(A) Monolayer HUVECs were wounded by manual scraping and incubated with 100 µg/ml patient HDL (P) or healthy human HDL (H) for 12 h. Migrated cells were photographed in low-power fields and quantified in 12 random wound widths per well ($n = 3$ wells). The percentage of migrated cells treated with HDLs compared to cells with PBS treatment (control; CON, 100%). The values are expressed as the mean + SD. N.S., not significant. *** $p < 0.01$. Correlation between wound healing cell migration and (B) the ratio of apoA-I/apoA-II heterodimer to total apoA-I, (C) the ratio of apoA-I/apoA-II heterodimer to total apoA-II, and (D) the ratio of *N*-Hcy apoA-I to total apoA-I ($n = 14$). The statistical tests were used ANOVA followed by post hoc Tukey's multiple comparison test.

involving in vascular endothelial repair can be functionally impaired at the same time (Borup et al. 2015; Christoffersen et al. 2011). Therefore, it has not been elucidated yet whether apoA-I/apoA-II heterodimer is the key driver of the effects in this study. In either case, the dimerization is a dramatic change resulting from HDL oxidation because it

implies not only amino acid residues modification but also binding between proteins. Therefore, the detection of apoA-I/apoA-II heterodimer is useful for comprehensive evaluation including other oxidative modifications. Future studies should investigate the interaction between EC migration and HDL modification.

Conclusion

In conclusion, our findings provide novel evidence that oxidized HDL with MPO oxidation products, apoA-I/apoA-II heterodimers, weakens endothelial repair capacity. Our results suggest that measuring apoA-I/apoA-II heterodimers, which reflect MPO activity in atherosclerotic lesions, may be useful to estimate HDL-mediated endothelial healing. The current study shows that modified HDLs indicate the extent of endothelial healing in vascular injuries, such as cardiovascular intervention. Moreover, measuring heterodimer formation may be more useful than measuring MPO protein levels, and may be a biomarker for atherosclerotic disease.

Materials and methods

Antibodies

Goat anti-human apoA-I and apoA-II polyclonal antibodies were purchased from Academy Biomedical (Texas, USA). Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG polyclonal antibody was purchased from Medical and Biological Laboratories (Nagoya, Japan).

Blood samples

Plasma samples from seven patients with varying troponin I levels were obtained from residual blood samples obtained for laboratory analyses at the Clinical Laboratory of the Medical Hospital at the Tokyo Medical and Dental University. Control blood samples were obtained from seven healthy volunteers who gave written informed consent at Tokyo Medical and Dental University. This study was approved by the ethics committee of the Faculty of Medicine, Tokyo Medical and Dental University (No. M2015-546 and M2016-049), and performed from June to November 2017.

Lipoprotein isolation

HDL ($1.063 < d < 1.21$ g/ml) were isolated from plasma or serum by ultracentrifugation as described previously (Havel et al. 1955). Isolated HDL were dialyzed against phosphate buffered saline (PBS) by using Cellulose Tubing (20/32) with a molecular weight cutoff of 12–14 kDa (Viskase Companies Inc, Illinois, USA). The samples were stored at -80°C until use in experiments, and that all samples were stored at 4°C during all analyses.

Treatment of HDL with MPO

HDL (100 μl at 10 mg protein/ml in PBS) isolated from pooled healthy serum was incubated with 100 μl 50 mM PBS (pH 7.4) containing 0.2 mM hydrogen peroxide, 0.2 mM diethylene triamine pentaacetic acid, 0.4 mM L-tyrosine, and 20 nM MPO at 37°C for 24 h. After MPO treatment, HDL was dialyzed with 3×2 l PBS by using Cellulose Tubing (8/32) with a molecular weight cutoff of 12–14 kDa (Viskase

Companies Inc, Illinois, USA). Then, MPO-oxidized HDL was sterilized with a 0.22- μm filter and stored at 4°C in the dark. MPO-oxidized HDL was analyzed by SDS-PAGE and 8% nondenaturing PAGE followed by immunoblotting or with Coomassie Brilliant Blue (CBB) R250 staining to assess the profile of HDL proteins.

Immunoblotting

Western blot analysis was performed as described previously (Kameda et al. 2012). Briefly, proteins were separated by SDS-PAGE using 14% polyacrylamide gels under reducing conditions and transferred to PVDF membranes (Millipore, Massachusetts, USA). The membranes were incubated with goat anti-apoA-I or anti-apoA-II polyclonal antibodies, followed by incubation with HRP-conjugated rabbit anti-goat IgG. The antibody incubations were performed at room temperature for 1 h. Then, the bands containing apoA-I and apoA-II were visualized with 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2 . The relative amounts of apoA-I/apoA-II heterodimers were semi-quantified with CS Analyzer4 software (ATTO CORPORATION, Tokyo, Japan).

Evaluation of HDL particle size

HDL isolated by ultracentrifugation from blood samples were dialyzed against buffer A (20 mM Tris/HCl, 1 mM EDTA-2K, pH 7.4). After MPO treatment, HDL was dialyzed with 3×2 l buffer A by using Cellulose Tubing (8/32) with a molecular weight cutoff of 12–14 kDa (Viskase Companies Inc). MPO-oxidized HDL was subjected to native-gel electrophoresis (native-PAGE) using 8% polyacrylamide gels. Separated proteins were stained with CBB or transferred to polyvinylidene fluoride membrane (Millipore) for immunoblotting. The membranes were incubated with goat anti-apoA-I or anti-apoA-II polyclonal antibodies, followed by incubation with HRP-conjugated rabbit anti-goat IgG. The antibody incubations were performed at room temperature for 1 h. Then, the bands containing apoA-I and apoA-II were visualized with 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2 .

N-homocysteinylation of HDL

N-homocysteinylation of HDL was performed according to a previously reported method (Ishimine et al. 2010). HDL isolated from pooled healthy serum was incubated with 0, 1, or 10 mM HcyT (MP Biomedicals, California, USA) at 37°C for 24 h. Then, the treated samples were dialyzed against with 3×2 l PBS by using Cellulose Tubing (20/32) (Viskase Companies Inc, Illinois, USA). HcyT-treated HDL (50 μl at 2 mg protein/ml in PBS) were mixed with a 2-aminoethanethiol hydrochloride solution (dissolved in 20 mM Tris-HCl [pH 10.4] to adjust the pH to near neutral) at a ratio of HDL protein: 2-aminoethanethiol 1:6.7 (w/w) and incubated at 37°C for 12 h. To confirm the production of N-Hcy apoA-I in HDL, 2-aminoethanethiol-treated samples were isolated by isoelectric focusing (IEF) in a pH range from 4.0 to 6.5 and transferred onto PVDF membranes, which were then incubated with anti-apoA-I antibody. Then, the membranes were incubated with HRP-conjugated rabbit anti-goat IgG. The antibody incubations were performed at room temperature for 1 h. The bands containing apoA-I were visualized with 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2 . The relative amount of N-Hcy apoA-I were analyzed with CS Analyzer4 software (ATTO CORPORATION). To

calculate the ratio of *N*-Hcy apoA-I to total apoA-I, we defined *N*-Hcy apoA-I bands by a higher positive charge than intact apoA-I.

SAA quantification

HDL was isolated from plasma samples from patients with varying troponin I levels and healthy human plasma. The isolated HDL was analyzed by SDS-PAGE with CBB-R250 staining. Single band at the 14 kDa position was regarded as serum amyloid A (SAA) and the relative amount of SAA to total HDL protein was quantified with CS Analyzer4 software (Atto Corporation).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Promo Cell (Heidelberg, Germany). Cells were cultured on gelatin-coated culture flasks in EGM-2 medium (Promo Cell) supplemented with 2% fetal bovine serum (FBS).

Wound healing assay

Wound healing assays were conducted as described previously (Lv et al. 2016). HUVECs were plated in endothelial cell medium with 2% FBS in 24-well plates (2×10^4 cells/well) and cultured until monolayer formation. The cell monolayers were wounded by manual scraping with a 200- μ l micropipette tip. Then, the cells were washed with PBS and incubated with endothelial cell medium containing 0.5% FBS alone or 100 μ g/ml HDL, MPO-oxidized HDL, *N*-Hcy HDL, or patient HDL for 12 h. Cells were fixed with methanol and stained with Mayer's hematoxylin solution. Cells that migrated past the wound edge were photographed in low-power fields (8 \times). The gap width between cells (12 random scraped areas per well) were measured with a BZ-X710 instrument (Keyence Corporation, Osaka, Japan) using lens (plan Apo λ , 4 \times) and digital zoom ($\times 2.0$). All assays were performed in triplicate. The results were confirmed in at least three independent experiments. The length of cell migration was determined by the sum of the differences of wound width before and after HDL incubation. The results are expressed as the percentage of migrated cells treated with HDLs compared to cells with PBS treatment (control, 100%).

Statistical analysis

All data were expressed as mean \pm SD. Differences between treatment groups were analyzed using unpaired *t*-test and ANOVA followed by post hoc Tukey's multiple comparison test. The correlation between wound healing cell migration and quantified HDL modification products was tested by Pearson correlation coefficient. These statistical analyses were performed by using IBM SPSS 25.0 (Illinois, USA). Significance was set at $p < 0.05$ (two-sided).

Author contributions: TK, RO, and MT designed the experiments. TK, SS, YH, KY, and SL performed the experiments and analyzed the data. TK, RO, and MT wrote this paper. YH, NI, ST, YK, RO, and MT contributed to

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