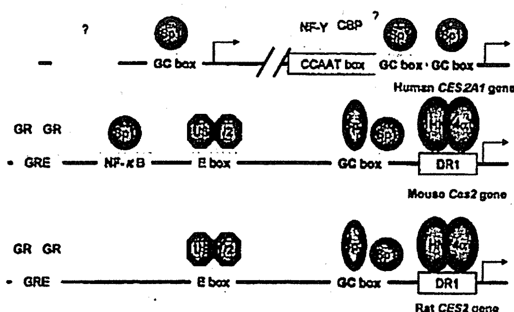

Fig. 6. Structure of the 5′ flanking region of mammal and human CES2 genes.

hepatocyte nuclear factor (HNF)-4α binding sites.

We have found that *mCES2* is expressed in various tissues with higher levels of expression in the liver, kidney and small intestine. It was shown that three transcription factors, Sp1, Sp3 and USF1, could bind to the promoter region of the *mCES2* gene, leading to synergistic transactivation of the promoter.[57]

The mouse CES2 isozyme, *mCES2*, is thought to play important roles in lipid metabolism and is expressed in the liver, kidney, and small intestine at high levels; therefore, we examined the molecular mechanisms controlling this tissue-specific expression of *mCES2*. We found that HNF-4α could enhance transcription of the *mCES2* gene *in vitro* and *in vivo*, and its effect on *mCES2* promoter activity was repressed by small heterodimer partner (SHP) and chenodeoxycholic acid (CDCA) in luciferase assays (Fig. 7). Accordingly, *mCES2* gene transcription was repressed by CDCA treatment in mouse immortalized hepatocytes. The repression of *mCES2* gene transcription might result from the combined effects of both inhibition of the HNF-4α transactivation ability by SHP and reduction of the HNF-4α expression level. Thus, HNF-4α plays an important role in the regulation of *mCES2* gene transcription.[58]

We have also isolated and characterized two genes encoding human *CES1A1* (AB119997) and *CES 1A2* (AB119998),

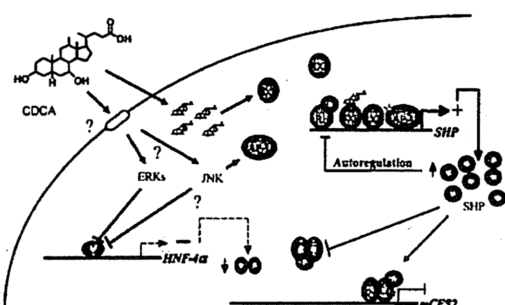

Fig. 7. Possible model for down-regulation of mCES2 gene transcription by CDCA treatment.

and also cloned and sequenced the 5′ flanking region of each gene in order to elucidate the structure of the promoter.[47] Only six nucleotide differences resulted in four amino acid differences in the open reading frame, and all of the differences existed in exon 1. Since exon 1 of the *CES1* gene encodes a signal peptide region, intracellular localization of the *CES1* gene product was preliminarily investigated using a signal peptide/EYFP-ER chimera protein-expressing system. It was interesting that the CES1A1 signal peptide/EYFP-ER chimera protein was localized to the endoplasmic reticulum, whereas the CES1A2 signal peptide/EYFP-ER chimera protein was distributed in the endoplasmic reticulum and cytosol. These results suggested that *CES1A1* and *CES1A2* have different intracellular localizations and different expression profiles in liver differentiation. We therefore investigated the transcriptional regulation of these two CES genes. Reporter gene assay and electrophoretic mobility shift assay demonstrated that Sp1 and C/EBPα could bind to each responsive element of the CES1A1 promoter but Sp1 and C/EBP could not bind to the responsive element of the CES1A2 promoter.[47]

Fukami *et al.*[59] reported that the sequences of the *CES1A2* gene downstream and upstream of intron 1 are identical with those of the *CES1A1* and *CES1A3* genes, respectively. A *CES1A1* variant in which exon 1 is converted with that of the *CES1A3* gene (transcript is CES1A2) has been identified. It was found that the *CES1A2* gene is a variant of the *CES1A3* pseudogene. The expression level of CES1A1 mRNA is much higher than that of CES1A2 mRNA in the liver.[47] Since *CES1A1* is highly variable in the individual liver,[60] it was thought that these results provided information on the inter-individual variation of human *CES1*.

For the first time, we reported that DNA methylation is involved in *CES1A1* gene expression in the human liver and kidney.[61] The tissue-specific expression of the *CES1A1* gene was examined using 5-aza-2′-deoxycytidine (5-aza-dC) and bisulfite sequencing. Treatment of HEK293 cells, human embryonic kidney cells not expressing the *CES1A1* gene, with 5-aza-dC caused marked expression of the *CES1A1* gene. Bisulfite sequencing revealed that the region around the transcription start site (TSS) of the *CES1A1* gene was almost entirely methylated in HEK293 cells, whereas the region was almost entirely unmethylated in HepG2 cells, human hepatoma cells. Hypo-methylated DNA molecules for the region were observed in HEK293 cells treated with 5-aza-dC. Furthermore, it was observed that the DNA methylation level differs in the TSS region of the *CES1A1*gene between human liver and kidney samples. From these findings, it can be concluded that DNA methylation in the TSS region is involved in the different expressions of *CES1A1* gene in the human kidney and liver.

8. Genetic Polymorphism

Recently, Geshi *et al.*[62] reported that *CES1A2*-816A/C poly-

morphism was significantly associated with the anti-hypertensive efficacy of imidapril medication. Imidapril is a prodrug ACE inhibitor, which requires hepatic activation by hCE1 to form an active metabolite. It was shown that −816C allele had higher transcriptional activity than the −816A allele. Since no putative transcription factor recognition site was found around the −816A/C region, it was speculated that this polymorphism was a marker of other functional polymorphism(s). Recently, our investigation into the CES1A2 promoter region (*ca.* 1 kB) in 100 Japanese hypertensive patients revealed ten SNPs at positions −816, −674, −427, −62, −47, −46, −41, −40, −37, and −32, and one I/D at −34.[63] Pairwise D′ and r^2 showed that all of these polymorphisms were in high linkage disequilibrium (LD). From all eleven polymorphisms spanning this region, four haplotypes were obtained as infer haplotypes which had frequencies of more than 1%, and they accounted for 96% of the alleles. Three consisted of the same SNPs between −62 to −32 and the −34 I/D as the most common haplotype (frequency of 54%), which accounted for 74% and residual 22% was the minor haplotype. Interestingly, −816A/C was in high LD with the major and minor haplotypes ($D'=0.92$, $r^2=0.85$).[63] In contrast to the major haplotype, the minor haplotype had higher transcription and Sp1 binding activity due to the presence of two putative Sp1 binding sites. The Sp1 binding site variation in the CES1A2 promoter affects the pharmacological effect and the −816A/C might be a good candidate for pharmacogenetic study of *CES1*-activated prodrugs.

More recently, Sai *et al.* reported a gene-dose effect of functional *CES1A* genes on SN-38 formation in irinotecan-treated Japanese cancer patients.[64] Irinotecan is a prodrug of SN-38 and is well hydrolyzed by hCE2 at high affinity in comparison with hCE1[23]; however, hCE1 also plays an important role in the hydrolysis of irinotecan in the human liver[65] because of its major expression. In this study, *CES1A* diplotypes [combination of haplotypes A (*1A3-1A1*), B (*1A2-1A1*), C (*1A3-var1A1*) and D (*1A2-var1A1*)] (Fig. 8) and the major SNPs (−75T>G and −30G>A in *1A1*, and −816A>C in *1A2* and *1A3*) were determined in 177 Japanese cancer patients. The associations of *CES1* genotypes, the number of functional *CES1* genes (*1A1*, *1A2* and *var1A1*) and major SNPs, with an AUC ratio of (SN-38+SN-38G)/irinotecan, a parameter of *in vivo* CES activity, were analyzed for 58 patients treated by irinotecan monotherapy. The median AUC ratio of patients having 3 or 4 functional *CES1* genes (diplotypes A/B, A/D or B/C, C/D, B/B and B/D; N=35) was 1.24-fold of that in patients with 2 functional *CES1* genes (diplotypes A/A, A/C and C/C; N=23) [median (25th–75th percentiles): 0.31 (0.25–0.38) *vs.* 0.25 (0.20–0.32), $P=0.0134$]; however, it was interesting that no significant effects of *var1A1* and the major SNPs examined were observed.[64] In addition, comprehensive haplotype analysis of the *CES2* gene, which encodes hCE2, was performed[66] and twenty haplotypes were identified in 262 Japanese subjects. Patients with

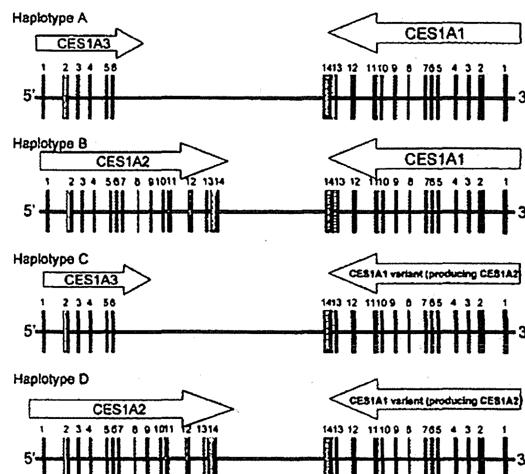

Fig. 8. CES1 gene structure and haplotype.

nonsynonymous SNPs, 100C>T (Arg[34]Trp: allele frequency 0.002) or 1A–T (Met[1]Leu: allele frequency 0.002), showed low AUC ratios.[66] Both haplotypes are important for hCE2 to activate prodrugs, but hepatic hydrolase activity might be kept at a certain level due to the compensatory activity of hCE1. Thus, prodrugs are hydrolyzed even in a subject with a variant *CES* gene, and show a pharmacological effect. There is no report that severe toxicity of a prodrug is caused by genetic polymorphism of the *CES* gene; however, understanding genetic polymorphisms is important to confirm the safety and effectiveness of noble prodrugs.

Conclusion

CESs are widely distributed in all mammalian species, and play an important role in the bioconversion of prodrugs. Major human CES isozymes, hCE1 and hCE2, show different substrate specificity, resulting in tissue-specific hydrolysis. Therefore, successful prodrug design will be improved by further detailed analysis of the substrate recognition and expression of human CES isozymes. Furthermore, detailed analysis of the species difference of tissue activity mediated by CES might help in the preclinical study of prodrug development.

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Editorial

Are Non-human Primates Useful Experimental Animals for Pre-clinical Study?

Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Recently, genome assemblies for several primate species have become available, and sequencing projects are underway or have been approved for other primates. Preliminary genome assemblies, with various levels of sequencing coverage, are also available for the gorilla, marmoset, bushbaby, mouse lemur, and tarsier genomes, and work is underway to sequence the gibbon and baboon genomes. Moreover, additional primate species have been approved for sequencing by the National Human Genome Research Institute (NHGRI). These new genome sequences will help to identify the genetic basis of differences between primate species, including genomic features that differentiate humans from non-human primates, to identify and characterize functional sequences present in primates but not in other mammals and to catalog genomic similarities and differences between humans and non-human primates widely used in biomedical research, such as the baboon and rhesus macaque (Rhesus Macaque Genome Sequencing and Analysis Consortium 2007).[1] Research on these non-human primates will also help to clarify the molecular evolutionary context for human drug metabolism.

Experimental animals have been commonly used in the pre-clinical development of new drugs to predict the metabolic profiles of new compounds in humans. It is, however, important to realize that humans differ from animals with regard to isozyme composition, tissue-specific expression and catalytic activities of human kinds of drug-metabolizing enzymes. In the present manuscript, I describe similarities and differences in the major drug-metabolizing enzymes among non-human primates and humans. Information presented in this manuscript may be helpful for drug development to choose the most relevant non-human primates in which the metabolism of drugs can be studied for extrapolating the results to humans.

Rhesus and cynomolgus monkeys are used in studies on drug metabolism and toxicity due to their evolutionary closeness to humans compared with other non-human primate species. A recent mRNA cloning study of cytochrome P450 (CYP) subfamilies from cynomolgus monkeys showed a high degree of homology in cDNA and amino acid sequences with corresponding human CYPs (more than 90%), and cynomolgus monkey CYPs catalyzed typical enzyme reactions of corresponding human CYPs . However, one member of the cynomolgus monkey CYP2C subfamily, CYP2C76, exhibited lower homology in amino acid sequences with other cynomolgus monkey and human CYP2C subfamilies. CYP2C76 does not correspond to any human CYP isozymes and is partly responsible for the difference in pitavastatin metabolism between cynomolgus monkeys and humans.[2] We should pay attention to data for CYP2C76 catalyzed enzyme reactions when extrapolating results for cynomolgus monkeys to humans.

Glucuronidation by UDP glucuronosyltransferase(UGT) 1A enzymes (UGT1As) is a major pathway for elimination of drugs and endogenous substances, such as bilirubin. Aligning the human and baboon UGT1 loci revealed rearrangements that have been occurring since the divergence of baboons and humans. Baboon UGT1A cDNAs were cloned and shown to have an orthologous relationship with several genes in the human UGT1A family. Activities of the baboon UGT1As resembled those of their human counterparts in glucuronidating endobiotics, such as serotonin, bilirubin, and various xenobiotics.[3] Gluclonidation by UGT1Bs is also a major pathway for elimination of drugs, such as morphine. Morphine, a probe drug for UGT2B7, is metabolized to morphine-3-β-glucuronide (M3G) and morphine-6-β-glucuronide (M6G) in humans. Baboon UGT2Bs also metabolized morphine to both M3G and M6G metabolites. Although there are considerable duplications and deletions among primates, a close relationship exists among human, baboon and cynomolgus monkey UGT2B enzyme families. Similarities between the primary structures of human, baboon and cynomolgus monkey UGT2B proteins provided further evidence that there is a close relationship among UGT2B family enzymes in these species.[4] UGT2Bs together with the close relationship shown between human and baboon UGT1A enzyme families further indicate that the baboon is an excellent model for studying clinically relevant aspects of drug metabolism.

Hydrolysis by the carboxylesterases (CESs) is a major metabolic pathway for bio-activation of pro-drugs, such as CPT-11, temocapril and oseltamivir. CESs and UGTs, the catalytic domains of which are localized in the luminal sides of the endoplasmic reticulum (ER) membrane, are two major enzyme groups responsible for phase I and II reactions. Products hydrolyzed by CESs, such as SN-38 from CPT-11, are also good substrates for UGT. Thus, I

speculate that CES-UGT interaction in the luminal side of the ER membrane is important for drug metabolism. It has been suggested that CESs can be classified into five major groups denominated CES1-CES5, according to the homology of the amino acid sequence, and the majority of CESs that has been identified belong to the CES1 or CES2 family. Substrate specificities of CES1 and CES2 are significantly different. The CES1 isozyme mainly hydrolyzes a substrate with a small alcohol group and large acyl group, but its wide active pocket sometimes allows it to act on structurally distinct compounds of either a large or small alcohol moiety. In contrast, the CES2 isozyme recognizes a substrate with a large alcohol group and small acyl group, and its substrate specificity may be restricted by the capability of acyl-enzyme conjugate formation due to the presence of conformational interference in the active pocket.[5] Alignments of baboon CES1 with human CES1 and baboon CES2 with human CES2 showed 94% and 90% sequence identities, respectively, while other primate CES1 amino acid sequences have identities of 93% or more with human CES1, whereas other primate CES2 sequences have identities of 86% or more with human CES2. Non-human primate CES1 and CES2 have amino acid sequences that are very similar to those of the corresponding human CES isozymes, and share key conserved sequences and structures that have been reported for human CES1 and have family-specific sequences consistent with their multimeric and monomeric subunit structures respectively. Predicted secondary and tertiary structures for baboon CES1 showed a high degree of conservation with human CES1. Phylogeny studies using primate and other mammalian CES1 and CES2 amino acid sequences showed that these two CES classes underwent sequence divergence during mammalian and primate evolution, with primate CES2 showing higher amino acid substitution rates than these for primate CES1.[6] In addition, catalytic substrate specificity of rhesus monkey and cynomolgus monkey CES1 and CES2 hydrolyzed typical enzyme reactions of corresponding human CES1 and CES2, respectively. However, tissue-specific expression of the CES1 family is different in monkeys and humans: cynomolgus and rhesus monkey CES1 is expressed in the small intestine, but human CES1 is not expressed in the small intestine. We should pay attention to data for CES1-catalyzed enzyme reactions when extrapolating results for rhesus and cynomolgus monkeys to humans for drug development.

More recently, rhesus and cynomolgus monkeys are being used for evaluation of *in vivo* drug interaction. The recent cloning of rhesus monkey CYP3A64 revealed 93% homology in the amino acid sequence with human CYP3A4 and 83% homology to CYP3A5. More interestingly, amino acid sequences of ligand binding domains between rhesus monkey and human PXR are 96% similar. With the use of midazolam as an *in vivo* probe for CYP3A64, the pharmacokinetic consequences of *in vitro* findings and their corresponding *in vitro-in vivo* relationships were demonstrated in rhesus monkey. In rhesus monkey, the pharmacokinetics of midazolam can be significantly altered with rifampicin co-administration, resulting in reduced systemic exposure and hepatic bioavailability, similar to humans.[7] *In vitro* and *in vivo* rhesus monkey models, when used in conjunction with *in vitro* human systems, may serve as valuable preclinical tools to help provide a basis for extrapolating *in vitro* human data for clinical evaluation.

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DMPK Associate Editor

The Critical Role of Neutral Cholesterol Ester Hydrolase 1 in Cholesterol Removal From Human Macrophages

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Rationale: **Hydrolysis of intracellular cholesterol ester (CE) is the key step in the reverse cholesterol transport in macrophage foam cells. We have recently shown that neutral cholesterol ester hydrolase (Nceh)1 and hormone-sensitive lipase (Lipe) are key regulators of this process in mouse macrophages. However, it remains unknown which enzyme is critical in human macrophages and atherosclerosis.**

Objective: **We aimed to identify the enzyme responsible for the CE hydrolysis in human macrophages and to determine its expression in human atherosclerosis.**

Methods and Results: **We compared the expression of NCEH1, LIPE, and cholesterol ester hydrolase (CES1) in human monocyte-derived macrophages (HMMs) and examined the effects of inhibition or overexpression of each enzyme in the cholesterol trafficking. The pattern of expression of NCEH1 was similar to that of neutral CE hydrolase activity during the differentiation of HMMs. Overexpression of human NCEH1 increased the hydrolysis of CE, thereby stimulating cholesterol mobilization from THP-1 macrophages. Knockdown of NCEH1 specifically reduced the neutral CE hydrolase activity. Pharmacological inhibition of NCEH1 also increased the cellular CE in HMMs. In contrast, LIPE was barely detectable in HMMs, and its inhibition did not decrease neutral CE hydrolase activity. Neither overexpression nor knockdown of CES1 affected the neutral CE hydrolase activity. NCEH1 was expressed in CD68-positive macrophage foam cells of human atherosclerotic lesions.**

Conclusions: **NCEH1 is expressed in human atheromatous lesions, where it plays a critical role in the hydrolysis of CE in human macrophage foam cells, thereby contributing to the initial part of reverse cholesterol transport in human atherosclerosis. (*Circ Res.* 2010;107:1387-1395.)**

Key Words: neutral cholesterol ester hydrolase ■ reverse cholesterol transport ■ macrophage ■ atherosclerosis ■ KIAA1363

Atherosclerotic cardiovascular diseases are the leading cause of mortality in industrialized countries, despite advances in the management of coronary risk factors. Heart attacks arise from the thrombotic occlusion of coronary arteries following the rupture of plaques. Lipid-rich plaques, which are characterized by a plethora of cholesterol ester (CE)-laden macrophage foam cells, are prone to rupture.[1] Esterification of cholesterol in macrophages is mediated by acyl-coenzyme A cholesterol acyltransferase 1 or sterol O-acyltransferase 1 (SOAT1).[2] Conflicting results have been reported as to the effects of genetic ablation of SOAT1 on atherosclerosis in mice.[3,4] Furthermore, it has not been successful to demonstrate the efficacy of nonselective inhibitors of SOAT to clinically prevent the atherosclerosis in humans.[5,6] On the other hand, the hydrolysis of intracellular CE is the initial step of reverse cholesterol transport.[7] As the hydrolysis of CE preceding reverse cholesterol transport takes place at neutral pH, the enzymes catalyzing it have been collectively called neutral CE hydrolases. Because this step is rate-limiting, particularly in macrophage foam cells,[8,9] it is important to clarify the mechanisms that mediate the hydrolysis of CE in foam cells.

To date, 3 enzymes have been proposed to serve as neutral CE hydrolases in macrophages: hormone-sensitive lipase (LIPE)[10]; cholesteryl ester hydrolase (CEH),[11] which is identical to human liver carboxylesterase 1 (CES1, hCE-1)[12] or

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Non-standard Abbreviations and Acronyms	
acLDL	acetylated low-density lipoprotein
CE	cholesterol ester
GM-CSF	granulocyte/macrophage colony-stimulating factor
HDL	high-density lipoprotein
HMM	human monocyte-derived macrophage
LDL	low-density lipoprotein
M-CSF	macrophage colony-stimulating factor
MPM	murine peritoneal macrophage
moi	multiplicity of infection
PNPB	*p*-nitrophenyl butyrate
SOAT1	sterol O-acyltransferase 1
WAT	white adipose tissue

macrophage serine esterase 1 (HMSE1),[13] also known as a human ortholog of triacylglycerol hydrolase[14]; and neutral cholesterol ester hydrolase 1 (NCEH1),[15] which is also known as KIAA1363 or AADACL1 (arylacetamide deacetylase-like 1).[16] Lipe is expressed in mouse macrophages and its overexpression inhibits the accumulation of CE in THP-1 macrophages.[17,18] However, mouse peritoneal macrophages (MPMs) of Lipe-deficient (*Lipe*$^{-/-}$) mice in a mixed genetic background still retain substantial neutral CE hydrolase activity,[19,20] indicating the presence of additional neutral CE hydrolase(s). Ghosh reported CES1 as a promising candidate for a neutral CE hydrolase,[11] because its overexpression reduced CE contents in macrophage foam cells.[21,22] Moreover, its macrophage-specific overexpression driven by the promoter of macrophage scavenger receptor-1 protected against diet-induced atherosclerosis in low-density-lipoprotein receptor-deficient mice.[23] However, the effects of loss-of function of CES1 on neutral CE hydrolase activity in macrophages have not been reported. Furthermore, a mouse ortholog of *CES1*, triacylglycerol hydrolase, was barely detectable in MPMs[15] and possessed negligible neutral CE hydrolase activity.[24] In contrast, Nceh1 is robustly expressed in MPMs as well as in atherosclerotic lesions. Its overexpression inhibits the accumulation of CE in THP-1 macrophages[15] and its knockdown or knockout significantly reduces neutral CE hydrolase activity of MPMs.[15,25] We have also shown that Nceh1 is more responsible for the hydrolysis of CE in MPMs than in immortal cell line such as RAW 264.7.[15] Furthermore, ablation of Nceh1 accelerated atherosclerosis in mice.[25] Therefore, Nceh1 is more likely to be involved in the hydrolysis of CE in mouse macrophages including MPMs. However, NCEH1 in human macrophages has yet to be characterized. Furthermore, although Lipe contributes to neutral CE hydrolase activity in MPMs,[25] previous reports showed that expression of LIPE in human macrophages is extremely low.[15,26,27] Thus, there seems to be great differences in the hydrolysis of CE among macrophages from different species, and it is unknown which enzyme is the dominant neutral CE hydrolase in human macrophages. To solve this question and translate the findings to clinical application, we aimed to identify the enzyme responsible for CE hydrolysis in human macrophages and to determine its expression in human atherosclerotic lesions.

In the present study, we demonstrate for the first time that NCEH1 is expressed in macrophage foam cells in human atheromatous plaques and accounts for the majority of the neutral CE hydrolase activity of human monocyte-derived macrophages (HMMs). These findings should provide a novel paradigm for understanding the pathogenesis of human atherosclerosis as well as for developing new drugs for its treatment.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Western Blot Analyses

Western blotting analyses were performed essentially as described previously.[15]

Enzyme Assays

p-Nitrophenyl butyrate (PNPB)-hydrolyzing activity was determined as described previously.[11,15] Neutral CE hydrolase activity was determined as described by Hajjar et al,[28] using a reaction mixture containing 6.14 μmol/L cholesterol [1-^{14}C]oleate (48.8 μCi/μmol: 1 μCi=37 kBq).

Cholesterol Determination

Cellular cholesterol contents were determined by enzymatic fluorometric microassay.[4]

Cholesterol Formation

CE formation from [^{14}C]oleate was measured.[29]

Samples for Immunohistochemistry

Tissue samples for immunohistochemistry analysis were obtained from autopsy cases. Arteriosclerotic lesions of the aorta were from 20 autopsy cases (Table). After macroscopic inspection of the intimal surface, several tissue specimens were removed from the thoracic or abdominal aorta of these cases. Biopsy specimens were fixed in buffered formalin, embedded in paraffin wax, and serially sectioned onto 4-μm-thick microscopic slides.

Immunohistochemistry

The method is described in detail in the Online Data Supplement.

Results

To determine which enzyme(s) is closely related to the neutral CE hydrolase activity at various stages in the differentiation of human macrophages, we compared the pattern of expression of NCEH1, CES1, and LIPE with that of neutral CE hydrolase activity during the differentiation of HMMs and 2 immortal lines: THP-1 and U937 (Figure 1). Human NCEH1 protein was recognized as duplets with molecular mass of 40 and 45 kDa. HMMs showed a robust increase in neutral CE hydrolase activity during the differentiation from monocytes up to day 8 (14.9-fold) (Figure 1A). PNPB hydrolase activity in HMMs showed a similar, but less robust, increase (3.2-fold) (Figure 1B). The manner of its induction of the neutral CE hydrolase and PNPB hydrolase activity was similar to that of the expression of NCEH1, but not to the expression of CES1 (Figure 1C). We quantified the amounts of endogenous NCEH1 and CES1 in HMMs at day 8 of differentiation by estimating the density of band of NCEH1

Table. Clinicopathological Findings of the Aortic Samples Used for Histology

No.	Age, y	Sex	Diagnosis	Pathology	CD68 (%)	NCEH1 (%)
1	63	M	Pancreatic cancer, DM	AP	35	30
2	50	M	Diabetic cardiomyopathy, DM	AP	15	20
3	63	M	Bladder cancer	FS	35	15
4	93	F	AMI, DM	AP	30	15
5	58	F	AML+GVHD	AP	40	30
6	76	M	Lung cancer, HT	AP	35	15
7	69	M	Lung cancer, HT, HL	AP	25	20
8	85	F	Vulvar cancer, HT	FS	10	10
9	73	F	Acute pancreatitis, HT, HL	AP	25	5
10	69	M	Renipelvic cancer	AP	30	10
11	51	M	AML	DIT	5	0
12	52	M	Pancreatic cancer	DIT	0	0
13	76	M	Prostate cancer, Cerebral infarction	AP	45	35
14	65	F	Polyarteritis nodosa, DM	DIT	0	0
15	75	F	Malignant lymphoma	FS	10	5
16	89	M	Rectal cancer	AP	40	30
17	50	M	DCM	FS	5	0
18	66	M	Lung cancer, DM	AP	40	25
19	53	F	HCC, DM	AP	40	25
20	82	M	Renal cancer, DM	FS	30	20

AMI indicates acute myocardial infarction; AML, acute myelogenous leukemia; AP, atheromatous plaque; DCM, dilated cardiomyopathy; DIT, diffuse intimal thickening; DM, diabetes mellitus; FS, fatty streak; GVHD, graft-vs-host disease; HCC, hepatocellular carcinoma; HL, hyperlipidemia; HT, hypertension. Ratio of CD68- or NCEH1-positive cells in 200 nucleated cells of representative view was calculated.

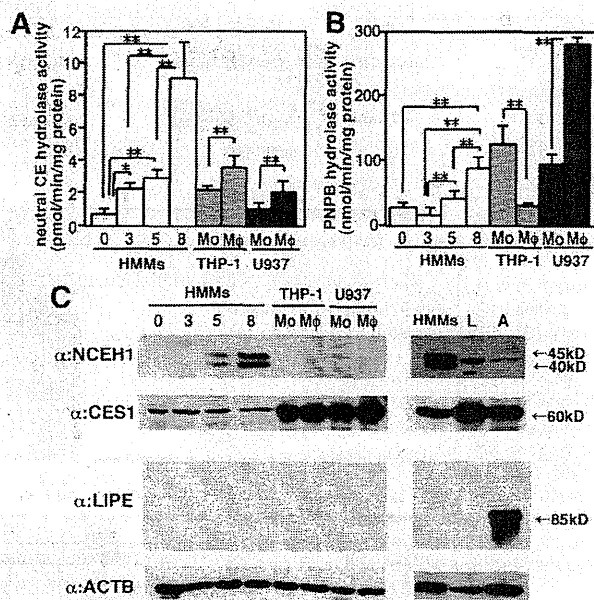

Figure 1. Endogenous neutral CE hydrolase in human macrophages. Neutral CE hydrolase activity **(A)**, PNPB hydrolase activity **(B)**, and protein expression of NCEH1, CES1, and LIPE **(C)** in peripheral blood monocytes, THP-1, and U937, before and after differentiation, was examined. Mo indicates monocytes; Mϕ, macrophages. Human liver (L) and adipose tissue (A) were used for comparison. Ten micrograms of cell lysate were subjected to Western blotting. Data are presented as means±SD of 6 measurements. $^{*}P<0.05$, $^{**}P<0.01$ (determined by ANOVA followed by the Bonferroni post hoc analysis).

or CES1 in HMMs, using GST-fused proteins, which were also used as antigens to produce anti-NCEH1 or CES1 antiserum, as standards (Online Figure I, A). The molar ratio of NCEH1 to CES1 was calculated to be 10. However, NCEH1 was barely detectable, even at the differentiated stage, in either THP-1 cells or U937 cells, although the differentiation of these cells accompanied increases in neutral CE hydrolase activity. The level of NCEH1 was much lower in the liver or white adipose tissue (WAT) than in HMMs. LIPE was specifically expressed in WAT. LIPE protein was undetectable in HMMs and liver on Western blots using 3 different LIPE antibodies (Figure 1C). LIPE mRNA was detectable in HMMs and liver by quantitative real-time PCR, but the level was much lower compared that in WAT in accordance with the previous report (Online Figure I, B).[27] In contrast to the relatively specific expression of these 2 enzymes, CES1 was expressed in the liver and WAT at a level comparable to that in HMMs, indicating the ubiquitous nature of its expression. The essential difference in expression profile between HMMs and immortal cell lines supports the idea that there are great differences in the hydrolysis of CE among macrophages from different species. We mainly focused on HMMs in subsequent experiments, because HMMs are more relevant to the pathogenesis of human atherosclerosis.

We examined whether differences in cytokines used for differentiation of macrophages affect the expression of NCEH1, CES1, and LIPE and neutral CE hydrolase activity in HMMs. Treatment by macrophage colony-stimulating factor (M-CSF) or granulocyte/macrophage (GM)-CSF greatly increased the amounts of NCEH1 protein (M-CSF, 2.2-fold; GM-CSF, 3.4-fold). In parallel, it increased neutral CE hydrolase activity (M-CSF, 2.4-fold; GM-CSF, 3.5-fold) as reported previously (Online Figure II).[30] GM-CSF slightly increased CES1 protein but LIPE was not affected.

To compare the ability of the overexpressed enzyme to remove CE from macrophage foam cells, we used an adenoviral vector to overexpress NCEH1, CES1, or LIPE in THP-1 cells that had been loaded with CE by incubation with acetylated low-density lipoprotein (acLDL) (Figure 2). Infection with increasingly higher doses of the adenoviral vectors resulted in the expression of the enzymes in a dose-dependent manner (Figure 2A). Neutral CE hydrolase activity in the whole cell lysate was increased robustly by Ad-*LIPE* (34-fold) and by Ad-*NCEH1* to a lesser degree (3.2-fold). However, it was not affected by Ad-*CES1*, even at a multiplicity of infection (moi) of 300 (Figure 2B). PNPB hydrolase activity was increased by all 3 enzymes (Ad-*NCEH1*, 2.2-fold; Ad-*CES1*, 14-fold; Ad-*LIPE*, 4.6-fold), with the effect of Ad-*CES1* most pronounced. The increased activity of neutral CE hydrolase, which was attained by infection with Ad-*NCEH1* or Ad-*LIPE*, was associated with a decrease in

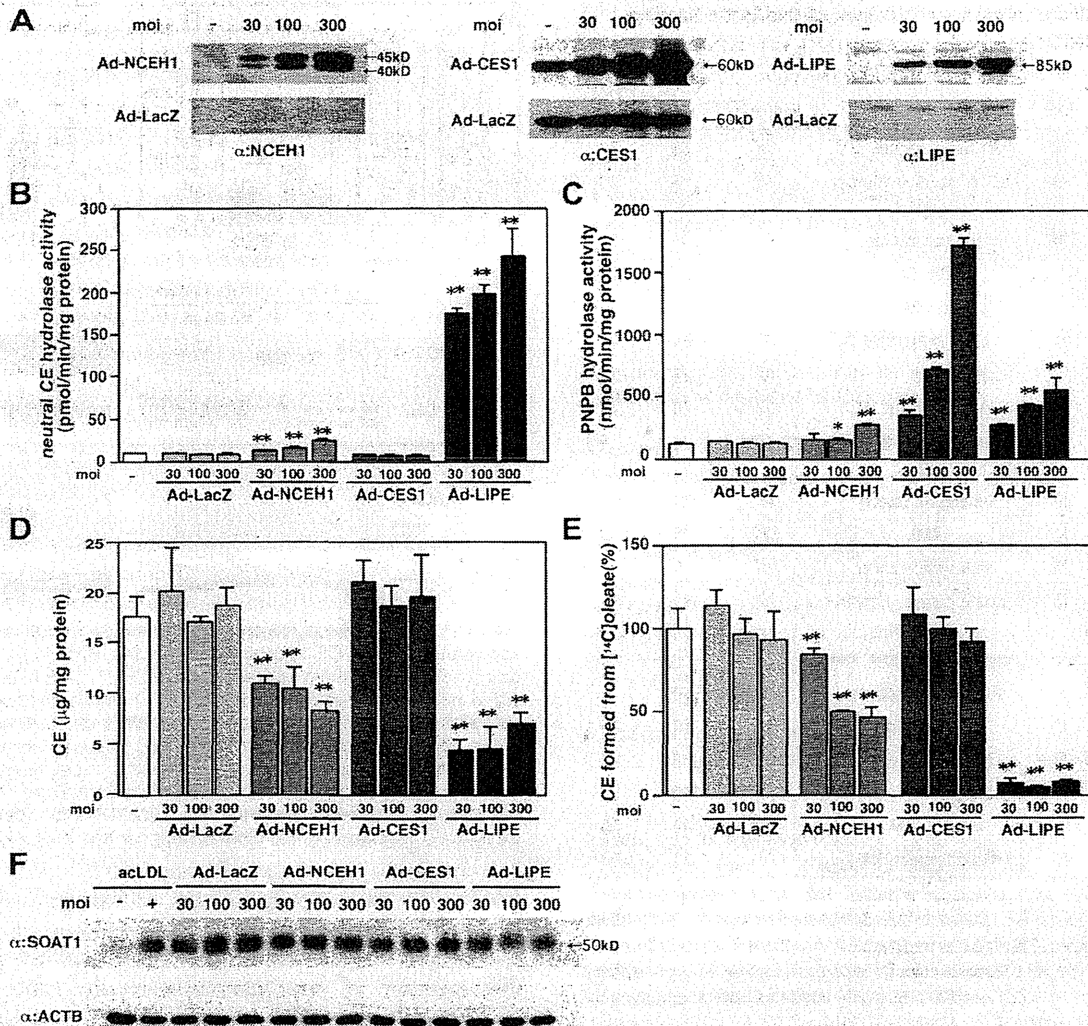

Figure 2. Enzymatic activity and cholesterol trafficking in cholesterol-loaded THP-1 macrophages overexpressing NCEH1, CES1, or LIPE. THP-1 macrophages were incubated with 100 μg/mL of acLDL for 24 hours. After infection with the recombinant adenovirus constructed to express LacZ (Ad-*LacZ*), NCEH1 (Ad-*NCEH1*), CES1 (Ad-*CES1*), or LIPE (Ad-*LIPE*), the cells were incubated with a medium containing 100 μg/mL acLDL and 250 μg/mL HDL. Three days after the infection, the cells were used for Western blot analyses for NCEH1, CES1, LIPE **(A)**, or SOAT1 **(F)**, measurements of neutral CE hydrolase **(B)** or PNPB hydrolase **(C)** activity, CE mass **(D)** and the formation of CE from [^{14}C]oleate **(E)**. Data are presented as the means±SD of 3 **(B, C, and E)** or 4 **(D)** measurements. *$P<0.05$, **$P<0.01$, Ad-*NCEH1* vs Ad-*LacZ*, Ad-*CES1* vs Ad-*LacZ*, or Ad-*LIPE* vs Ad-*LacZ* (determined by the 2-tailed Student's *t* test for **B and C** and by ANOVA followed by the Bonferroni post hoc analysis for **D and E**).

the cellular CE content (Figure 2D), as well as in the rate of formation of CE from oleate (Figure 2E). However, overexpression of CES1 did not significantly reduce the cellular CE accumulation. The decreased CE formation was not accompanied by changes of level of SOAT1 protein (Figure 2F). Similarly, overexpression of NCEH1 or LIPE significantly decreased CE content in THP-1 cells, which had been loaded with oxidized LDL, aggregated LDL, or β-very-low-density lipoprotein (Online Figure IV).

Cholesterol efflux was examined in THP-1 macrophages that overexpressed NCEH1, CES1, or LIPE (Online Figure V). Overexpression of NCEH1 and LIPE significantly promoted cholesterol efflux in the presence of high-density lipoprotein (HDL) (Online Figure V, B) or apolipoprotein A-1 (Online Figure V, C). Addition of 10 μmol/L CS-505, a SOAT1 inhibitor, inhibited the CE formation completely (Online Figure V, A) but did not affect cholesterol efflux in the cells overexpressing NCEH1, CES1, or LIPE (Online Figure V, B). Dibutyryl cAMP promoted cholesterol efflux in the cells overexpressing LIPE as described previously[18,31] but did not affect cholesterol efflux in the cells overexpressing NCEH1 or CES1, in accordance with our previous report (Online Figure V, D).[32] Overexpression of NCEH1, CES1, or LIPE did not affect the expression of ABCG1 protein, whereas overexpression of NCEH1 or LIPE slightly increased the expression of ABCA1 protein (Online Figure V, E). These results indicate that human NCEH1 is primarily involved in CE hydrolysis and that its overexpression promotes cholesterol efflux without affecting SOAT1 activities probably by increasing the expression of ABCA1.

To determine whether LIPE or NCEH1 is involved in the hydrolysis of CE in HMMs, we used 76-0079, a LIPE

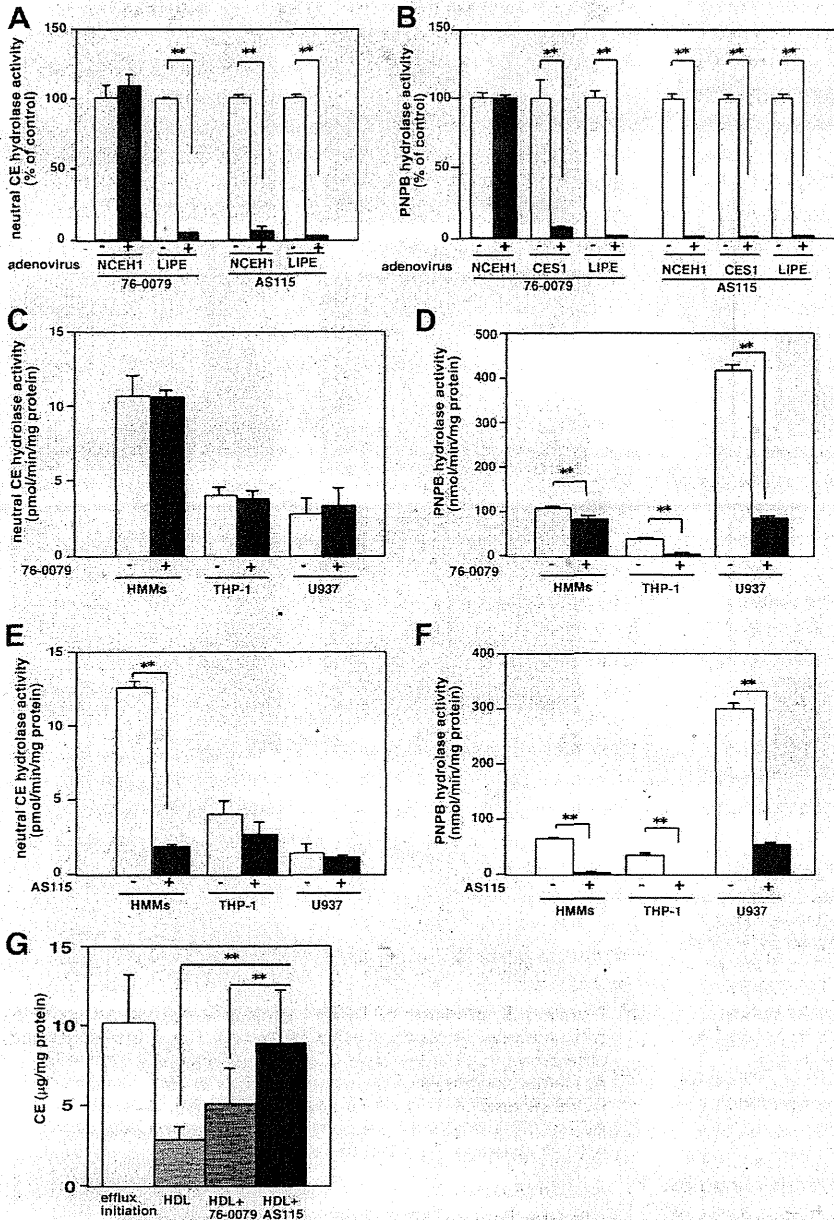

Figure 3. Effects of 76-0079 or AS115 on enzymatic activity. HEK293 cells were transfected with with Ad-*LacZ*, Ad-*NCEH1*, Ad-*CES1*, or Ad-*LIPE*, and whole-cell lysate was used for measurements of neutral CE hydrolase activity **(A)** or PNPB hydrolase activity **(B)** in the absence or presence of 5 μmol/L 76-0079 or 10 μmol/L AS115. Results are shown as percentages of the activity measured in the absence of 76-0079 or AS115. Whole-cell lysate was prepared from HMMs, THP-1 macrophages, and U937 macrophages and used for measurements of neutral CE hydrolase activity **(C and E)** or PNPB hydrolase activity **(D and F)** in the absence or presence of 5 μmol/L 76-0079 **(C and D)** or 10 μmol/L AS115 **(E and F)**. Data are presented as the means±SD of 3 measurements. $^*P<0.05$ and $^{**}P<0.01$ (as determined by the 2-tailed Student's *t* test). **G,** Human monocytes were differentiated into HMMs in the presence of 15 ng/mL M-CSF. HMMs were loaded with cholesterol by incubating the cells with 100 μg/mL acLDL. After 24 hours, cholesterol efflux was initiated by the addition of 250 μg/mL HDL in the presence of CS-505 with or without 50 μmol/L 76-0079 or 10 μmol/L AS115 and continued for 12 hours. Lipids were extracted after termination of efflux, and cellular CE mass was measured. Data are presented as the means±SD of 5 measurements. $^*P<0.05$, $^{**}P<0.01$ (determined by ANOVA followed by the Bonferroni post hoc analysis).

inhibitor,[33] or AS115, a KIAA1363 inhibitor.[34] 76-0079 was an inhibitor of both LIPE and CES1, and AS115 was an inhibitor of LIPE, NCEH1, and CES1 (Figure 3A and 3B). Although 76-0079 and AS115 are nonspecific inhibitors, we can estimate the contribution of NCEH1 by the difference between the effects of those inhibitors. First, we examined the effects of 76-0079 and AS115 on the neutral CE hydrolase (Figure 3C and 3E) or PNPB hydrolase (Figure 3D and 3F) activity in the whole cell lysate of HMMs, THP-1 macrophages, and U937 macrophages. 76-0079 did not significantly inhibit the neutral CE hydrolase activity of HMMs. In contrast, AS115 inhibited the neutral CE hydrolase activity by 85%. 76-0079 inhibited the PNPB hydrolase activity of HMMs, THP-1 cells, and U937 macrophages by 23%, 96%, and 80%, respectively. On the other hand, AS115 inhibited the PNPB hydrolase activities of these macrophages by 98%, 100% and 83%, respectively. Furthermore, whereas AS115 significantly decreased cholesterol efflux from HMMs that had been loaded with CE, 76-0079 did not (Figure 3G). These results support the notion that the neutral CE hydrolase activity of HMMs is primarily mediated by NCEH1 but not by CES1 or LIPE.

To determine whether NCEH1 or CES1 is involved in the hydrolysis of CE in HMMs, we used an RNA-silencing technique (Figure 4). Infection with Ad-sh*NCEH1* reduced the amounts of NCEH1 protein as compared with Ad-sh*LacZ* (by 41% at 250 mois and by 66% at 750 mois). In parallel, it decreased neutral CE hydrolase activity as compared with Ad-sh*LacZ* (by 47% at 250 mois and by 50% at 750 mois). To the contrary, although infection with Ad-sh*CES1* reduced the amounts of CES1 protein as compared with Ad-sh*LacZ* (by 60% at 250 mois and by 51% at 750 mois), it did not decrease

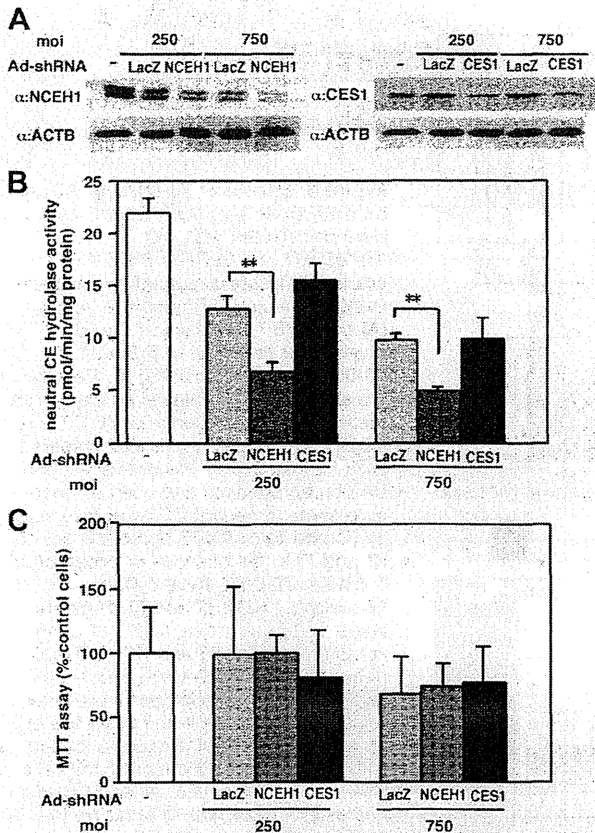

Figure 4. Effects of knockdown of NCEH1 or CES1 on the neutral CE hydrolase of HMMs. Recombinant adenoviruses coding for short hairpin RNA (shRNA) against LacZ, NCEH1, or CES1 were used to infect HMMs at 250 and 750 mois. Two days after infection, whole-cell lysate was subjected to Western blotting **(A)** and the measurement of neutral CE hydrolase activity **(B)**. **C,** MTT assay was performed to assess cell viability of HMMs infected with recombinant adenoviruses coding for shRNA against LacZ, NCEH1, or CES1. Data are expressed as percentages to control cells. Data are presented as the means±SD of 3 **(B)** or 4 **(C)** measurements. $^*P<0.05$, $^{**}P<0.01$ (determined by ANOVA followed by the Bonferroni post hoc analysis).

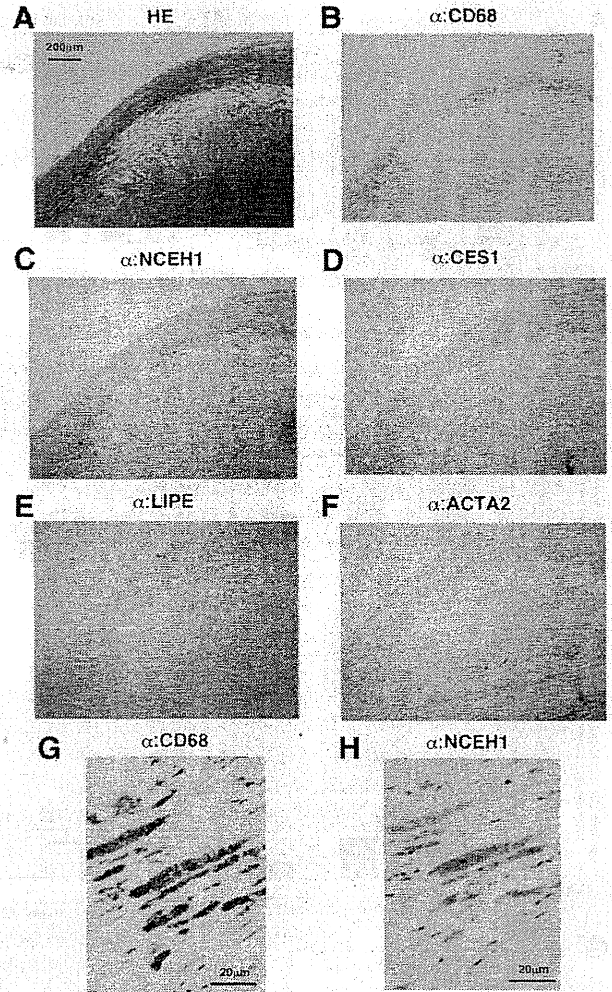

Figure 5. Expression of NCEH1 protein in human atherosclerotic lesions. Sections of aorta from case 1 or 7 that contained atheromatous plaques were used for histological examination. **A,** Hematoxylin/eosin (HA) staining. Immunohistochemistry for CD68 **(B and G)**, NCEH1 **(C and H)**, CES1 **(D)**, LIPE **(E)**, or ACTA2 **(F)** in the serial sections of **A**. Original magnifications: ×40 **(A through F)**; ×400 **(G and H)**.

the neutral CE hydrolase activity further from that attained with Ad-sh*LacZ*. Infection with increasingly higher doses of Ad-sh*LacZ* nonspecifically reduced the protein expression (Figure 4A) and neutral CE hydrolase activity (Figure 4B). To rule out the possibility that infection of Ad-sh*LacZ*, Ad-sh*NCEH1*, or Ad-sh*CES1* is cytotoxic, we performed MTT assay (Figure 4C). There was no significant difference in MTT activity among the cells infected with 3 viruses. Viability of cells infected 750 mois of adenovirus slightly decreased compared with no treatment cells, but the difference was not significant. Although there is a nonspecific effect of adenovirus infection, these results support that neutral CE hydrolase activity in HMMs is primarily mediated by NCEH1, not by CES1 or LIPE, in combination with the results of inhibition of human NCEH1 in HMMs by AS115 (Figure 3).

Finally, we investigated the expression of NCEH1 in human aortas (Table). We stained sections of aortas from 20 cases of autopsy whose clinical characteristics are summarized in Table. The lesions were morphologically classified into 3 types: diffuse intimal thickening, fatty streak lesions, and atheromatous plaques.[35] Atheromatous plaques were rich in cells positive for CD68, a pan-macrophage/dendritic cell marker, as well as NCEH1-positive cells compared with fatty streak lesions or diffuse intimal thickening. NCEH1-positive cells were absent in diffuse intimal thickening. Figure 5 shows representative sections from atheromatous plaques. This region contains acellular necrotic core (Figure 5A). The subintimal area between the intima and the necrotic core contained many CD68-positive cells (Figure 5B). A majority of the CD68-positve cells were also positive for NCEH1 (Figure 5C and the Table). The colocalizing characteristics of CD68 and NCEH1 were more obvious at a higher magnification (Figure 5G and 5H). In contrast, CES1 was weakly positive for some CD68-positive cells (Figure 5D). LIPE protein was barely detectable in HMMs in the same condition (Figure 5E). ACTA2 was strongly positive in most of the

cells in the media and weakly positive for some of the cells in the intima (Figure 5F). Furthermore, we show representative sections from fatty streak lesions (Online Figure VII, A through C). Although the expression of CD68 and NCEH1 in fatty streak lesions decrease compared with atherosclerotic plaque, many of the CD68-positve cells were also positive for NCEH1 (Online Figure VII, B and C). CES1 was weakly expressed and LIPE was not expressed (data not shown). Thus, NCEH1 is the major enzyme that is specifically expressed in macrophages of human atherosclerotic lesions.

Discussion

Previously, we have reported that both Lipe and Nceh1 are involved in the hydrolysis of CE to a comparable degree in mice.[25] Circumstantial evidence, however, suggests that there are great differences in the hydrolysis of CE between human and mouse macrophages.[15,26,27] To understand the pathogenesis of atherosclerosis in humans, therefore, it is essential to determine the major enzyme that mediates the hydrolysis of CE hydrolysis in foam cell macrophages of human atherosclerotic lesions. In the present study, we show for the first time that NCEH1 is primarily involved in the hydrolysis of CE in human macrophages, thereby constituting the initial step toward reverse cholesterol transport in atherosclerotic lesions. We also show that contribution of CES1 and LIPE is trivial. Involvement of NCEH1 in atherosclerosis is further supported by its expression in situ in CD68-positive macrophages, which are abundant in human atheromatous plaques with cholesterol crystal in its necrotic core.

Recently, we have identified Nceh1 as a microsomal enzyme that mediates the hydrolysis of CE in mouse macrophages.[15,32] In that report, we showed that the mRNA of a human ortholog of *Nceh1* was markedly increased during the differentiation from human monocytes to mature macrophages. The present results confirm that the expression of NCEH1 protein was also markedly increased (Figure 1C). This level of induction was similar to the pattern of increase in neutral CE hydrolase activity (Figure 1A). Adenovirus-mediated overexpression of human NCEH1 in CE-loaded THP-1 macrophages decreased the cellular CE content by stimulating cholesterol efflux along with the expression of ABCA1 (Figure 2D; Online Figure V). On the other hand, inhibition of human NCEH1 by AS115, a KIAA1363 inhibitor, or RNA interference in HMMs significantly decreased neutral CE hydrolase activity (Figure 3E and 4B). Furthermore AS115 significantly decreased cholesterol efflux from HMMs which had been loaded with CE (Figure 3G). Thus, NCEH1 primarily regulates the hydrolysis of CE in HMMs.

Is NCEH1 the only enzyme with neutral CE hydrolase activity in human macrophages? This is a pressing question, because at least 2 other enzymes have been proposed to regulate neutral CE hydrolase activity in macrophages: LIPE and CES1. LIPE was the first enzyme proven to contribute to neutral CE hydrolase activity in macrophages. LIPE is indeed expressed in several macrophage cell lines including MPMs and preferentially catalyzes the hydrolysis of CE.[36,37] Although some groups reported the expression of LIPE in human THP-1macrophages,[27,38] other groups doubted its role as a neutral CE hydrolase because of an extremely low level of expression as compared with that in adipose tissues.[31] The negligible expression of LIPE in HMMs was supported by the finding that efflux of cholesterol was not stimulated by cAMP,[31] which is known to stimulate lipolysis by activating LIPE in adipocytes.[39] Consistent with the claim of the latter groups, we failed to detect significant expression of LIPE in HMMs (Figure 1C). The negligible role of LIPE as a neutral CE hydrolase in human macrophages is also supported by the finding that 76-0079, a reportedly specific inhibitor of LIPE, did not significantly inhibit neutral CE hydrolase activity in HMMs (Figure 3C) and cholesterol efflux from HMMs which had been loaded with CE (Figure 3G). This can be extrapolated to other immortal cell lines of human macrophages: THP-1 and U937 macrophages. In mice, on the other hand, Lipe contributes to neutral CE hydrolase activity in MPMs.[25] Thus, there seems to be a species difference in terms of the relative role of LIPE in the hydrolysis of CE in macrophages.

The rediscovery of CES1 in human macrophages provided a second twist in the history of the investigation of neutral CE hydrolase in macrophages. CES1 was originally identified as a human carboxylesterase in both liver[12] and macrophages.[13] The use of different names for CES1 has complicated matters. Ghosh found neutral CE hydrolase activity in CES1.[11] Gosh and colleagues subsequently reported that overexpression of CES1 inhibits the accumulation of CE in macrophages.[21,22] We tried to reproduce their findings in our model. So far, we have been unable to detect neutral CE hydrolase activity when overexpressed either in HEK293 cells (Online Figure III) or in THP-1 macrophages (Figure 2B). Overexpression of CES1 did not reduce the amounts of CE in the cells even in the presence of the SOAT inhibitor (Online Figure V, B). Thus, it is unlikely that CES1 stimulate reesterification of cholesterol directly or indirectly, thereby mitigating the effect of increased hydrolysis of CE on the cholesterol efflux. Although CES1 expression was recognized to a level comparable to that in the liver or WAT (Figure 1C), its silencing did not reduce the neutral CE hydrolase activity of HMMs (Figure 4B). Crow et al recently reported similar results: a recombinant CES1 protein did not possess neutral CE hydrolase activity.[40] Interestingly, they found that treatment of cholesterol-loaded THP-1 macrophages with ether paraoxon (a nonspecific CES inhibitor) or benzil (a specific CES inhibitor) caused enhanced retention of intracellular CE. This puzzling finding led them to speculate that the retention of CE is due in part to inhibition of enzymes other than CES1. Indeed, KIAA1363, an ortholog of *NCEH1*, is potently inhibited by many of organophosphates (OP) including paraoxon.[16,41]

There is a great difference in the ability to mobilize cholesterol between different types of macrophages. For example, THP-1 cells are known to be relatively ineffective at mobilizing cholesterol, primarily owing to slow hydrolysis of CE.[8,42] In addition to the weak expression of LIPE, the absence of NCEH1 may explain the characteristics of THP-1 macrophages (Figure 1C). There are many other examples of differences in neutral CE hydrolase activity.[9,43–45] It is tempting to speculate that these differences are attributable to the difference in the expression level of NCEH1. Furthermore, we examined the difference of the expression of NCEH1, CES1 and LIPE by M-CSF or GM-CSF treatment. NCEH1

protein and neutral CE hydrolase activity proportionally increased (Online Figure II). In those M-CSF– or GM-CSF–treated HMMs, it is thought that NCEH1 greatly contributes to neutral CE hydrolase. It is further study to investigate difference of the contribution of NCEH1, CES1, and LIPE in macrophages stimulated by other cytokines or in different macrophages subclasses.

Finally, the expression of NCEH1 in CD68-positive macrophages in human atherosclerotic lesions in situ substantiates the role of NCEH1 in the development of atherosclerosis proposed above (Figure 5). NCEH1 was expressed in CD68-positive macrophages, but not in other cells such as smooth muscle cells and endothelial cells. This pattern of expression is very similar to that of SOAT1.[35] The number of NCEH1-positive cells was generally larger in atheromatous plaques, which contain a necrotic center with a fibrous cap (Table). In contrast, samples with diffuse intimal thickening lacked NCEH1-positive cells. The abundance of these cells was moderate in fatty streak lesions (Online Figure VII, A through C). In spleen as representative of other organs, NCEH1 was abundantly expressed in CD68-positive cells in the marginal zone around white pulp (Online Figure VII, D and E). Interestingly, the expression level of NCEH1 was not homogeneous in CD68-positive macrophages in those atherosclerotic lesions and spleen. Some CD68-positive macrophages weakly expressed NCEH1. Does this heterogeneity represent the presence of distinct subpopulations of macrophages? Does it just reflect the different stages of differentiation? These questions need to be answered to elucidate the precise pathogenesis of atherosclerosis.

In conclusion, we demonstrate for the first time that NCEH1 is quantitatively the most important neutral CE hydrolase in human macrophages and atherosclerosis. We also clearly show that the contribution of LIPE and CES1 is negligible compared with that of NCEH1, implying that we should be careful when findings in cholesterol metabolism of mice are extrapolated to those of humans. These findings indicate that NCEH is the only enzyme that requires attention when dealing with neutral CE hydrolase activity in human macrophages. Given its high levels in CD68-positive macrophages in initial fatty streaks, as well as in more advanced atheromatous plaques of human aortas, NCEH1 is a promising target for the treatment of atherosclerosis.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Intracellular hydrolysis of cholesterol ester (CE) is the initial and rate-limiting step for removal of cholesterol from macrophage foam cells that predominate in fatty streak atherosclerotic lesions.
- Hormone-sensitive lipase (LIPE) and neutral cholesterol ester hydrolase (NCEH1) have comparable roles in CE hydrolysis in mouse macrophages, but the relative contribution of the hydrolytic enzymes in human macrophages is unknown.

What New Information Does This Article Contribute?

- NCEH1 is the principal enzyme that performs CE hydrolysis in human monocyte-derived macrophages (HMMs).
- The contributions of both cholesterol ester hydrolase (CES1) and LIPE to CE hydrolysis are trivial compared to NCEH1.
- NCEH1 is abundantly expressed in CD68-positive macrophages in cholesterol crystal–rich regions of human atherosclerotic lesions.

There is circumstantial evidence of differing contributions of NCEH1, LIPE, and CES1 to CE hydrolysis in macrophages of differing species. As we have reported previously, in mice, both LIPE and NCEH1 are involved in CE hydrolysis in macrophages. Although at least 3 enzymes are able to hydrolyze CE in nonlysosomal compartments in human macrophages, the relative contribution of these enzymes is controversial. The present study established the predominant role of NCEH1 compared with CES1 and LIPE in CE hydrolysis in human macrophages. Furthermore, we also demonstrated the expression of NCEH1 in macrophages in human atherosclerotic lesions. These findings facilitate the focus on NCEH1, a promising therapeutic target for promoting reverse cholesterol transport, and provide a novel paradigm for understanding the pathogenesis of human atherosclerosis.

アドメノート

薬物動態研究における実験材料及び評価系開発の最近の動向

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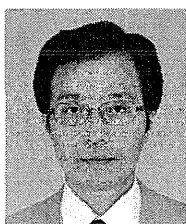

肝細胞，腎尿細管上皮細胞，小腸上皮細胞，脳血管内皮細胞，心筋細胞，神経細胞などのヒトの試料は，創薬研究の薬物動態試験や毒性試験において極めて有用な実験材料であるが，入手が困難な場合が多く，たとえ入手できたとしてもロット間差が大きいうえに，量も限られている．近年，胚性幹細胞(ES 細胞)や人工多能性幹細胞(iPS 細胞)などの多能性幹細胞がヒトより樹立され，生体を構成する全ての細胞に分化する能力を秘めていることから，創薬研究における新しい実験材料として注目されている．特に，肝細胞への分化については数多くの研究が行われており，薬物動態試験や毒性試験への利用が大いに期待されている[1,2]．一方，生体の様々な組織にある組織幹細胞(体性幹細胞，成体幹細胞)は，分化の方向性がある程度決まっているため多能性幹細胞と比較して分化能は限られているが，高い増殖性を維持しており，目的とする細胞への分化が比較的容易であるという利点がある．その中で，間葉系幹細胞(mesenchymal stem cell)は，本来間葉系に属する細胞(骨細胞，心筋細胞，軟骨細胞，腱細胞，脂肪細胞など)への分化能を持っているが，最近グリア細胞(外胚葉由来)や肝細胞(内胚葉由来)など，胚葉の差をこえて中胚葉性でない組織にまで分化できる可塑性を持っていることが示された[3]．間葉系幹細胞から分化誘導された肝細胞様細胞は，薬物代謝活性や薬物代謝酵素の誘導能を有しており，薬物動態研究の有望な材料の1つとして興味が高まっている[4,5]．

肝臓は再生能力に優れた代表的な臓器である．しかし，成人の肝細胞を体外で培養した場合，増殖能力が殆ど無いばかりか，肝細胞特異的な機能が急激に低下し，それを維持することさえ困難である[6]．種差があることから，臨床でのヒトでの薬物の挙動を予測するためにヒト細胞を用いた試験が必要不可欠とされている．そのためヒト凍結肝細胞を使用するケースが年々増加している．しかしながら，通常の培養方法あるいは装置でヒト肝細胞を培養すると，プレートに接着しにくく，生体に近い機能を維持することができないことから長期の薬物代謝や毒性試験には不向きである．これまで，三次元細胞培養装置，浮遊(スフェロイド)培養法，サンドイッチ培養法など生体環境に少しでも近づけるための培養技術の開発が進んできた[7-9]．このような技術は，貴重で高価なヒト試料の有効利用に繋がるだけでなく，幹細胞の肝細胞への分化誘導において，肝特異的機能を獲得・維持することにも利用できる大変貴重な技術である．

一方，細胞培養技術を用いた *in vitro* 研究ではなく，ヒト化動物を作成することによる *in vivo* での薬物動態試験を行う系も確立されている．その中で最も代表的なものがヒト肝細胞を持つキメラマウスであろう．このマウスは，肝臓に障害を持つ albumin enhancer/promoter urokinase plasminogen activator トランスジェニックマウス(uPA マウス)と免疫不全の SCID マウスを掛け合わせ，どちらの形質もホモ接合体である uPA/SCID マウスであり，ヒトの肝細胞を移植することで80％がヒト肝細胞に置換した肝臓を持つ[10]．このヒト肝細胞を持つキメラマウスは，既に薬物動態研究において高い評価を得ている[11-13]．一方，ヒト人工染色体(HAC)技術を基にさまざまな遺伝子の機能を解析するツールとして HAC ベクターが開発された．この HAC ベクターシステム技術を利用し，ヒトの医薬品代謝において最も重要な酵素である CYP3A 遺伝子クラスターを導入した CYP3A ヒト型マウスが作製された．本マウスは，肝臓と小腸にヒト CYP3A を発現したモデル動物としてヒトにおける CYP3A を介した薬物相互作用や薬物代謝が血中動態に及ぼす影響を *in vivo* で予測する新規モデルとして注目されている．

薬物の多くは複数の代謝酵素により代謝を受けると共に，薬物や代謝物の細胞内への取り込みや排泄にはトランスポーターが関与している．また，発現には多くの転写因子が複雑に関与しており，受容体には種差があることも知られている．ヒト肝細胞を持つキメラマウスはヒト肝細胞そのものであることから，薬物動態試験や毒性試験において薬物代謝酵素や薬物トランスポーター等の関与や相互作用を総合的に評価できる実験材料として優れている．しかし，少量残っているマウスの肝細胞の影響を無視できない場合もあることが知られているし，*in vivo* と言っても経口投与で問題となる小腸での評価は出来ない．一方，CYP3A-HAC マウスの場合では薬物代謝で重要な CYP3A が小腸と肝臓に発現していることから，小腸での代謝も解析できる特徴がある．しかし，HAC マウスでは人工染色体を導入して発現した酵素により解析することになり，その他の代謝酵素との関わりや誘導評価における転写因子あるいは受容体については今後の課題かと思われる．

各種オミックス技術は，創薬研究において重要な地位を占めてきた．その中でメタボロミクスは，大規模な発現プロファイリングやプロテオーム研究を論理的に補完するものとして急速に浸透している手法である．この技術は，あ

る遺伝子，または生理的・病理的環境が異なった複数の系において多数の構成分子を網羅的・包括的に分析し，そのプロファイルを比較することにより，細胞のある瞬間の生理的・病理的現象に最も関与する可能性の高い因子を明らかにすることができる[14,15]．しかし，代謝物は化学的にきわめて多様であることから，メタボロミクスには分析上の大きな困難が伴う．その困難を克服する技術として，高感度と高分離能を兼ね備えたLC-MS/MSなど質量分析装置があげられる．

新しい実験材料や評価系の開発は，これまで困難だった薬物動態研究を容易にすることで，その予測精度を増すことになり，延いては安全で有効な医薬品の開発に資することになる．そこで，本シリーズは各分野の専門の先生により，第1回「多能性幹細胞と組織幹細胞」，第2回「3次元培養・ミクロ組織形成技術」，第3回「ヒト化モデル動物」，第4回「質量分析装置」で紹介して頂く予定である．

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実験材料及び評価系開発の最近の動向(第1回)：幹細胞の分化と薬物動態研究への応用—1 ヒトESおよびiPS細胞から肝細胞様細胞および腸管組織への分化誘導

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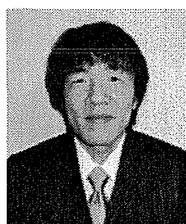

1. はじめに

近年，創薬研究において薬物動態試験や毒性試験の必要性はますます高まってきている．その理由のひとつとして，医薬品の効果や毒性は体内動態に大きく左右されることがあげられる．肝臓や小腸はそれに関与する主要な臓器であり，そこでの動態をより正確かつ簡便に予測・評価することは極めて重要である．従来，薬物動態試験には実験動物が多用されてきたが，種差の問題があり，ヒトへの外挿は容易ではない．それに伴い，ヒトの細胞やオルガネラが薬物動態試験に使用されるようになった．しかし，ヒトの試料は予測が容易である反面，ロット間差が大きく，新鮮な細胞を入手することが困難なうえに非常に高価であることなどから，容易には利用し難いのが現状である．

胚性幹細胞(ES細胞)は，1981年Evansらによって胚盤胞にある内部細胞塊から多能性の細胞株として初めてマウスより樹立された[1]．また，1998年Thomsonらは不妊治療で生じる余剰胚を用いてヒトES細胞を樹立した[2]．ES細胞の特性は，①自己複製能，②高い増殖能，③多分化能を有することである．したがって，本細胞は原理的にほぼ無限に増殖し，三胚葉(外胚葉，中胚葉，内胚葉)由来のあらゆる細胞群に分化することが可能であるため，ES細胞由来の肝細胞や小腸上皮細胞などの薬物動態試験への利用が期待されている．すなわち，ヒトES細胞は受精卵という限られた材料から作られ，その使用においても倫理的問題のため厳しい規制が設けられている[3,4]．

京都大学の山中らは，体細胞に山中ファクターと呼ばれる4つの因子(OCT3/4, SOX2. c-MYC, KLF4)を導入することにより人工多能性幹細胞(iPS細胞)を樹立した[5]．このiPS細胞は，間葉系幹細胞や組織幹細胞など他の幹細胞に比べてES細胞に近い性質を有する[6](表1)．ES細胞における分化誘導法はiPS細胞にも応用可能であり，現在，ヒトiPS細胞から膵細胞[7,8]，神経細胞[9]，心筋細胞[10]などさまざまな細胞への分化誘導が報告されている．患者の体細胞から同じ遺伝子を持つiPS細胞の樹立は，再生医療において免疫抑制剤を使用する必要が無くなると注目されているが，創薬研究においても極めて重要な可能性を秘めている．またヒトiPS細胞は体細胞から樹立されることから，特定胚研究(クローン技術を含む)[11,12]や生殖細胞の作製を行う研究[13]以外取り扱いに関する規制は普通の細胞と基本的に変わらない．さらに，薬物動態に重要な酵素の遺伝子多型の影響も，遺伝的背景が異なる細胞からヒトiPS細胞を樹立し，機能性体細胞を分化誘導することで解析が可能となる．

そこで本稿では，ヒトESおよびiPS細胞の薬物動態試験への応用という観点から，薬物動態において重要な細胞である肝細胞様細胞および小腸上皮細胞様細胞への分化誘導に関して，最近の報告に著者らの結果を踏まえて紹介したい．

表1　幹細胞の特性(文献[6]より引用)

	Established artificial stem cells		Adult stem cells	
	ES	iPS	MSC	TSC
Autologous transplantation	No	No (possibly Yes)	Yes	Yes
In vitro differentiation	Yes	Yes	Yes	Partial commitment
In vivo differentiation	Yes	Yes	Yes	Yes
Differentiation potency	Very high	Very high	High (limited)	Limited
Growth *in vitro*	Infinite	Infinite	Semi-infinite	Limited
Ethical issues	Yes	Low or No	No	No
Availability in medicine	No	Yes	Yes	Potentially Yes
Legislative/governmental law	Yes	No	No	No
Tumorigenesis	Yes	Yes	Low or No	No
Rejection	Yes	No	No	No
Trophic activity *in vivo*	No	No	Yes	?

ES：ES細胞；iPS：iPS細胞；MSC：間葉系幹細胞；TSC：組織幹細胞.

2. ヒトESおよびiPS細胞から肝細胞様細胞への分化誘導法

ES細胞の肝細胞への分化誘導法は最初にマウスで確立された[14-16]．この方法の多くは，胚様体(Embryoid body, EB)を作成し，それを接着培養することで分化させるEB形成法である．EB形成法は，ES細胞を浮遊培養することによりマウス初期胚にみられる円筒胚に類似した形態を示し，かつその後三胚葉のいずれの細胞も発生することを利用したものである[17]．EB形成法においても，多くは胚発生に準じて成長因子やサイトカインなどの液性因子が加えられるが，無秩序に分化するために再現性に乏しく，雑多な細胞群からなるため解析が困難となる欠点がある．ヒトESあるいはiPS細胞の肝細胞への分化誘導はマウスでの方法が応用されているが，近年ではEBを形成せず，未分化コロニーの単層培養系の細胞に直接分化誘導因子を添加する方法が主流になっている．

ヒトES細胞から肝細胞様細胞への分化誘導は，2003年にRambhatlaらによって初めて報告された[18]．その後，より効率的に分化させるために改良された方法が多数報告されている[19-23]．また，ヒトiPS細胞からの分化については，2009年SongらがES細胞での分化誘導と類似の方法にて行ったのが最初であり[24]，それに引き続いていくつか報告がなされた[25-27]．肝細胞様細胞への分化誘導法はほぼ共通しており，複数の因子を段階的に添加していくことで分化誘導を行っている．分化誘導を大きく分けると，胚体内胚葉への分化(ステップ1)，肝芽細胞様細胞への分化(ステップ2)，肝細胞様細胞への分化・成熟(ステップ3)の3つのステップに分類される(**表2**)．

肝臓は内胚葉系の臓器であることから，まず胚体内胚葉へ分化させる必要がある(ステップ1)．以前はヒストン脱アセチル化酵素阻害剤であるsodium butyrateが用いられることもあったが，最近はtransforming growth factor-β (TGF-β) superfamilyのひとつであるactivin Aが用いられることがほとんどである．このとき，Wnt3aが併用される場合もある．Activin Aは，アクチビン受容体に結合して，Smadタンパク質をリン酸化することによってシグナルを細胞内に伝達する．機能的な特徴としては，アフリカツメガエルのアニマルキャップを用いた方法により，胚発生の段階で濃度依存的にさまざまな臓器への分化を制御することが知られている[28]．そこで，高濃度のactivin A (100 ng/mL)を処理したところ，効率的に胚体内胚葉へ誘導されることが明らかになったこと[29]が，汎用される大きな理由となっている．

表2　肝細胞への分化過程で用いられる因子類

ステップ1	ステップ2	ステップ3
Activin A	BMP2, 4	HGF
Sodium butyrate	FGF2, 4	Oncostatin M
Wnt3a	DMSO	Dexamethasone Insulin

BMP, bone morphogenetic protein; FGF, fibroblast growth factor; DMSO, dimethyl sulfoxide; HGF, hepatocyte growth factor.

Sodium butyrateを使用する場合は，ほとんど細胞が死滅するような条件で行わなければうまくいかず，残った細胞が肝細胞に分化する．Activin Aはsodium butyrateほどではないが，それでも非常に多くの細胞死がおこるため培地が濁り，最初は細菌が混入したのかと間違えるほどである．また，Wnt familyのひとつであるWnt3aは内胚葉や中胚葉の分化に重要であり，activin Aと併用することで効率よくかつ速やかに胚体内胚葉への分化が進むことから[30]，これが用いられることもある．

ステップ2は，胚体内胚葉から肝芽細胞様細胞へ分化の過程である．肝臓の初期発生において，腹側前腸内胚葉が近接する心臓中胚葉からのfibroblast growth factor (FGF)シグナルや横中隔からのbone morphogenetic protein (BMP)シグナルによって肝芽細胞への分化が誘導されることが知られていることから[31]，FGFやBMPが用いられる．また，dimethyl sulfoxide (DMSO)も肝芽細胞様細胞や肝細胞様細胞へ分化させることが知られている[21]．

このステップは研究者によりさまざまで，多くは複数ある上記因子のfamilyをいろいろ組み合わせて使用されている．しかし，その組み合わせは，あまり分化効率に影響しないように思われる．著者らは多くの方法を試みたが，DMSOのみと複数の液性因子の添加で大きな差は認められなかった．それよりむしろ，培養方法，培地および細胞株の違いが大きく影響する．それで，著者らは高価な液性因子でなくDMSOのみで行っている．この方法により，ヒトES細胞において25日の分化誘導で成人様の薬物応答性を示す肝細胞様細胞を得ることができた[32]．

最後は，hepatocyte growth factor (HGF), interleukin-6 (IL-6) familyのサイトカインであるoncostatin M (OSM), dexamethasone (DEX)を用いて肝細胞の成熟を行う(ステップ3)[33-35]．

HGFは，肝再生において成熟肝細胞の増殖因子として発見されたものであるが[36,37]，肝臓形成中間期(mid-stage hepatogenesis)において胎児肝細胞の増殖・維持に関係している[38,39]．また，初代培養肝細胞系でOSMがDEX存在下マウス胎仔肝細胞の成熟を誘導することが示された[33]．さらに，ShirahashiらはI型コラーゲン上にて培養されたEBにインスリンとDEXを添加することにより種々の内胚葉系遺伝子の発現が増加することを明らかにして

いる[40]．このようなことから，成熟段階のステップ３においてほとんどの研究者はHGF, OSM, DEX，インスリンを添加している．しかし，この組み合わせで胎児のレベルまでは誘導されるが，それ以降の成熟は難しい．成人様の肝細胞に成熟させるために必要な因子類はまだ解明されておらず，今後薬物動態試験に使用するために克服しなければならない必須の課題である．

最近，これまでとは異なる方法で分化誘導する２つの報告がなされた．その１つは大阪大学の水口らのグループによるもので，肝発生において重要な転写因子のひとつであるhematopoietically expressed homeobox (HEX)[41,42]を，ファイバー改良型アデノウイルスベクターを用いてヒトiPS細胞に導入することで，従来の方法に比べ，短期間かつ高効率に肝細胞様細胞を作成している[43]．また，Si-Tayebらは胚体内胚葉からの成熟の前段階までを低酸素状態(4% O_2)にして培養することで肝細胞様細胞への分化を行っている[26]．このように，肝発生において重要な役割を果たしている転写因子類の導入や，その過程を模倣した環境で分化誘導することで効率的な肝細胞様細胞の作成が試みられている．

このように，肝細胞への分化誘導は，再生医療への応用もあいまって熾烈な競争が行われており，ヒト初代肝細胞に匹敵する細胞を創薬研究に使える日も近いと思われる．ただし，そのためには評価が確立していなければならず，産業応用するならばもとになるiPS細胞の標準化と誘導された肝細胞の規格化が必須であろう．

3.　ヒトESおよびiPS細胞由来肝細胞の特徴

形態学的な特徴として，未分化なiPS細胞はES細胞と同様細胞質がほとんどなく細胞のサイズも小さいが，肝細胞様細胞への分化を進めていくにつれ徐々にサイズは大きくなり，空胞を形成して敷石状となって，分化の最終段階では肝細胞に特徴的な多核の細胞が出現する(図１)．

遺伝子の発現に関しては，ステップ１のactivin Aの処理により，NANOGやOCT4などの未分化マーカーの発現が低下し，肝細胞への分化に重要なファクターであるforkhead box A2 (FOXA2), sex determining region Y box 17 (SOX17)の発現が誘導される．さらにステップ２，ステップ３と分化が進むと，α-fetoprotein (AFP), cytokeratin (CK) 8, CK18, albumin (ALB), α-1-antitrypsin (AAT), tyrosine aminotransferase (TAT), tryptophan 2,3-dioxygenase (TDO2), CYP3A4, CYP7A1など多くの肝細胞マーカーの発現量が増加する．

最終的に分化させた肝細胞様細胞はALBの分泌，transthyretin (TTR), fibronectin, fibrinogenの産生，indocyanine green (ICG)やlow-density lipoprotein (LDL)の取り込み，glycogenの蓄積，urea産生など肝細胞に特徴的な機能も有する．しかし，遺伝子の発現レベルはある程度高く，成人肝細胞に特徴的な機能を有しているものの，その機能的なレベルはまだまだ低く，十分には成熟していない．

HEXを導入して分化させた場合においても，CCAAT/enhancer binding protein α (C/EBPα), prospero homeobox 1 (PROX1)など肝細胞への分化に重要な転写因子や，hepatocyte nuclear factor (HNF) 1α, HNF1β, HNF4α, HNF6など肝に豊富に存在する転写因子の発現が認められ，これらの転写因子が協調的に働くことでより効率的に分化が進む．しかし，ALB, CYP3A4, CYP2D6, CYP7A1の発現量は低い．

4.　ヒトESおよびiPS細胞由来肝細胞の薬物代謝活性

ヒトESおよびiPS細胞から誘導した肝細胞様細胞におけるCYPの発現やその代謝活性は非常に低い．CYPの代謝活性は成人肝細胞のおおよそ10%未満である．これは，CYPのmRNA発現量の比ともほぼ一致する．この分化誘導した肝細胞様細胞は，methylcholanthrene (CYP1Aの誘導剤), phenobarbital (CYP2Bの誘導剤), rifampicin (CYP3A4の誘導剤)により誘導され，代謝活性の上昇がみられる[18,24,43]．その程度はCYP分子種によっても異なるが，2-5倍程度である．Bufuralolを基質としてヒトiPS細胞から分化誘導した肝細胞様細胞の代謝特性について検討を行った報告では，primary hepatocytesと同様の代謝物が生成され，その代謝物はCYPによるものだけでなく，UDP-glucuronosyltransferase (UGT)やglutathione S-transferase (GST)などphase IIの代謝酵素によって生成されるグルクロン酸抱合体やグルタチオン抱合体なども検出されている[22]．また，pregnane X receptor (PXR), constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AhR), liver X receptor α (LXRα)などの核内受

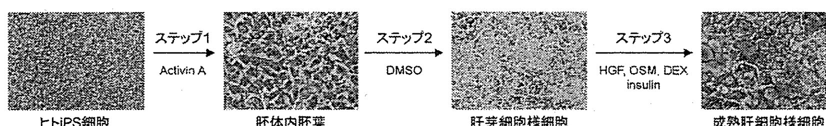

図１　肝細胞への分化法と各段階におけるヒトiPS細胞の形態学的変化