Table 2. Baseline characteristics of patients in secondary prevention group

Characteristics	Secondary prevention No. (%) of patients			*p*
	All *n*=88	Exposed *n*=74 (84.1)	Unexposed *n*=14 (15.9)	
Age, mean (range)	52 (23-71)	51 (29-70)	53 (23-71)	0.62
Men, No. (%)	53 (60.2%)	46 (62.2%)	7 (50.0%)	0.55
BMI ≥25	21 (25.3%)	17 (24.3%)	4 (30.8%)	0.73
Smoker	42 (50.0%)	38 (53.5%)	4 (30.8%)	0.23
Drinker	39 (46.4%)	33 (46.5%)	6 (46.2%)	1.00
Xanthoma	75 (85.2%)	63 (85.1%)	12 (85.7%)	1.00
Tendon xanthoma	71 (80.7%)	61 (82.4%)	10 (71.4%)	0.46
Nodular xanthoma	7 (8.0%)	6 (8.1%)	1 (7.1%)	1.00
Palpebral xanthoma	8 (9.1%)	5 (6.8%)	3 (21.4%)	0.11
PAD	2 (2.3%)	2 (2.7%)	0 (0.0%)	1.00
Hypertension	36 (40.9%)	30 (40.5%)	6 (42.9%)	1.00
Diabetes	14 (15.9%)	9 (12.2%)	5 (35.7%)	0.04
Lipid profile, (mg/dL)				
TC†	332 (191-469)	334 (191-469)	322 (229-444)	0.41
TG†	128 (37-636)	128 (37-636)	136 (63-318)	0.85
HDL-C†	42 (20-90)	42 (20-90)	39 (26-73)	0.91
LDL-C†	249 (117-381)	256 (117-381)	245 (138-354)	0.57
Blood Pressure, mmHg				
SBP†	129 (90-180)	128 (96-180)	136 (90-166)	0.97
DBP (mmHg)†	80 (52-114)	80 (52-114)	78 (60-104)	0.33
FBS (mg/dL)†	96 (72-252)	97 (72-197)	94 (79-252)	0.96
HbA1c (%)†	5.8 (4.1-10.6)	5.5 (4.1-8.1)	6.4 (5.3-10.6)	0.06
Tendon xanthoma thickness (mm)†	14.5 (5.8-25.0)	15.0 (5.8-25.0)	10.0 (8.5-18.8)	0.09
Prior CV events				
Angina Pectoris	45 (51.1%)	36 (48.6%)	9 (64.3%)	0.39
Myocardial Infarction	34 (38.6%)	33 (44.6%)	1 (7.1%)	<0.01
Stroke	7 (8.0%)	4 (5.4%)	3 (21.4%)	0.08
Heart failure	2 (2.3%)	2 (2.7%)	0 (0.0)	1.00
TIA	2 (2.3%)	1 (1.4%)	1 (7.1%)	0.29
Treatment				0.08
Cholesterol-lowering drugs (non-probucol)	81 (92.0%)	70 (94.6%)	11 (78.6%)	
LDL-apheresis	13 (14.8%)	11 (14.9%)	2 (14.3%)	1.00
Anti-platelet drugs	50 (56.8%)	44 (59.5%)	6 (42.9%)	0.38
Anti-hypertensive drugs	47 (53.4%)	42 (56.8%)	5 (35.7%)	0.24
Diabetic drugs	6 (6.8%)	3 (4.1%)	3 (21.4%)	0.05

†Data are the median (range). All data are numbers (%) unless otherwise indicated. Each percentage is related to the total number with measurement data. TIA indicates transient ischemic attack.

147 (124-197) and 33 (17-70) mg/dL. Sub-analysis of changes in the lipid profile after probucol treatment detected significant three predictors of CV event risk: higher baseline TC (HR 2.74, 95% CI 1.05-7.16; p=0.04) in the primary prevention group; reduction in TG (HR 0.22, 95% CI 0.06-0.86; p=0.03); and reduction in LDL-C (HR 0.17,95% CI 0.03-0.90; p=0.04) after treatment in the subset of the secondary prevention group on stable doses of probucol. Neither TC nor HDL-C after treatment was associated with CV event risk in the probucol-exposed group, which indicates that reduction of the HDL-C level after probucol treatment is not related to CV event risk for probucol-exposed patients.

We evaluated the safety of probucol for all collected data from 541 patients, and found 56 adverse

Table 3. Incidence of cardiovascular events

		Cardiovascular Event		No event	Total	*p*
Primary prevention ($n=322$)	Exposed ($n=233$)		27 (11.6%)	206	233	
		MI	4			
		AP	18			
		Str.	3			
		TIA	1			
		PAD	1			0.058
	Unexposed ($n=89$)		4 (4.5%)	85	89	
		AP	1			
		Str.	2			
		TIA	1			
Secondary prevention ($n=88$)	Exposed ($n=74$)		20 (27.0%)	54	74	
		MI	6			
		AP	12			
		HF	1			
		Str.	1			0.012
	Unexposed ($n=14$)		9 (64.3%)	5	14	
		MI	2			
		AP	6			
		Str.	1			

MI, myocardial infarction; AP, angina pectoris; HF, heart failure; Str., stroke; TIA, transient ischemic attack; PAD, peripheral artery disease.
†One of the 4 patients died after 12 months of probucol termination.

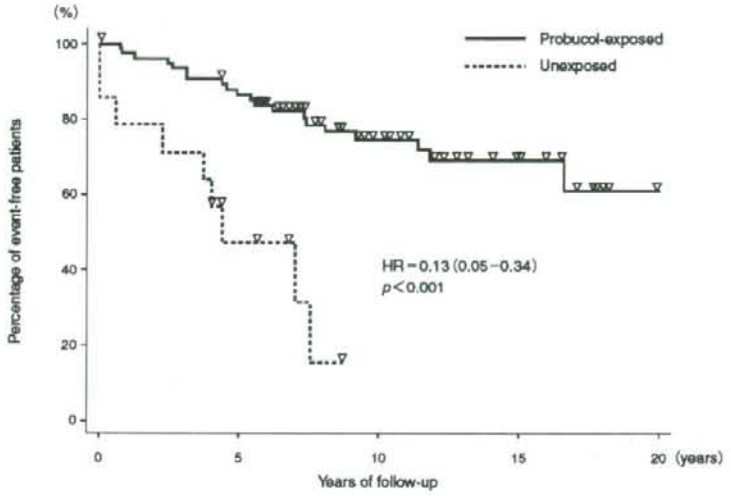
events in 18 patients. Malaise, pruritus, macrocytic anemia and pain in the extremities were recorded as adverse drug reactions associated with probucol. We noted and reported gastric cancer stage III immediately to the Ministry of Health and Welfare as an unexpected serious event, because of an unknown drug relation due to many concomitant drugs, although probucol was found to be non-carcinogenic alone[21]. Six deaths were observed in the population not taking probucol or stopping probucol. There was no other difference in the incidence of adverse events, including serious events, between probucol exposure and non-exposure.

Discussion

Many data from large-scale randomized controlled trials have overwhelmingly demonstrated the clinical benefits of lowering cholesterol with statins[22, 23], yet the rapid and extensive prophylactic use of cholesterol-lowering drugs remains controversial. Few studies have addressed the clinical risks and benefits of long-term treatment of hyperlipidemia among women[24] or elderly patients[25]. The safety of long-term cholesterol-lowering therapy, including the issue of associated cancer risk or benefit, remains inconclusive because of conflicting clinical evidence[26]. More importantly, conclusions from the results of randomized controlled trials are limited by their relatively short follow-up periods (generally less than 5 years) in the analyzed studies.

In long-term treatment for FH, probucol was used with other cholesterol lowering drugs in over 80% of the secondary prevention group–those with a more severe clinical outlook than the primary prevention group: a higher prevalence of hypertension and diabetes, significant thicker tendon xanthoma, more combined therapy with LDL-apheresis, anti-platelet drugs, and anti-hypertensive drugs. The high rate of probucol use in FH was surprising, different from expected. This might partly reflect the prescription behavior of experts with the result that intractable patients responded to the regimen.

In the secondary prevention, the higher-risk group, probucol exposure was associated with a reduction in the risk of cardiovascular events (HR 0.13; 95% CI 0.05–0.34) with high significance ($p<0.001$), while it was not significant in the primary prevention group. This result was also contrary to our expectation that probucol exposure would likely be associated with increased event risk due to a confounding indication–that patients considered more severe at diagnosis would receive more treatment, including probucol. We did not collect the details of non-probucol drugs



Number at risk

Years	0				5					10					15					20
Exposed	74	71	70	68	66	62	54	50	42	38	34	30	25	19	17	13	12	8	3	1	0
Unexposed	14	11	11	10	9	5	4	2	1	0	0	0	0	0	0	0	0	0	0	0	0

Estimates of event-free rates are according to whether patients received probucol. The cumulative probability of remaining without events was higher in patients treated with probucol ($p < 0.001$; log-rank test).

Fig. 2. Kaplan-Meier Estimates of Event-free Rate.

For secondary prevention, the incidence of cardiovascular events was 27.0% in the exposed group and 64.3% in the unexposed group. An event-free survival curve for the secondary prevention group is given.

Table 4. The results of multivariate analysis using Cox regression procedure

Factor	Primary prevention			Secondary prevention		
	HR	95% CI	*p*	HR	95% CI	*p*
Baseline variables						
Total cholesterol	1.58	1.06–2.33	0.02	–	–	–
Drinking	2.43	1.09–5.44	0.03	–	–	–
Peripheral artery disease	5.27	0.51–54.63	0.16	–	–	–
Palpebral xanthoma	–	–	–	2.94	1.02–8.47	0.05
Diabetes	–	–	–	2.58	0.76–8.76	0.13
Treatment in follow-up						
Probucol use	1.50	0.48–4.67	0.49	0.13	0.05–0.34	<0.001
Anti-platelet drug use	–	–	–	2.48	1.00–6.17	0.05

to simplify the study procedure. However, we would likely exclude underused statins because of the reduced use of non-probucol drugs from the possible factors of the higher event rate in the unexposed group, because statins were available when all of the 9 recurrent patients (**Table 3**) started and the patients continued on cholesterol-lowering drugs. We suppose, therefore, that the reasons for this unanticipated great risk reduction include some antioxidant and anti-atherogenic actions[3, 4, 27] of probucol. The finding in second prevention may be suggested by the report[27] that probucol significantly decreased *in vitro* LDL oxidizability measured under typically strong oxidative conditions, and that long-term treatment with probucol had an

anti-atherogenic effect in Watanabe Heritable Hyperlipidemic rabbits. From the observation that the baseline lipid profile was not different between the two groups of exposure and non-exposure in secondary prevention, the drug might exhibit greates effectiveness in post-cardiovascular disease patients, in possibly advanced lipid accumulation and inflammation, which are associated with the circulation of oxidized LDL[28].

In primary prevention, we observed an almost significant increase of events in the exposed group (**Table 3**), and an apparently increased risk (HR 1.5), although not statistically significant after adjustment (**Table 4**). We suppose, however, that the ideal effects of probucol might be concealed by the following factors noted in primary prevention. The exposed group had a worse lipid profile (TC, LDL-C and HDL-C levels), higher HbA_{1c}, and thus definitely a higher risk than the unexposed group. Furthermore, 8 (nearly 30%) of the 27 patients experiencing cardiovascular events in the exposed group discontinued probucol when they had events. This was consistent with the different finding between primary and secondary preventions in the exposed group: less than half of the patients (113 of 233) in primary prevention continued on probucol, while 53 (72%) of 74 patients continued in secondary prevention. This estimation might be conservative.

The controversial and paradoxical action of probucol– lowering HDL-C– level was not associated with the risk of CV events in the cohort, therefore, the association between low levels of HDL-C and an increased risk for CV events or death indicated by the early Framingham Heart Study[29] may not be extrapolated to probucol-treated patients. This proposition is consistent with recent findings that a lowered HDL-C level is not always atherogenic, but that the quality or function of HDL-C is more important than the HDL-C levels[30]. In fact, increased levels of HDL-C with torcetrapib, a CETP inhibitor, were not associated with a significant clinical benefit in patients with coronary disease[31], FH[32] or mixed dyslipidemia[33].

We speculate that enhanced reverse cholesterol transport by CETP activation as a result of probucol treatment also contributed to the detected risk reduction in the cohort. The observed positive outcome of probucol, a CETP activator, might be a mirror image of the negative clinical trial results for the CETP inhibitor[34]. Reports[35, 36] of increased coronary heart disease in CETP deficiency despite increased HDL-C levels, and the molecular approach to review CETP deficiency[37] support our hypothesis, at least in Japanese genealogy. Interestingly, a recent basic research reports that human CETP expression enhances the mouse survival rate in an experimental systemic inflammation model[38], indicating for the first time a role for CETP in the defense against the exacerbated production of proinflammatory mediators.

For the safety evaluation, we found no cardiotoxic adverse drug reaction including QT/QTc prolongation or torsade de pointes, in this study, although probucol can cause them[16, 39, 40].

We obtained these results from an observational study with no control for inaccuracy, unexpected bias or confounding factors. We could not assure the precision of the baseline measurements due to unrecorded data. The participant centers were major hospitals for FH, but not all hospitals in Japan, because the study was conducted as part of a post-marketing study by a pharmaceutical manufacturer within the framework of the Japanese government regulations. Some restrictions on collecting data might have resulted in unexpected small numbers in the unexposed group in secondary prevention, although we think that the study cohort represents nearly a nationwide population of heterozygous FH in Japan. The results derived from patient data in Japan can not necessarily be generalized to patients in western countries.

Despite these limitations of the study, however, we could evaluate the outcome of long-term probucol treatment in the medical practice setting for FH, a high-risk population, for as long as 20 years in Japan. The significant risk reduction of CV events observed in the secondary prevention group holds clinical significance and suggests some beneficial therapeutic actions of this drug in arteriosclerotic diseases. The hypothesis from the findings warrants a randomized controlled trial for verification of the secondary prevention, and needs further research into the molecular mechanisms or roles of CETP in pathogenesis.

Author Contributions

Dr. Yamashita had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Matsuzawa, Kita, Saito, Fukushima, Matsui. Acquisition of data: Yamashita, Bujo, Arai, Harada-Shiba, Saito, Kita, Matsuzawa. Analysis and interpretation of data: Yamashita, Bujo, Arai, Harada-Shiba, Matsui, Saito, Fukushima, Kita, Matsuzawa.

Drafting of the manuscript: Yamashita, Bujo, Arai, Harada-Shiba, Matsui, and Fukushima. Critical revision of the manuscript for important intellectual content: Yamashita, Matsui, Fukushima, Kita, Saito,

and Matsuzawa. Statistical analysis: Matsui and Fukushima. Administrative, technical, or material support: Fukushima, Matsui, Kita, Saito, and Matsuzawa. Study supervision: Yamashita, Fukushima, Matsui, Kita, Saito, and Matsuzawa.

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From the formerly Daiichi and Otsuka, Dr. Matsui, Dr. Fukushima, Dr. Matsuzawa, and Dr. Kita received fees and expenses for meetings related to protocol design, statistical and clinical interpretation of the data; Dr. Bujo, Dr. Arai, Dr. Harada-Shiba received honoraria and travel expenses for lectures, Dr.Yamashita, Dr. Bujo, Dr. Arai received fees and travel expenses for a meeting related to clinical interpretation of the data. Dr. Yamashita received consultancy fees from Otsuka. Dr. Matsuzawa is contracted as a short-term adviser to Otsuka in medical science. Dr. Saito received travel expenses only.

POSITIVE Investigators

Osaka University Hospital, Suita (S. Yamashita, T. Maruyama); National Cardiovascular Center, Suita (M. Harada-Shiba); Sumitomo Hospital, Osaka (Y. Minami); Chiba University Hospital, Chiba (H. Bujo); Asahi General Hospital, Asahi (N. Hashimoto); Kawatetsu Chiba Hospital, Chiba (M. Takahashi); Nishifuna Naika, Funabashi (M. Shinomiya); Kashiwado Hospital, Chiba (K. Kosuge); Numazu City Hospital, Numazu (Y. Hayashi); Toho University Sakura Medical Center, Sakura (K. Shirai, Y. Miyashita); Matsudo City Hospital, Matsudo (T. Oeda); Kyoto University Hospital, Kyoto (M.Yokode, H. Arai); Hiroshima General Hospital of West Japan Railway Company, Hiroshima (K. Takata); Maizuru Kyosai Hospital, Maizuru (R. Tatami); Kido Hospital, Niigata (T. Miida)

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Adiponectin prevents atherosclerosis by increasing cholesterol efflux from macrophages

Kazumi Tsubakio-Yamamoto [a,1], Fumihiko Matsuura [a,1], Masahiro Koseki [a], Hiroyuki Oku [a], Jose C. Sandoval [a], Miwako Inagaki [a], Kazuhiro Nakatani [a], Hajime Nakaoka [a], Ryota Kawase [a], Miyako Yuasa-Kawase [a], Daisaku Masuda [a], Tohru Ohama [a], Norikazu Maeda [d], Yumiko Nakagawa-Toyama [c], Masato Ishigami [b], Makoto Nishida [c], Shinji Kihara [d], Iichiro Shimomura [d], Shizuya Yamashita [a,*]

[a] Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2, Yamamdaoka, Suita, Osaka 565-0871, Japan
[b] Department of Biomedical Informatics, Division of Health Sciences, Osaka University Graduate School of Medicine, 1-7, Yamamdaoka, Suita, Osaka 565-0871, Japan
[c] Health Care Center, Osaka University, 1-17, Machikaneyama, Toyonaka, Osaka 560-0043, Japan
[d] Department of Metabolic Medicine, Osaka University Graduate School of Medicine, 2-2, Yamamdaoka, Suita, Osaka 565-0871, Japan

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ABSTRACT

Plasma high density lipoprotein (HDL)-cholesterol levels are inversely correlated to the risk of atherosclerotic cardiovascular diseases. Reverse cholesterol transport (RCT) is one of the major protective systems against atherosclerosis, in which HDL particles play a crucial role to carry cholesterol derived from peripheral tissues to the liver. Recently, ATP-binding cassette transporters (ABCA1, ABCG1) and scavenger receptor (SR-BI) have been identified as important membrane receptors to generate HDL by removing cholesterol from foam cells. Adiponectin (APN) secreted from adipocytes is one of the important molecules to inhibit the development of atherosclerosis. Epidemiological studies have revealed a positive correlation between plasma HDL-cholesterol and APN concentrations in humans, although its mechanism has not been clarified. Therefore, in the present study, we investigated the role of APN on RCT, in particular, cellular cholesterol efflux from human monocyte-derived and APN-knockout (APN-KO) mice macrophages. APN up-regulated the expression of ABCA1 in human macrophages, respectively. ApoA-I-mediated cholesterol efflux from macrophages was also increased by APN treatment. Furthermore, the mRNA expression of LXRα and PPARγ was increased by APN. In APN-KO mice, the expression of ABCA1, LXRα, PPARγ, and apoA-I-mediated cholesterol efflux was decreased compared with wild-type mice. In summary, APN might protect against atherosclerosis by increasing apoA-I-mediated cholesterol efflux from macrophages through ABCA1-dependent pathway by the activation of LXRα and PPARγ.

Plasma HDL levels are inversely correlated to the risk of atherosclerotic cardiovascular diseases. High density lipoprotein (HDL) plays a crucial role to remove excess cholesterol in atherosclerotic plaques and transport it back to the liver in the protective system, so-called "reverse cholesterol transport (RCT)" [1]. The cellular cholesterol efflux by HDL or a major apolipoprotein of HDL, apolipoprotein A-I (apoA-I) is the initial phase in RCT system. The ATP-binding cassette transporters (ABCA1 and ABCG1), which are expressed in the liver, small intestine and peripheral tissues, have been identified as key regulator molecules of cellular cholesterol efflux from macrophages [2–4]. ABCA1 promotes both free cholesterol and phospholipids efflux from macrophages to apoA-I [2]. ABCG1 also facilitates cellular cholesterol efflux to large, mature HDL, but not lipid poor apoA-I [3]. Scavenger receptor, class B, typeI (SR-BI) is expressed in hepatocytes to mediate selective cholesteryl ester uptake. It is also expressed in macrophages and is thought to be associated with HDL-mediated cholesterol efflux [4].

Adiponectin (APN) secreted from adipose tissues is the important molecule to prevent from development of atherosclerosis *in vivo*. Numerous epidemiological studies have revealed that male patients with hypoadiponectinemia had an increase in coronary artery disease (CAD) prevalence [5]. Furthermore, we and other

Abbreviations: *ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; APN, adiponectin; APN-KO, adiponectin knockout; ApoA-I, apolipoprotein A-I; BSA, bovine serum albumin; CAD, coronary artery disease; HDL, high density lipoprotein; LDL, low density lipoprotein; PCR, polymerase chain reaction; PBS, phosphate buffered saline; RCT, reverse cholesterol transport; SR-BI, scavenger receptor, class B, type I; LXRα, liver X receptor α; PPARγ, peroxisome proliferator-activated receptor γ.*

* Corresponding author. Fax: +81 6 6879 3739.
E-mail address: shizu@imed2.med.osaka-u.ac.jp (S. Yamashita).
[1] These authors equally contributed to this work.

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laboratories have demonstrated that plasma HDL-cholesterol levels are positively correlated to APN concentrations in humans [6,7]. Although these findings suggest that APN might have an ability to prevent the development of atherosclerosis by the acceleration of RCT system, the underlying mechanism for it has not been clarified yet. It has been reported that *in vitro* APN suppresses atherosclerotic vascular changes, for example, the expression of adhesion molecules in vascular endothelial cells [8,9] and inhibits the formation of foam cells [10]. Recently, we reported that human recombinant APN enhanced the expression of ABCA1 and accelerated the synthesis of apoA-I in a human hepatoma cell line, HepG2 cells, suggesting that APN might increase the HDL assembly through ABCA1 in the liver [11]. Furthermore, we demonstrated that the expression levels of apoA-I in plasma and the liver were decreased in APN-knockout (APN-KO) mice and that the ABCA1 expression was also reduced in APN-KO mice, compared to wild-type mice [12].

In the present study, to clarify further the role of APN in RCT system, we investigated the effect of APN on cellular cholesterol efflux from human monocyte derived- and APN-KO or wild-type mice peritoneal macrophages.

Materials and methods

Cell culture. Human mononuclear cells (monocytes and lymphocytes) were isolated from peripheral venous blood by density gradient centrifugation using NycoPrep™, with a density of 1.077 g/ml (Nycomed Pharma, Oslo, Norway). The isolated cells were plated in 24-well culture plates (FALCON, Becton-Dickinson Labware, USA), according to the standard condition in RPMI1640 medium containing 10% human AB-type serum in a humidified 5% CO_2 controlled incubator at 37 °C. The medium was changed twice a week, and the attached cells obtained after 7 days were used as human monocyte-derived macrophages. For the assay, macrophages were incubated for 24 h in RPMI1640 medium containing 0.1% BSA with the indicated concentrations of recombinant human APN as previously prepared [11]. Liver X receptor (LXR) agonist [TO-901317 (TO), Sigma-Aldrich, USA] was added to the medium with the concentration of 3 μM to induce ABCA1 and ABCG1 in macrophages. Peritoneal macrophages of APN-KO and wild-type mice were isolated after injection of 4% thioglycollate.

RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction (PCR). Total RNA from macrophages was purified by using RNeasy Mini Kit (Qiagen, USA) followed by treatment with DNase I (Qiagen). One microgram of total RNA was primed with 50 pmol of oligo(dT) 20 and reverse-transcribed with SuperScript III (Invitrogen, USA) for first strand cDNA synthesis, according to the protocol of the manufacturer. Real-time quantitative PCR was performed according to the protocol of DyNamo HS SYBR Green quantitative PCR kit.

Primers used in this study. The primers for human ABCA1 were ABCA1-forward: 5′-GCACTGAGGAAGATGCTGAAA-3′ and ABCA1-reverse: 5′-AGTTCCTGGAAGGTCTTGTTCAC-3′, for human LXRα, LXRα-forward: 5′-CTGTGCCTGACATTCCTCCT-5′ and LXRα-reverse: 5′-CATCCTGGCTTCCTCTCTGA-3′, for human PPARγ, PPARγ-forward: 5′-AACTCTGGGAGATTCTCCTGTTGA-3′ and PPARγ-reverse: 5′-TGGTAATTTCTTGTGAAGTGCTCATA-3′, for human GAPDH, GAPDH-forward: 5′-GAGTCAACGGATTTGGTCGT-3′ and GAPDH-reverse: 5′-TTGATTTTGGAGGGATCTCG-3′, for mouse LXRα, LXRα-forward: 5′-GCTCTGCTCATTGCCATCAG-3′, and LXRα-reverse: 5′-TGTTGCAGCCTCTCTACTTGGA-3′, for mouse PPARγ, PPARγ-forward: 5′-TTCCCCTGGAGATGAGTGTC-3′, and PPARγ-reverese: 5′-CAGTAGGCCACAGGGAACAT-3′, for mouse GAPDH, GAPDH-forward: 5′-ACTCCACTCACGGCAAATTC-3′ and GAPDH-reverse: 5′-TCTCCATGGTGGTGAAGACA-3′

Western blotting. Macrophages were solubilized with 0.5% CHAPS: [3-(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (Pierce Chemical, USA) and 1 mM EDTA in phosphate buffered saline (PBS). Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad, Germany). Incubations of antibodies with the membranes were performed in TBS including 0.1% Tween 20 and 2% skimmed milk at 4 °C overnight. Detection of the immune complexes was carried out by ECL Advance Western Blot Detection System (Amersham Bioscience, UK). Anti-ABCA1 (Novus Biologicals, USA), anti-ABCG1 antibody (Novus Biologicals, USA) and anti-SR-BI antibody were used for the assay as previously described [11].

Cellular cholesterol efflux. Human macrophages were cultured in RPMI1640 medium (serum free) containing [^{3}H]-cholesterol (12 h), and then incubated in RPMI1640 medium containing 0.1% BSA with the indicated concentrations of human-recombinant APN (24 h) and in the presence of acceptor [apoA-I (10 μg/ml) or HDL (50 μg/ml)]. Cellular cholesterol efflux was determined as the percentage of radioactivity of [^{3}H]-cholesterol in the efflux medium to total cell-labeled radioactivity. The radioactivity was determined by liquid scintillation counting. The background for the efflux was in the presence of 0.1% BSA alone. Specific apoA-I or HDL-mediated cholesterol efflux was calculated by subtracting the background from total cellular cholesterol efflux. Mouse peritoneal macrophages were cultured for 3 h in DMEM medium (serum free) containing [^{3}H]-cholesterol, and then incubated in DMEM medium containing 0.1% BSA with the acceptor [apoA-I (10 μg/ml) or HDL (50 μg/ml)].

Statistical analysis. Values were expressed as the means ± SD. Statistical significance was assessed by Student's *t* test for paired values and set at $p < 0.05$.

Results

Effects of APN on ABCA1 expression level in and apoA-I-mediated cholesterol efflux from human macrophages

To investigate the effect of APN on ABCA1 expression level, human monocyte-derived macrophages were incubated for 24 h in RPMI1640 medium containing 0.1% BSA with the indicated concentrations of recombinant APN. The mRNA and the protein levels of ABCA1 were analyzed by real-time quantitative PCR and Western blot. APN increased the mRNA level of ABCA1 in a dose-dependent manner (Fig. 1A) and also enhanced the protein level of ABCA1 (Fig. 1B). To study the effect of APN on apoA-I-mediated cellular cholesterol efflux, the macrophages were incubated for 24 h in RPMI1640 medium containing apoA-I (10 μg/ml). As expected, APN accelerated apoA-I-mediated cholesterol efflux by 5-fold compared with that without treatment (Fig. 1C).

Effects of APN on ABCG1 and SR-BI expression levels in and HDL-mediated cholesterol efflux from human macrophages

The protein levels of ABCG1 and SR-BI were analyzed by Western blot after incubation. The protein level of ABCG1 was slightly, but not significantly, increased by APN (Fig. 2A). Similarly, HDL-mediated cholesterol efflux was not significantly enhanced by APN (Fig. 2B). Regarding SR-BI expression, the protein levels of SR-BI were not significantly influenced by APN (Fig. 2A).

Expression of ABCA1, ABCG1, SR-BI in and apoA-I-mediated or HDL-mediated cholesterol efflux from peritoneal macrophages of APN-KO and wild-type mice

The mRNA and the protein levels of ABCA1, ABCG1, SR-BI were analyzed by real-time quantitative PCR and Western blot. The

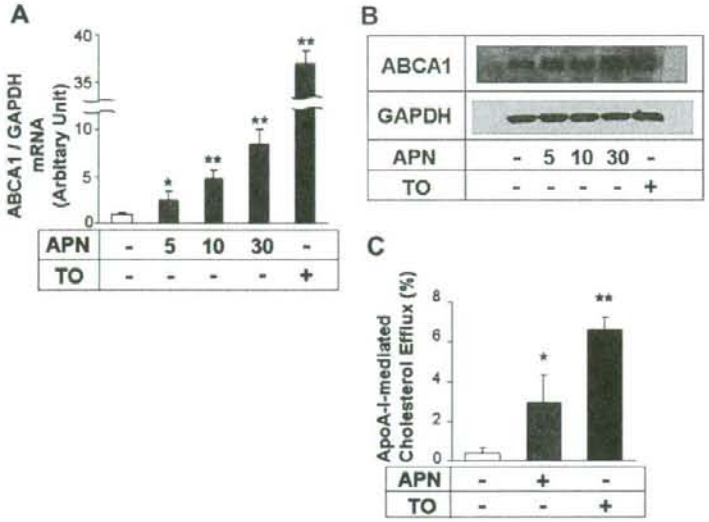

Fig. 1. Effects of APN on the expression of ABCA1 and apoA-I-mediated cholesterol efflux from human macrophages. Human monocyte-derived macrophages were incubated for 24 h in RPMI1640 medium containing 0.1% BSA with the indicated concentrations (0–30 μg/ml) of recombinant human adiponectin or with 3 μM of TO. (A) APN increased the mRNA level of ABCA1. Data are expressed as means ± SD of triplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.01$ vs 0 μg/ml of APN. (B) APN enhanced the protein level of ABCA1 in a dose dependent manner. (C) For cholesterol efflux assay, macrophages labeled with [^{3}H]-cholesterol were incubated in RPMI1640 medium containing 0.1%BSA with APN (10 μg/ml) and apoA-I (10 μg/ml) for 24 h. APN significantly increased apoA-I-mediated cholesterol efflux from cells. Values are means ± SD of triplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.01$ vs 0 μg/ml of APN.

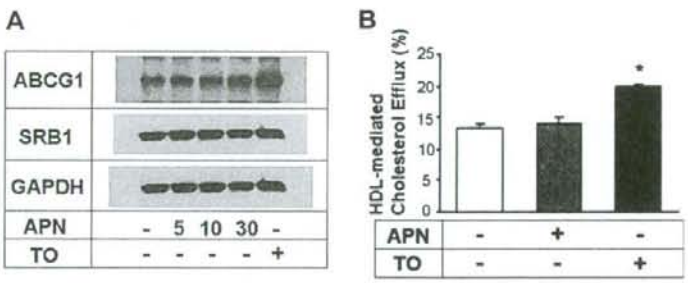

Fig. 2. Effects of APN on the expression of ABCG1 and HDL-mediated cholesterol efflux from human macrophages. Macrophages were incubated for 24 h in RPMI containing 0.1% BSA with the indicated concentrations (0–30 μg/ml) of recombinant human adiponectin or with 3 μM of TO. (A) APN slightly enhanced the protein level of ABCG1, but not SR-BI. (B) Macrophages labeled with [^{3}H]-cholesterol were incubated for 24 h in RPMI1640 medium containing 0.1%BSA with APN (10 μg/ml) and HDL (50 μg/ml). APN did not increase HDL-mediated cholesterol efflux from macrophages. Values are means ± SD of triplicate determinations. $^{*}P < 0.05$ vs 0 μg/ml of APN.

mRNA levels of ABCA1 and ABCG1, but not SR-BI were decreased in macrophages of APN-KO mice macrophages compared with wild-type mice (data not shown). The protein levels of ABCA1 and ABCG1 were also decreased in APN-KO mice compared with wild-type mice (Fig. 3A). To study apoA-I-mediated and HDL-mediated cellular cholesterol efflux, the macrophages were incubated for 24 h in DMEM medium containing apoA-I (10 μg/ml) and HDL (50 μg/ml). As expected, apoA-I-mediated cholesterol efflux was decreased in APN-KO compared with wild-type mice (Fig. 3B). In contrast, HDL-mediated cholesterol efflux was slightly but not significantly reduced in APN-KO mice (Fig. 3C).

Expression of LXRα and PPARγ

To investigate the mechanism of APN on cellular cholesterol efflux, we examined the mRNA levels of LXRα and PPARγ, analyzed by real-time quantitative PCR. In human monocyte-derived macrophages, APN significantly increased the mRNA levels of LXRα and PPARγ (Fig. 4A,B). As expected, in APN-KO mice the expressions of LXRα and PPARγ were significantly decreased compared with those of wild-type mice (Fig. 4C,D).

Discussion

In the present study, we for the first time demonstrated that APN induced ABCA1 expression and enhanced apoA-I-mediated cellular cholesterol efflux in human monocyte-derived macrophages. APN might accelerate RCT system by increasing cellular cholesterol efflux from macrophages through ABCA1 pathway.

It is well known that both ABCA1 and ABCG1 are target genes of a nuclear receptor, LXR. However, unexpectedly, the protein level of ABCG1 was not significantly changed by APN. Furthermore, HDL-mediated cholesterol efflux was not significantly influenced by APN as well. Recently, Ranalletta et al. reported

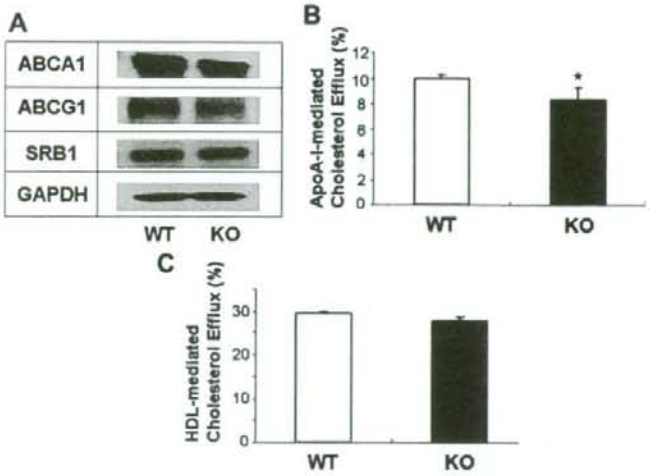

Fig. 3. Expression of ABCA1, ABCG1, SR-BI, and apoA-I- or HDL-mediated cholesterol efflux in peritoneal macrophages of APN-KO (KO) and wild-type (WT) mice. Peritoneal macrophages of APN-KO (KO) and wild-type (WT) mice were isolated after injection of 4% thioglycollate. (A) The protein levels of ABCA1 and ABCG1 were also decreased in APN-KO mice. (B,C) Macrophages labeled with [^{3}H]-cholesterol were incubated for 24 h in DMEM medium containing 0.1%BSA with apoA-I (10 μg/ml) and HDL (50 μg/ml). (B) ApoA-I-mediated cholesterol efflux was decreased in APN-KO compared with wild-type mice. Data are expressed as means ± SD of triplicate determinations. $^*P < 0.05$ vs wild-type mice. (C) HDL-mediated cholesterol efflux was slightly but not significantly reduced in APN-KO mice.

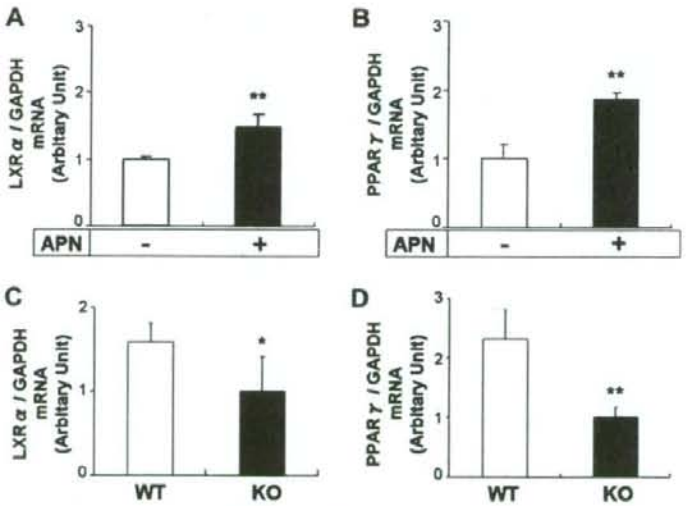

Fig. 4. Expression of LXRα and PPARγ mRNA in monocyte-derived macrophages (A,B) Human monocyte-derived macrophages were incubated for 24 h in RPMI1640 medium containing 0.1% BSA with recombinant human adiponectin (10 μg/ml). Data are expressed as means ± SD of triplicate determinations. $^*P < 0.05$, $^{**}P < 0.01$ vs 0 μg/ml of APN. (A) APN increased the mRNA levels of LXRα. (B) APN enhanced the mRNA levels of PPARγ. (C,D) Peritoneal macrophages of APNKO and wild-type mice were isolated after injection of 4% thioglycollate. Values are expressed as means ± SD of triplicate determinations. $^*P < 0.05$, $^{**}P < 0.01$ vs wild-type mice. (C) The mRNA level of LXRα was reduced in APN-KO compared with wild-type mice. (D) The mRNA level of PPARγ was also decreased in APN-KO mice compared with wild-type mice.

that the protein level of ABCA1 was definitely increased in ABCG1-deficient mouse macrophages [13]. Furthermore, Charvet et al. also showed that apoA-I-mediated cholesterol efflux was significantly increased in the peritoneal macrophages from ABCG1 knockout mice compared with wild-type mice and that HDL-mediated cholesterol efflux was enhanced in the macrophages from ABCA1-knockout mice [14]. These data suggested that the deficiency of ABCG1 in macrophages might lead to the compensatory induction of ABCA1. There might be an inverse relationship between ABCA1 and ABCG1 expressions in macrophages. Therefore, in this study, although the protein level of ABCA1 in human macrophages was up-regulated by APN, the level protein of ABCG1 might not be increased by the mechanism of compensatory reduction. These issues need to be investigated in future studies. Otherwise, Ling Tian et al. have recently reported that HDL-mediated cholesterol efflux was significantly induced by APN [15]. In their report, the adiponectin-conditioned medium, in which adiponectin gene transfected THP-1 cells were

incubated, was used to study the HDL-mediated cholesterol efflux from THP-1 foam cells. Therefore, the discrepancy in the results between ours and their study might be due to the difference of APN concentration in the medium or cell lines used for the assay.

In our previous report, it has been shown that APN did *not* enhance the expression level of SR-BI in HepG2 cells [11]. In the current study, APN did *not* increase the expression of SR-BI nor accelerate HDL-mediated cholesterol efflux from human macrophages. Although SR-BI is known to be one of the very important molecules to promote cellular cholesterol efflux, APN might not enhance the SR-BI-mediated cholesterol efflux.

Recently, we have reported that APN deficiency might impair the HDL synthesis in the liver [11,12]. In APN-KO mice, the synthesis of apoA-I in the liver was reduced compared with wild-type mice. Furthermore, the ABCA1 expression in the liver was also decreased in APN-KO mice. *In vivo*, HDL is mainly synthesized in the liver. However, partially, the cellular cholesterol efflux from foam cells is also associated with HDL generation. In this study, the ABCA1 expression in macrophages and apoA-I cholesterol efflux were also decreased in APN-KO mice.

We demonstrated that APN influenced the expression of ABCA1 and ABCG1 in macrophages. The expression of these transporters was reported to be regulated by LXR. In the present study, the mRNA level of LXRα was significantly enhanced by APN. APN also increased the expression of PPARγ. Furthermore, in APN-KO mice, the expression of LXRα and PPARγ mRNA was significantly reduced compaired with that in wild-type mice. Therefore, APN might enhance the expression of ABCA1 and ABCG1 in macrophages by the activation of LXRα and PPARγ.

Taken together, the current study has demonstrated that APN might have an ability to accelerate RCT and protect against atherosclerosis by increasing apoA-I-mediated cholesterol efflux through enhancing ABCA1 pathway in macrophages, as well as enhancing the HDL assembly in the liver.

Acknowledgments

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Identification of Neutral Cholesterol Ester Hydrolase, a Key Enzyme Removing Cholesterol from Macrophages*[S]

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Hiroaki Okazaki[‡1], Masaki Igarashi[‡1], Makiko Nishi[‡], Motohiro Sekiya[§], Makiko Tajima[§], Satoru Takase[‡], Mikio Takanashi[‡], Keisuke Ohta[‡], Yoshiaki Tamura[‡], Sachiko Okazaki[‡], Naoya Yahagi[‡], Ken Ohashi[‡], Michiyo Amemiya-Kudo[‡¶], Yoshimi Nakagawa[‖], Ryozo Nagai[‡], Takashi Kadowaki[‡], Jun-ichi Osuga[‡], and Shun Ishibashi[‡2]**

*From the [‡]Departments of Metabolic Diseases and [§]Cardiovascular Diseases, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, [¶]Okinaka Memorial Institute for Medical Research and Toranomon Hospital, Tokyo 105-0001, [‖]Metabolism, Endocrinology, and Atherosclerosis, Institute of Clinical Medicine, University of Tsukuba, Ibaraki 305-8575, and **Division of Endocrinology and Metabolism, Department of Medicine, School of Medicine, Jichi Medical University, Tochigi 329-0498, Japan*

Unstable lipid-rich plaques in atherosclerosis are characterized by the accumulation of macrophage foam cells loaded with cholesterol ester (CE). Although hormone-sensitive lipase and cholesteryl ester hydrolase (CEH) have been proposed to mediate the hydrolysis of CE in macrophages, circumstantial evidence suggests the presence of other enzymes with neutral cholesterol ester hydrolase (nCEH) activity. Here we show that the murine orthologue of KIAA1363, designated as neutral cholesterol ester hydrolase (NCEH), is a microsomal nCEH with high expression in murine and human macrophages. The effect of various concentrations of NaCl on its nCEH activity resembles that on endogenous nCEH activity of macrophages. RNA silencing of NCEH decreases nCEH activity at least by 50%; conversely, its overexpression inhibits the CE formation in macrophages. Immunohistochemistry reveals that NCEH is expressed in macrophage foam cells in atherosclerotic lesions. These data indicate that NCEH is responsible for a major part of nCEH activity in macrophages and may be a potential therapeutic target for the prevention of atherosclerosis.

Atherosclerotic cardiovascular diseases are the leading causes of mortality in industrialized countries, despite advances in the management of coronary risk factors. Heart attacks arise from thrombotic occlusion of coronary arteries following the rupture of plaques. Lipid-rich plaques, which are characterized by a plethora of CE[3]-laden macrophage foam cells, are prone to rupture (1). Thus, it is important to clarify the mechanism that eliminates CE from macrophage-derived foam cells.

Foam cells are generated by the unlimited uptake of modified lipoproteins through scavenger receptors (2). Cholesterol in the lipoproteins is stored in lipid droplets as CE after re-esterification by acyl-CoA:cholesterol acyltransferase 1 (ACAT1) (3). Hydrolysis of CE is the initial step toward elimination of cholesterol from foam cells (4). Free cholesterol thus generated is re-esterified or is released from the cells primarily through ATP-binding cassette transporters (5). Thus, the balance between synthesis and hydrolysis of CE conceivably governs the level of CE in macrophages.

Hydrolysis of CE in macrophages has been known for over 40 years (6). However, its molecular mechanism has yet to be fully understood. Circumstantial evidence suggests that the hydrolysis of CE in foam cell macrophages is mediated by hormone-sensitive lipase (HSL), a multifunctional enzyme that catalyzes the hydrolysis of triacylglyerol (TG), diacylglycerol, CE, and retinyl ester in various organs such as adipose tissue, muscle, and testis (7, 8). This belief is supported by the following facts. First, it has been demonstrated that various lines of macrophages express HSL (9–12). Second, HSL expression is regulated coordinately with nCEH activity in murine macrophages (13). Third, we (14) and others (15) demonstrated that overexpression of HSL is associated with increased hydrolysis of CE stores in THP-1 and RAW264.7 macrophages.

However, recent studies by us (16) and others (17) have challenged this notion by finding that peritoneal macrophages isolated from HSL-deficient mice retained almost a normal level of nCEH activity, suggesting that enzyme(s) other than HSL are responsible for nCEH activity in macrophages. Moreover, HSL is expressed at a low level (11, 12) or is undetectable (17) in human macrophages. Recently, Ghosh *et al.* (18) reported cloning of cholesteryl ester hydrolase (CEH) which mediates the hydrolysis of CE in human monocyte-derived macrophages. They have further shown that macrophage-specific transgenic expression of CEH significantly reduced atherosclerosis in low

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Division of Endocrinology and Metabolism, Dept. of Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan. Tel.: 81-285-58-7355; Fax: 81-285-40-6035; E-mail: ishibash@jichi.ac.jp.

[3] The abbreviations used are: CE, cholesterol ester; HSL, hormone-sensitive lipase; CEH, cholesteryl ester hydrolase; nCEH, neutral cholesteryl ester hydrolase; Ad, adenovirus; BSA, bovine serum albumin; LDL, low density lipoprotein; acLDL, acetylated LDL; DMEM, Dulbecco's modified Eagle's medium; MPM, murine peritoneal macrophage; shRNA, short hairpin RNA; PBS, phosphate-buffered saline; TGH, triacylglycerol hydrolase; GST, glutathione *S*-transferase; m.o.i., multiplicity of infection; TG, triacylglycerol.

density lipoprotein receptor knock-out mice (19). However, the CEH gene is identical to the human orthologue of TGH, which was originally reported as a microsomal TG lipase in rat liver by Lehner *et al.* (20). In our hands, both the mouse orthologue of TGH, designated as TGH-1, and its paralogue with 70.4% identity to TGH-1, designated as TGH-2, have negligible nCEH activity (21). These considerations have prompted us to employ a bioinformatic approach to identify the enzyme responsible for nCEH activity in macrophages.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate, triolein, lecithin, bovine serum albumin fraction V (BSA), *p*-nitrophenyl butyrate, and leupeptin were purchased from Sigma. Dexamethasone, 3-isobutyl-2-methylxanthine, fatty acid-free BSA, cholesterol esterase from *Pseudomonas* sp., cholesterol oxidase, horseradish peroxidase, *p*-hydroxyphenylacetic acid, and sodium taurocholate were purchased from Wako Pure Chemicals (Osaka, Japan). Pioglitazone was provided by Takeda Pharmaceutical (Osaka, Japan). Tri[^{3}H]oleoylglycerol, cholesterol [1-^{14}C]oleate, and [1-^{14}C]oleic acid were purchased from GE Healthcare. LDL was isolated by ultracentrifugation and acetylated to prepare acetylated LDL (acLDL) as described previously (14).

Cells—HEK293 or RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose containing 10% (v/v) fetal bovine serum without or with 1 mM sodium pyruvate, respectively. THP-1 cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and differentiated to THP-1 macrophages by treatment with 100 nM phorbol 12-myristate 13-acetate for 48 h. Murine peritoneal macrophages (MPM) were harvested as described previously (16). In brief, 1 ml of 5% thioglycolate broth was injected into the peritoneal cavities of mice. After 4 days, the peritoneal cavities were lavaged with 16 ml of ice-cold saline. The cells were washed three times with PBS and resuspended in DMEM to give a concentration of 10^6 cells per ml, and 10 ml per dish was plated in 10-cm dishes. After incubation at 37 °C for 2 h, the nonadherent cells were removed by washing three times with warmed PBS. Cells were incubated in DMEM containing 10% fetal calf serum for 24 h and harvested for the experiments. 3T3-L1 cells were cultured in medium A (DMEM containing 10% calf serum, supplemented with calcium pantothenate and biotin). To induce differentiation of 3T3-L1 cells into adipocytes, 2 day postconfluent preadipocytes (day 0) were incubated with 1 μM dexamethasone and 0.5 mM isobutylmethylxanthine, 5 μg/ml insulin, 1 μM pioglitazone for 48 h, following treatment with 5 μg/ml insulin and 1 μM pioglitazone for 48 h. After the incubation period, cells were switched to medium A, and the medium was renewed every other day. Mononuclear cells were isolated from a normal volunteer using Lymphoprep (NYCOMED, Roskilde, Denmark), and cells that attached to plastic dishes were cultured in RPMI 1640 medium containing 10% fetal calf serum and used as human monocyte-derived macrophages.

Mice—C57BL/6J and apoE knock-out mice were purchased from Clea (Tokyo, Japan) and The Jackson Laboratories (Bar Harbor, ME), respectively. Mice were maintained and cared for according to the regulations of the Animal Care Committee of the University of Tokyo. For the preparation of atherosclerotic aortas, mice were caged separately with 12-h light/dark cycles and given free access to standard chow diet containing 0.075% cholesterol (MF; Oriental Yeast Co., Ltd.; Osaka, Japan).

Data Base Search and Amino Acid Sequence Analysis—The search for a novel lipase containing lipase consensus motifs and α/β-hydrolase folds was performed in the GENES protein data base of Kyoto Encyclopedia of Genes and Genomes by using the MOTIF search program (22), followed by prediction of secondary structure by PSIPRED and the PREDICT PROTEIN PHDsec program (23). The search for orthologues or paralogues of NCEH was performed by Sequence Similarity Database in the Kyoto Encyclopedia of Genes and Genomes Database (22). Alignment of the deduced protein sequence with other lipases, calculation of the percent identity, and generation of a phylogenetic tree were performed using the ClustalW program. Prediction of the transmembrane domain was performed using SOSUI and the PREDICT PROTEIN PHDhtm program (23).

cDNA Cloning for the Expression of Recombinant Proteins—Total RNA isolated from murine peritoneal macrophages was reverse-transcribed into cDNA using reverse transcriptase following the manufacturer's protocol (Thermoscript RNaseH-reverse transcriptase (Invitrogen)). The coding sequences of murine NCEH were amplified by PCR from macrophage cDNA using cDNA polymerase (AmpliTaq DNA polymerase (Roche Applied Science)). The primers used for PCR were as follows: murine NCEH forward 5′-CCGCCTGGGGACAATGAG-3′ and murine NCEH reverse 5′-TCTGAGGAGTTTCCTCCG-TCA-3′.

The construction of cDNA for HSL was described previously. The products containing the complete open reading frame were ligated into compatible sites of pGEM T easy vector (Promega).

Recombinant Adenovirus for NCEH and HSL Expression (Ad-NCEH, Ad-HSL)—Murine NCEH or HSL cDNA fragment was amplified by PCR from mouse cDNA containing plasmids (described above) using forward and reverse primers. LacZ cDNA was amplified by PCR from SV40 β-galactosidase vector (Promega). The PCR products were ligated to pENTER4 vector (Invitrogen). Subsequently, murine NCEH or HSL cDNA fragment was subcloned into pAd/CMV/V5-DEST vector by Gateway Technology (Invitrogen). The adenoviral vectors were linearized using restriction enzyme and transfected into HEK293 using Superfect reagent (Promega). Large scale production of high titer recombinant Ad-NCEH, Ad-HSL, or Ad-LacZ was performed as described elsewhere (14). The purified viruses were stored in 10% (v/v) glycerol/phosphate-buffered saline (PBS) at −80 °C.

Recombinant Adenovirus Expressing Short Hairpin (sh) RNA Directed against Murine NCEH—Recombinant adenovirus expressing shRNA for NCEH (Ad-shNCEH) was produced by the Gateway Technology (Invitrogen). The sense and antisense templates of NCEH shRNA were produced from nucleotides 136 to 154 of the NCEH coding sequence.

The following oligonucleotides were used: shRNA for NCEH sense template, 5′-GT GAG TAA TCT GAT ACG TTA CGT GTG CTG TCC GTA ATG TAT CAG GTT ACT CAC-3′; shRNA for NCEH antisense template, 5′-GT GAG TAA CCT

GAT ACA TTA CGG ACA GCA CAC GTA ACG TAT CAG ATT ACT CAC-3'. An adenovirus containing shRNA for β-galactosidase (Ad-shLacZ) was used as a control.

Murine peritoneal macrophages were seeded in 6-well plates at a cell density of 1.7×10^6 cells/well. After transduction with Ad-shNCEH or Ad-shLacZ for 48 h at 37 °C, cells were harvested, and whole cell lysates were assayed for enzymatic activities.

Generation of Rabbit Polyclonal Antibodies against Murine NCEH—To prepare polyclonal anti-murine NCEH antiserum, amino acid residues containing the catalytic domain of murine NCEH (amino acids 99–250) were expressed in bacteria as a glutathione *S*-transferase (GST) fusion protein, which was purified by glutathione affinity chromatography, and used for immunization of rabbits according to a standard protocol as described previously (24). Serum of rabbits before immunization was used as a control. From serum samples with high antibody titers, IgG fractions were isolated using a protein G column (GE Healthcare).

Northern Blot Analyses—Total RNA was isolated from various tissues or cultured cells using TRIzol reagent according to the manufacturer's protocol (Invitrogen). RNA (10 μg) was subjected to formaldehyde/agarose gel electrophoresis and blotted to a Hybond-N membrane (GE Healthcare). After cross-linking, mouse NCEH, HSL, and TGH-1 mRNA were detected using ^{32}P-labeled probes. Probes for murine NCEH were originally prepared by reverse transcription-PCR from mouse peritoneal macrophage mRNA. Probes for HSL (exon 8 probe), TGH-1 were constructed from cDNA fragments amplified by reverse transcription-PCR using cDNA obtained from mouse adipose tissue as a template (14). After hybridization and washing, signals were visualized by exposure to a PhosphorImager Screen (FUJIFILM) and analyzed using BASTATION (FUJIFILM) software.

Western Blot Analysis—Cells were sonicated in buffer A and centrifuged at 40,000 or 100,000 × *g* for 45 min at 4 °C. The supernatant was used as S-40 or S-100 cytosolic fraction, and the precipitates were resuspended and used as the microsomal fraction (14). Ten micrograms of proteins of various cellular fractions were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. For detection of the proteins, the membranes were incubated with either anti-murine NCEH, anti-murine HSL antiserum, monoclonal anti-mouse ACAT1 antibody (25), or anti-F4/80 antibody (AbD serotec) at a dilution of 1:100–1,000. Specifically bound immunoglobulins were detected in a second reaction with a horseradish peroxidase-labeled IgG conjugate and visualized by enhanced chemiluminescence detection (ECL Plus, GE Healthcare) using a Kodak image system, and the intensity of immunoreactive bands were quantified by NIH-image software.

Assays for CE Hydrolase and TG Lipase Activities—Whole cell lysates were prepared from transfected HEK293, murine peritoneal macrophages, or THP1 cells and used for the enzyme activity assays. These cells were sonicated in buffer A (50 mmol/liter Tris-HCl, pH 7.0, 250 mmol/liter sucrose, 1 mmol/liter EDTA, 2 μg/ml leupeptin) and centrifuged at 100,000 × *g* for 45 min at 4 °C. The supernatant was used as S-100 cytosolic fraction, respectively. The precipitates at 100,000 × *g* were resuspended and used as the microsomal fraction (14). nCEH activity was measured essentially as described by Hajjar *et al.* (26), using a reaction mixture containing 6.14 μM cholesterol [1-^{14}C]oleate (48.8 μCi/μmol; 1 μCi = 37 kBq). Triacylglycerol lipase activity was measured according to a modification of the method of Hajjar *et al.* (26). In brief, the samples were incubated at 37 °C for 30 min in a final volume of 200 μl of a reaction mixture containing 105 μM tri[^{3}H]oleoylglycerol (99.4 μCi/μmol), 23.7 μM lecithin, 12.5 μM sodium taurocholate, 1 M NaCl, and 85 mM potassium phosphate, pH 7.0. The high concentration of NaCl was included to inactivate lipoprotein lipase.

In Vitro Adenovirus Experiments—Two days after THP-1 cells were treated with phorbol 12-myristate 13-acetate, THP-1 macrophages were incubated in RPMI 1640 medium containing 5 mg/ml BSA and transduced with recombinant adenovirus carrying NCEH, HSL, or LacZ as a control. After 24 h, acLDL was added to the medium at a final concentration of 100 μg/ml without or with 1% [^{14}C]oleate-BSA complex (14), and cells were incubated for 48 h at 37 °C. Cellular lipids were extracted using hexane/isopropyl alcohol (3:2), and cholesterol content was determined by enzymatic fluorometric microassay according to the method of Heider and Boyett (27), with minor modifications (25). The radioactivity of intracellular CE formed from [^{14}C]oleate was determined as described (14).

Immunohistochemical Localization—Cells were fixed with 3% paraformaldehyde after washing with PBS. Tissue samples were fixed in 10% formalin, pH 7.0, embedded in paraffin, cut into sections, and placed on glass slides. Tissue samples were deparaffinized and rehydrated before use. The samples were incubated in 3% H_2O_2 in PBS to quench endogenous peroxidase activity and washed with PBS, and nonspecific binding sites were blocked by incubation with 1% goat serum. The sections were then incubated with affinity-purified anti-NCEH polyclonal antibody. After washing with PBS, the sections were incubated with a biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA). The antibody-specific staining was visualized with a Vecstatin ABC reagent (Vector Laboratories) and diamine benzidine, resulting in a brown precipitate. Cell nuclei were counterstained with methyl green. Combined immunohistochemical staining was performed to identify the cell types expressing NCEH as described previously with minor modifications (28). NCEH was first localized by using the protocol above. Subsequently, the sections were treated with 0.1% pepsin (Roche Applied Science) in 0.1 N HCl for 30 min and incubated with rat monoclonal anti-murine F4/80 (1:10, serotec) for 30 min. The respective sections were incubated with a biotinylated rabbit anti-rat antibody (1:200, Vector Laboratories) for 30 min, followed by a 30-min incubation with an alkaline phosphatase-coupled ABC reagent (1:200, Vector Laboratories). Alkaline phosphatase activity was visualized by using the Alkaline Phosphatase Kit III (Vector Laboratories), resulting in a blue precipitate.

Statistical Analyses—Results are presented as means ± S.E. Student's *t* test was employed to compare the means. All calculations were performed with STAT view version 5.0 for Macintosh (SAS Institute Inc.).

RESULTS AND DISCUSSION

To search for enzymes with CE hydrolase activity, we screened a gene data base for murine and human proteins with homology to structures in known lipases, *i.e.* α/β-hydrolase folds (29), the GXSXG active serine motif for serine esterases, and the HG dipeptide motif that is present 70–100 amino acids N-terminal of the catalytic site serine in many lipases (29), yielding 53 candidates that included HSL, lipoprotein lipase, hepatic lipase, endothelial lipase, pancreatic lipase, carboxyl ester lipase, gastric lipase, lysosomal acid lipase, monoacylglycerol lipase, TGH-1, and TGH-2 (21). Thirty three genes had not been previously annotated. We expressed the candidate genes in HEK293 cells for measurement of nCEH activity. Northern blot analysis was used to verify the expression in murine peritoneal macrophages. Besides HSL, only one fulfilled these requirements.

The murine gene for NCEH (NCBI nucleotide entry AK045363) encodes a 408-amino acid protein (GenBank™ accession number NP_848887) with a calculated molecular mass of 45.7 kDa. KIAA1363, which is a human orthologue of murine brain chlorpyrifos oxon-binding protein (30) that was recently identified during our study, is the human orthologue of NCEH and encodes a 440-amino acid protein (GenBank™ accession number NP_065843) with 87.5% identity to the murine NCEH in the C-terminal 408 amino acids. The amino acid sequences of murine NCEH and its closely related proteins, murine, rat, and human HSL, are shown in Fig. 1. The structures of the predicted α-helix and β-sheet are well conserved between NCEH and HSL. NCEH contains domains that are present in HSL such as the catalytic core domain and lipid-binding domain (31, 32), and is thus apparently homologous to HSL in terms of domain structure. Based on the homology with HSL, it can be predicted that Ser-191, Asp-348, and His-378 of the murine NCEH protein form a putative catalytic triad, and His-113 and Gly-114 form an HG oxyanion motif. Despite the remarkably high structural homology between NCEH and HSL, NCEH possesses only 22.1% sequence identity to HSL. In addition to the absence of the regulatory domain of HSL, NCEH lacks the N-terminal region of HSL. The N-terminal region of NCEH instead contains a stretch of 23 hydrophobic amino acids, a putative transmembrane domain, which is absent in HSL, suggesting distinct subcellular localization of these two proteins.

Analysis using the ClustalW program supported the phylogenetic kinship between NCEH and HSL (supplemental Fig. S1). Neutral lipid hydrolases are clustered essentially into three large groups as follows: 1) an extracellular lipase superfamily comprising lipoprotein lipase, endothelial lipase, hepatic lipase, and pancreatic lipase; 2) a carboxylesterase family comprising carboxyl ester lipase, TGH-1, and TGH-2; and 3) a distinct "intracellular lipase" gene family comprising HSL and NCEH. Lysosomal acid lipase and adipose triglyceride lipase do not belong to any of these families. Arylacetamide deacetylase shows weak similarity (44.0%) to murine NCEH, and there is no other mouse protein with much sequence similarity to murine NCEH.

Northern blot analysis revealed that NCEH mRNA was expressed at high levels in peritoneal macrophages and kidney,

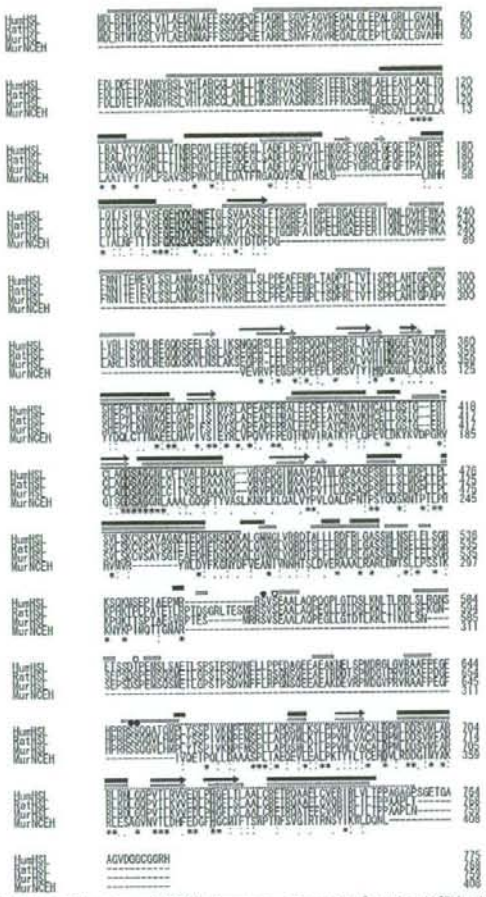

FIGURE 1. **Structure of NCEH.** Sequence alignment of murine NCEH with human, rat, and mouse HSL. The deduced amino acid sequence of murine NCEH protein (*NCEH*) is aligned and compared with murine, rat, and human HSL (*murHSL*, *ratHSL*, and *humHSL*). The first Met residue is numbered as 1. HSL has four functional domains as follows: 1) the N-terminal 300 residues domain, containing the ALBP-binding site (*boldface*); 2) catalytic core domain (*line*), containing the HG dipeptide oxyanion motif (*shaded box*) and GXSXG active serine motif (*shaded box*); 3) regulatory module domain, containing five serine residues, Ser-557, Ser-559, Ser-591, Ser-650, and Ser-65, which can be phosphorylated by cAMP-dependent protein kinase (*solid circle*) or other kinases such as AMP-activated protein kinase or extracellular signal-regulated kinase (ERK) (*open circle*); 4) lipid-binding domain (*dashed line*), containing a putative lipid-binding site and two of the catalytic triad, *i.e.* Asp-694 and His-724 (*shaded box*). The predicted secondary structure was shown as follows: α-helix is indicated by a *line*; β-sheet is indicated by an *arrow*; secondary structure of HSL and NCEH is shown in *gray* and *black*, respectively. The mature NCEH protein is highly conserved during evolution and has homology to HSL in these motifs and secondary structures, except for the regulatory domain and the N-terminal 300 residue domain. NCEH has an N-terminal transmembrane region (*outlined letters*). Identical amino acids are shown by *asterisks*. Amino acids considered as strongly conserved are as follows: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, and FYW, and are indicated by *colons*. Amino acids considered as weakly conserved are as follows: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, and HFY, and are indicated by *dots*.

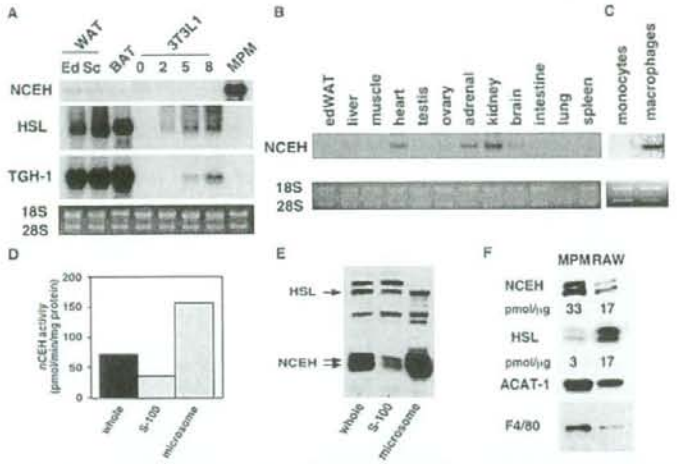

FIGURE 2. **Tissue and subcellular distribution of NCEH expression.** Total RNA (10 μg) from adipose tissues and 3T3-L1 adipocytes at various stages of differentiation and murine peritoneal macrophages (*A*), various murine tissues (*B*), and human monocytes/monocyte-derived macrophages (*C*) were subjected to Northern blot analysis. Specific mRNAs were detected with radiolabeled murine cDNAs for NCEH, HSL, and TGH-1. Ethidium bromide staining of the gels is shown. Abbreviations used are as follows: *WAT*, white adipose tissue; *BAT*, brown adipose tissue; *MPM*, murine peritoneal macrophages; *ed*, epididymal; *sc*, subcutaneous. Subcellular distribution of nCEH activity and NCEH and HSL proteins. MPM were sonicated and centrifuged at 100,000 × *g* and the supernatant (S-100) or microsomal (*Ms*) fractions along with whole cell lysate (*whole*) were subjected to the measurements of nCEH activity (*D*) and Western blot analysis (*E*) using anti-HSL and anti-NCEH antisera. *F*, quantification of NCEH or HSL proteins in macrophages. Ten micrograms of proteins of whole cell lysates from RAW264.7 and MPM were separated by SDS-PAGE on the same gel as the indicated amounts of GST fusion proteins (supplemental Fig. S2). Immunoblotting was performed, and the densities of bands from HSL or NCEH of RAW264.7 or MPM were quantified using NIH Image. Blots for ACAT1 and F4/80 are shown as controls. The moles of HSL or NCEH in 10 μg of protein from RAW264.7 or MPM were calculated from equations relating moles and densities of GST fusion proteins.

and to a lesser degree in heart and adrenal tissue (Fig. 2, *A* and *B*). Compared with other enzymes with nCEH activity expressed in macrophages, such as HSL and TGH-1, a murine orthologue of CEH reported by Ghosh (18), the expression of NCEH was the most abundant and specific for macrophages (Fig. 2*A*). Although NCEH expression was barely detectable in freshly isolated human monocytes, it was robustly induced during the differentiation to mature macrophages (Fig. 2*C*).

To determine the subcellular localization of endogenous nCEH activity and NCEH protein, we isolated S-100 and microsomal fractions from murine peritoneal macrophages, measured the nCEH activity (Fig. 2*D*), and performed Western blot analysis for either NCEH or HSL (Fig. 2*E*). The nCEH activity was distributed preferentially in the microsomal fraction. NCEH proteins, duplets with molecular mass of 45 and 50 kDa, were also distributed preferentially in the microsomal fraction. HSL was more equally distributed between the two fractions. Thus, the subcellular distribution pattern of nCEH activity is closer to that of NCEH protein than that of the HSL protein.

We compared the amounts of NCEH protein with those of HSL protein in murine peritoneal macrophages and RAW264.7 cells using recombinant proteins as standards for calculation (Fig. 2*F* and see supplemental Fig. S2). Here again, two bands were reacted with anti-NCEH antibody. Subsequent study showed that both bands are products of glycation of the 40-kDa protein.[4] The ratio of NCEH protein to HSL protein was calculated to be 11 in murine peritoneal macrophages and 1 in RAW264.7 cells, respectively.

The preferential localization of HSL and NCEH in the cytosol and microsomal fractions, respectively, was also confirmed by measurement of the enzymatic activity of each fraction in cells transiently transfected with plasmids expressing HSL or NCEH. Although most of the nCEH activity of HSL-transfected cells was recovered in the S-40 fraction, nCEH activity was barely detectable in the S-40 fraction of NCEH-transfected cells as well as murine peritoneal macrophages (data not shown). This subcellular localization pattern is compatible with the microsomal distribution of NCEH and its preponderance in murine peritoneal macrophages. These results are apparently contrary to those reported by Khoo *et al.* (33), who showed that 60% of the CE hydrolase activity in the homogenate of J774 cells was recovered in the S-40 fraction; this difference could be attributed to the high level of expression of HSL in J774 cells (34) and the high level of expression of NCEH in murine peritoneal macrophages.

To further determine enzymological characteristics of NCEH, we overexpressed NCEH in HEK293 cells (Fig. 3*A*) and measured enzymatic activities of their whole cell lysate at various concentrations of NaCl (Fig. 3*B*), pH (Fig. 3*D*), and concentrations of cholesterol oleate (Fig. 3*E*) or triolein (Fig. 3*F*) as substrates.

The nCEH activity in the whole cell lysates from NCEH-transfected cells was slightly stimulated by low concentrations of sodium chloride (6.5% at 0.05 M), and inhibited only by 31% at higher concentrations (1 M) (Fig. 3*B*). This pattern closely resembled that of murine peritoneal macrophages (Fig. 3*C*). The CE hydrolase activity of NCEH-transfected cells was optimal at neutral pH (7.2) (Fig. 3*D*), which is close to the optimal pH for HSL, suggesting that both enzymes are not active or at least not fully functional in lysosomes.

The whole cell lysate of HSL-transfected cells exhibited increased hydrolase activities for both TG and CE, with apparent K_m of 11.7 and 22.8 μM, respectively. On the other hand, whole cell lysate of NCEH-transfected cells hydrolyzed TG and CE with apparent K_m of 30.3 and 324 μM, respectively.

[4] M. Igarashi, manuscript in preparation.

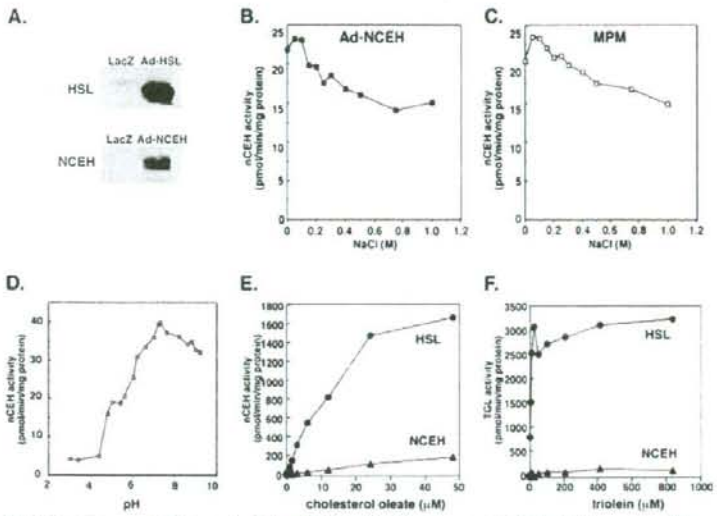

FIGURE 3. **Enzymological characteristics of NCEH.** HEK293 cells were infected with Ad-LacZ, Ad-HSL, or Ad-NCEH and used for the experiments. *A*, expression of NCEH or HSL was confirmed by Western blot analysis. Effects of various concentrations of NaCl on the nCEH activity of whole cell lysate from cells infected with Ad-NCEH (*B*) or of MPM (*C*). *D*, effects of various pH on the nCEH activity of whole cell lysate from cells infected with Ad-NCEH using 50 mM acetate buffer (pH ≦ 5), 50 mM phosphate buffer (5 < pH < 7.6), or 50 mM Tris-HCl buffer (pH ≧ 7.6). Shown are the effects of various concentrations of cholesterol oleate (*E*) or triolein (*F*) on the nCEH or triglyceride lipase activities, respectively, of whole cell lysate from cells infected with Ad-NCEH or Ad-HSL. Values for Ad-LacZ were subtracted from those for Ad-NCEH or Ad-HSL and plotted against the concentrations of substrates. K_m values were calculated by fitting lines in Lineweaver-Burk plots.

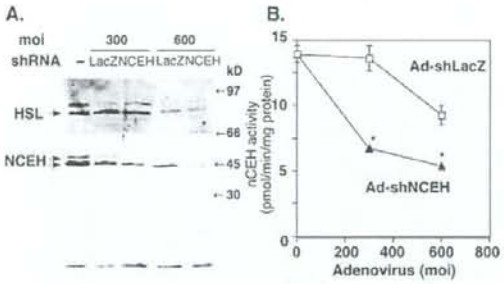

FIGURE 4. **Effects of RNA interference of NCEH to inhibit protein expression and nCEH activity in murine peritoneal macrophages.** Recombinant adenoviruses coding for shRNA against LacZ (*Ad-shLacZ*) or NCEH (*Ad-shNCEH*) were used to infect murine peritoneal macrophages at 300 and 600 m.o.i. Two days after infection, whole cell lysates were subjected to Western blotting (*A*) and the measurement of nCEH activity (*B*). This is a representative result of two independent experiments.

To determine more directly how much of the nCEH activity is accounted for by NCEH in murine peritoneal macrophages, we used RNA silencing technology (Fig. 4). Infection with increasing doses of Ad-shLacZ nonspecifically reduced the protein expression of both HSL and NCEH (Fig. 4*A*) as well as nCEH activities in the infected cells (Fig. 4*B*). Whereas infection with 300 and 600 m.o.i. of Ad-shNCEH did not specifically reduce the protein expression of HSL, it reduced the protein expression of NCEH by 51 and 41%, respectively, compared with Ad-shLacZ. In parallel with the inhibition of NCEH, nCEH activities were reduced by 49 and 42% by infection with 300 and 600 m.o.i. of Ad-shNCEH, respectively (Fig. 4*B*). These results indicate that at least half of the nCEH activity is mediated by NCEH in murine peritoneal macrophages.

To further examine whether NCEH inhibits accumulation of CE in macrophages, we infected THP-1 cells, which had been induced to differentiate to mature macrophages by incubation with phorbol ester, with Ad-NCEH, Ad-HSL, or Ad-LacZ, and incubated the cells with acetyl-LDL, and compared the amounts of CE in the cells (Fig. 5*C*) and CE formation from [^{14}C]oleate (Fig. 5*D*). The cells infected with Ad-HSL or Ad-NCEH expressed HSL or NCEH proteins, respectively, in a dose-dependent manner (Fig. 5*A*) and showed dose-dependent increases of nCEH activity in whole cell lysates (Fig. 5*B*). At 300 m.o.i., the nCEH activity in Ad-NCEH-infected cells was 14% that in Ad-HSL-infected cells (Fig. 5*B*). This increase in nCEH activity in cells overexpressing HSL or NCEH was associated with decreases in the intracellular CE content (Fig. 5*C*) as well as in the rate of CE formation from [^{14}C]oleate (Fig. 5*D*). More pronounced inhibition of CE accumulation by Ad-HSL than by Ad-NCEH may be explained by the higher nCEH activity attained by Ad-HSL.

To determine whether NCEH protein is expressed in atherosclerotic lesions, we performed immunohistochemistry (Fig. 6). Cultured peritoneal macrophages were positively stained with anti-NCEH antibody (*brown*, Fig. 6*B*) but not with nonimmune IgG (Fig. 6*A*). Next, we stained tissue sections of aorta from apoE$^{-/-}$ mice (Fig. 6*C*), which contained advanced atherosclerotic lesions filled with cholesterol cleft. Double staining with F4/80, a macrophage-specific antibody (*blue*, Fig. 6*E*), showed prominent expression of NCEH protein (*brown*, Fig. 6, *D* and *E*) in macrophages surrounding the cholesterol cleft in the subintimal space of apoE$^{-/-}$ aorta.

In summary, NCEH is substantially expressed in macrophages in atherosclerotic plaques and significantly contributes to nCEH activity of murine macrophages. This study is the first demonstration of the molecular identity of the dominant nCEH in macrophages, a long unidentified enzyme catalyzing the counter-reaction of acyl-CoA:cholesterol acyltransferase. Although our data do not exclude the possibility that HSL or possibly other enzymes account at least in part for the nCEH activity in macrophages, it is tempting to speculate that HSL evolves to have a more specialized role in adipocyte lipol-

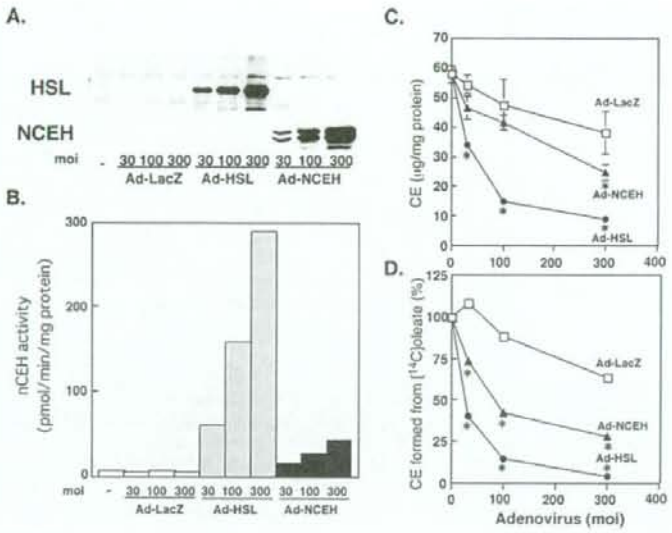

FIGURE 5. **Effects of overexpression of NCEH on cholesterol ester accumulation or cholesterol ester formation in THP-1 macrophages.** Recombinant adenoviruses coding for LacZ (Ad-LacZ), HSL (Ad-HSL), or NCEH (Ad-NCEH) were used to infect THP-1 macrophages, and experiments were performed 3 days after infection. The cells were sonicated and used for Western blot analysis (*A*) and measurement of nCEH activity (*B*). Twenty four h after THP-1 macrophages were infected with recombinant adenovirus carrying NCEH, HSL, or LacZ as a control, the cells were incubated with 100 μg/ml acLDL. On day 3 after infection, intracellular content of CE (*C*) and CE formation from [^{14}C]oleate (*D*) were measured. Data are presented as means ± S.E. of four measurements (*C* and *D*) (*, $p < 0.001$, Ad-HSL *versus* Ad-LacZ, or Ad-NCEH *versus* Ad-LacZ).

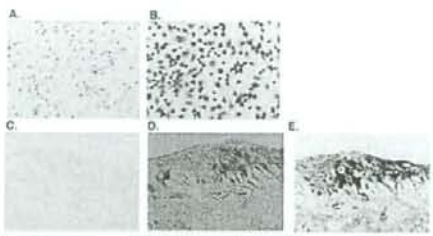

FIGURE 6. **Expression of NCEH in murine peritoneal macrophages and in foamy macrophages in atherosclerotic plaques.** Expression of NCEH protein in MPM (*A* and *B*) and the aorta of apoE$^{-/-}$ mice (*C* and *D*) was localized by immunohistochemistry using affinity-purified anti-NCEH rabbit IgG (visualized *in brown*) (*B* and *D*) or by combined immunohistochemical staining for NCEH (*brown*) and F4/80 (*in blue*, for macrophages) (*E*). Peroxidase activity was visualized using 3,3′-diaminobenzidine (*brown*) or Vector blue (*blue*), and sections were counterstained with 3% methyl green. As a control experiment, staining of MPM (*A*) and the aorta (*C*) with preimmune rabbit IgG as a primary antibody did not produce any horseradish peroxidase cross-reactivity. Objective magnifications are ×400 (*A* and *B*) and ×100 (*C–E*).

ysis and spermatogenesis (16), whereas NCEH serves in macrophages to hydrolyze CE or possibly other esters to maintain basic macrophage functions as scavengers. As mentioned above, the CEH gene is identical to the TGH gene in humans. Based on the negligible nCEH activity of overexpressed TGH-1 (21) and its marginal expression in murine peritoneal macrophages (Fig. 2*A*), it is unlikely that CEH plays a more significant role in CE hydrolysis than HSL or NCEH at least in mice. Further studies are needed to define the precise *in vivo* function of NCEH and its role in the development of atherosclerosis.

Given the relatively high expression of NCEH in human monocyte-derived macrophages (Fig. 2*C*), it is plausible that NCEH accounts for a major part of the CE hydrolysis in human macrophages, thereby contributing to the development of atherosclerosis. Although HSL (11) and CEH (18) have been reported to be expressed in human macrophages, there is no direct evidence for their relative contribution to the endogenous nCEH activity in human macrophages. It is necessary to clarify which enzymes are more relevant in human macrophages, before translating the current findings to clinical settings. Thorough characterization of these lipases in human macrophages, including relative expression and relative contribution to endogenous nCEH activity, deserves further study and is ongoing in our laboratory. Resolving this controversial issue would pave a way to the development of a new therapy targeted for the prevention and treatment of atherosclerosis.

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