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Review

(Special Topic)

Prodrug approach using carboxylesterases activity: catalytic properties and gene regulation of carboxylesterase in mammalian tissue

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A prodrug is a pharmacologically inactive derivative of an active parent drug, and is bioconverted to the active drug *in vivo*. Through chemical modification of a drug to a prodrug, we are able to deliver drugs to the target site, to optimize therapy and minimize toxicity. A major pathway for the bioconversion of prodrugs to the active parent drugs is *via* carboxylesterase (CES) activity. Among human CES isozymes, hCE1 and hCE2 predominantly participate in the hydrolysis of prodrugs in the liver and small intestine, respectively, although the substrate specificity is quite different between two isozymes; therefore, we can rationally design prodrugs based on the enzyme characteristics. However, since the expression levels of CES vary among individuals, there is a range of pharmacological responses following prodrug administration. Species differences are caused by tissue-dependent hydrolase activity mediated by CES, which makes it difficult to predict effectiveness in humans from a preclinical study using animals. Accordingly, understanding the regulation of CES expression and species difference of CES catalytic properties will be helpful in the design of prodrugs with increased specificity and enhanced physicochemical and biological properties.

Keywords: prodrug, carboxylesterase, substrate specificity, species difference, gene regulation.

Introduction

In the current drug development paradigm, it is necessary to design compounds with minimal or no side effects, and to specifically combat a target disease. The conversion of a drug to a prodrug that is pharmacologically inactive, but can become active via an enzymatic reaction, is an important strategy in targeting a drug to the site of action. By transformation of the drug to its prodrug, we can minimize or eliminate possible drug toxicity to optimize therapy. Chemotherapy using prodrugs was developed in the 1970s, and several prodrugs are now in clinical use. This approach is still accepted as an integral part of new drug design processes, because we can improve, delay, prolong, control, and specifically express the action of the parent drug using a prodrug.[1,2]

Prodrugs are mostly ester derivatives which are constructed from hydroxyl and carboxyl groups of the parent drug, because they can be enzymatically converted to parent drugs by hydrolases that widely exist *in vivo*. Carboxylesterase (CES, EC 3.1.1.1) is responsible for the activation of ester and amide prodrugs,[3] belongs to a super-family called the α,β-hydrolase-fold family, and is a member of the serine esterases, which are found in various mammalian tissue.[4,5] Mammalian CESs comprise a multigene family, and their isozymes are classified into five fundamental groups based on the homology of the amino acid sequence.[6] CES1 and CES2 families play a major role in the bioconversion of prodrugs. The expression levels of CESs and their tissue distribution profiles affect the fate of prodrugs in the body.[7,8] Furthermore, CES isozymes have inter-related substrate specificity but may be classified according to their hydrolase activities towards selected substrates; therefore, the prodrug can be rationally designed on the basis of the characteristics of human CES isozymes. For example, oseltamivir and temocapril are metabolized in the liver by human CES1 but not by human CES2 in the small intestine.[9,10]

In the design of prodrugs specifically susceptible to CES, it

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is important to evaluate the affinity of a prodrug to the corresponding CES as well as individual variations in the CES expression level at the target tissue. CES activity may show inter-individual variations due to both genetic polymorphisms as well as environmental factors, and these may influence toxicity induced by the parent ester.[11,12] Furthermore, the effectiveness and safety of prodrugs should be confirmed in a preclinical study using animal and cell culture models. The tissue distribution of CES1 and CES2 isozymes differs among animals, including humans, and their variation leads to species differences in tissue-specific hydrolase activity. It should also be noted that substrate specificity differs among orthologous CES isozymes in various species.

The present review discusses the development of prodrugs, which is an important issue in the design of a new drug, focusing on the varying substrate specificity of human CES isozymes, species differences in CES-specific tissue hydrolysis, and the genomic structure and regulation of CES genes.

1. General description of prodrug pharmacokinetics

Figure 1 shows the bioconversion processes which may follow the oral administration of a prodrug. As indicated by the conversion rate constants, the prodrug is converted to the parent drug at the absorption site (k_1), in the liver (k_2), in the blood (k_3), and other distribution sites in the body (k_4). The concentration profile of the prodrug and parent drug depends upon the aim of the prodrug. For example, a prodrug delivered to a specific tissue should be sufficiently converted at the fastest rate of k_4 at the target site, compared with other prodrug conversion rates (k_1 to k_3). When the bioavailable dose of a poorly absorbed drug is increased by administration of a suitable prodrug, its kinetics should be optimized to increase drug delivery to the systemic circulation. In this case, prodrug conversion takes place prior to arrival in the blood. The rate of prodrug conversion should be adequately fast, relative to the metabolism of the prodrug and parent drug. Prodrug excretion and metabolism cause a decrease in drug yield. Also, the slower conversion rate of the prodrug than the metabolism of the parent drug causes a decrease in drug yield due to sequential metabolism of the parent drug, and thus a reduction in the potential bioavailable dose of the drug. Figure 2A shows the typical blood concentration profile of a poorly converted prodrug. In order to obtain a high blood concentration of the parent drug, as shown in Fig. 2B and C, orally administered prodrug should be extensively converted to the parent drug in the intestine and liver through which they first pass before entering the systemic circulation. If the prodrug is completely converted to the parent drug during the first pass, then the pharmacodynamics and toxicity all depend on the original drug, provided that the disposable moiety is inert.

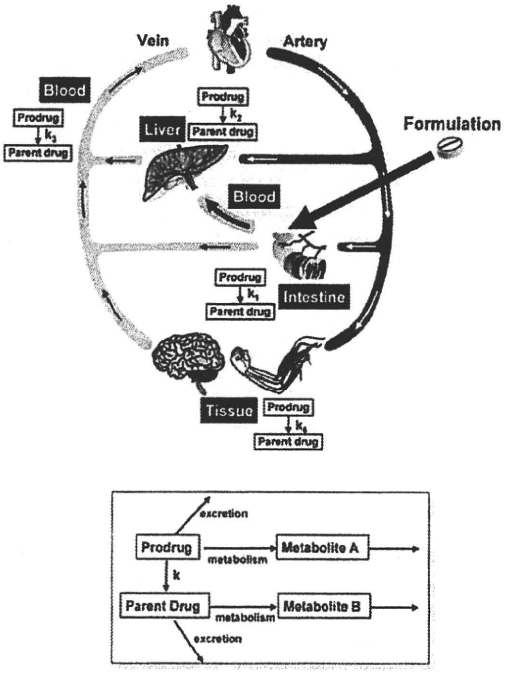

Fig. 1. Distribution of prodrug after oral administration and bioconversion of prodrug.

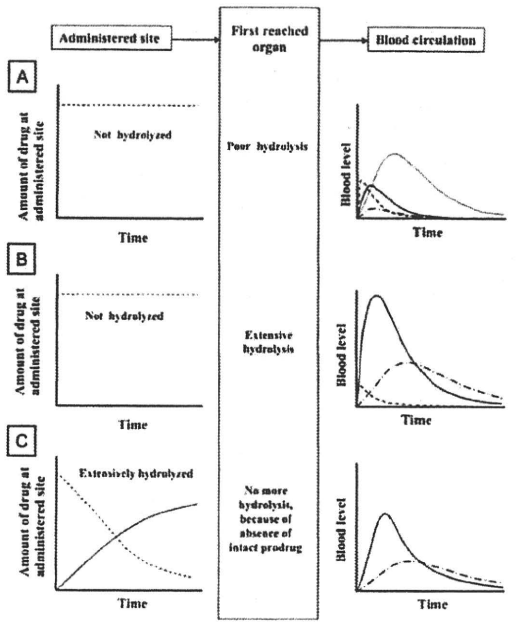

Fig. 2. Drug amount at administered site and blood after administration of prodrug. When prodrug is orally administered, the intestine is the administered site and the first organ reached is the liver. In cutaneous and intravenous administration, the administered site is the skin and veins, respectively, and the first organ reached is the lung in both cases. – – – –, prodrug; ———, parent drug; ———, metabolite of prodrug (Metabolite A); – · – · –, metabolite of parent drug (Metabolite B).

2. Prodrug Conversion Mediated by CES in the Intestine and Liver

Most drug-metabolizing enzymes present in the liver are also found in the small intestine; however, their levels are generally much lower in the small intestine. The importance of small intestinal metabolizing enzymes arises from the location of this organ, which can result in reduced systemic uptake of drugs. The small intestine and liver play a significant role in the metabolism of oral xenobiotics and drugs.

Hydrolase activity in the liver and small intestine in mammals is attributable to several esterase molecules.[13,14] The content of CES in rat liver is found to be about 1mg per g of fresh tissue while the microsomal fraction contains about 30 mg CES per g of microsomal protein.[15] In the liver and intestine, CES1 and CES2 isozymes are present, and play critical roles in prodrug bioconversion. In humans, CES2 isozyme, hCE2, predominantly presents in the small intestine, and the hydrolysis pattern for several substrates in the human small intestine microsomes is nearly the same as those of recombinant hCE2.[16] Although human liver microsomes express both CES1 isozyme (hCE1) and hCE2, the hepatic substrate specificity closely resembles recombinant hCE1. Furthermore, the anti-hCE1 antibody showed 80–95% inhibition of hepatic hydrolysis, and the residual hydrolase activity is due to hCE2.[6] hCE1 dominantly contributes to hepatic hydrolysis rather than hCE2; thus, the first-pass hydrolysis of the prodrug depends on the activity of hCE1 and hCE2 in the liver and intestine, respectively.

However, both hCE1 and hCE2 in the liver are inter-individually variable to a great extent. Furthermore, an age-dependent expression was observed. In general, the adult human liver expresses significantly higher hCE1 and hCE2 than the pediatric liver, which shows significantly higher expression than the fetal group.[12] Within the pediatric group (0–10 years), the hydrolysis of oseltamivir varies by 127-fold in agreement with the variation in the abundance of hCE1.[12] In adults, Hosokawa *et al.* reported a more than 8-fold range of variance in hCE1 protein levels among 12 human liver microsomes,[17] and Xu *et al.* reported a 3-fold range of variance for hCE2 among 13 human liver microsomes[18]; therefore, the prodrug should be designed by considering the variation in the CES expression level in the liver and intestine.

3. Intestinal Hydrolysis during the Process of Absorption

Most prodrugs aimed to improve oral bioavailability of their parent drugs possess adequate membrane permeability due to increasing lipophilicity by ester formation.[19] Therefore, the prodrug is easily taken up into epithelial cells and hydrolyzed to the parent drug, as shown in Fig. 3. The parent drug converted from the prodrug is present at the highest concentration in epithelial cells, and can be transported by passive and active transport into not only blood vessels but also the intestinal lumen. Therefore, it is difficult to achieve complete drug absorption. A prodrug hydrolyzed extensively in epithelial cells results in lower blood concentration of the parent drug, as shown in Fig. 2C. We studied the relation between prodrug absorption and its mucosal hydrolysis using *in situ* rat single-pass perfusion.[20,21] Isovaleryl propranolol (isovaleryl-PL) was well absorbed by epithelial cells by passive diffusion, and then was completely hydrolyzed to PL and isovaleric acid at the rate limited by the uptake of isovaleryl-PL into mucosal cells. The produced PL and isovaleric acid were passively transported to the luminal side and blood vessels according to the pH-partitioning theory. PL, a basic drug, was transported into intestinal lumen (pH 6.5), and isovaleric acid, an acidic compound, was absorbed into blood vessels (pH 7.4).[21] In contrast, when intestinal CES was inhibited by a specific inhibitor, hydrolysis of isovaleryl-PL during absorption was inhibited by about 80%, resulting in increased absorption of the intact prodrug.[21] Okudaira *et al.*[22] reported that an ester-type prodrug, ME3229, is taken up into mucosal cells at a rate compatible with its lipophilicity, and then completely hydrolyzed. The parent drug produced in mucosal cells was predominantly pumped out by an active efflux transporter. When a prodrug is scarcely hydrolyzed in the human small intestine, it is transported into blood vessels in an intact form. If an intact prodrug is taken up into the liver and then rapidly hydrolyzed, it shows an ideal blood concentration profile, as shown in Fig. 2B. Thus, intestinal hydrolysis is markedly important in determining prodrug bioavailability. Extensive hydrolysis degrades a prodrug intended to improve intestinal membrane permeability of the drug. It is therefore necessary to consider the hydrolysis susceptibility of prodrugs and the transport properties of the parent drug in a prodrug design.

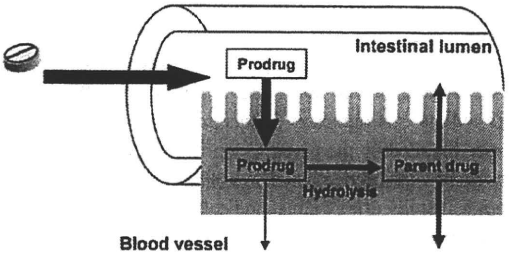

Fig. 3. Absorption and hydrolysis of prodrug in the intestinal epithelial cell.

4. Substrate Specificity of Human CES1 and CES2

The major hydrolase in the human liver and small intestine is hCE1 and hCE2, respectively. hCE1 and hCE2 exhibit 48% homology, and their distinct substrate specificity has been reported.[5,7,16] The major intestinal CES, hCE2, mainly hydrolyzes prodrugs into which an alcohol group of a pharmacological active drug is modified with a small acyl group.[5,7] Prodrugs grouped in this category include CPT-11[23] and as-

pirin.[24] In contrast, prodrugs into which the carboxyl group of the pharmacologically active drug is modified with a small alcohol group are preferentially hydrolyzed by hCE1, and have been numerously developed as pharmaceutical medicines, for example, oseltamivir,[9] meperidine,[25] capecitabine,[26] oxybutynin,[27] camostat mesilate[28] and angiotensin-converting enzyme (ACE) inhibitors[29] such as temocapril and enalapril. A number of successful prodrugs are stable in the human intestine and rapidly hydrolyzed in the liver; therefore, prodrugs can be designed by taking advantage of the markedly different substrate specificity between hCE1 and hCE2.

Thus, the distinct substrate specificity of hCE1 and hCE2 might be related to the structure of their reaction sites. Catalysis of ester cleavage by CES is achieved *via* a triad of catalytic amino acids (Ser203, His450 and Glu336). Carboxylesterases cleave the ester *via* a two-step reaction, as shown in Fig. 4. At neutral pH, the active site, Glu336, exists as the charged form, which facilitates the removal of a proton from His. This loss subsequently results in transfer of a proton from the adjacent Ser203 to the opposing nitrogen of His, generating an oxygen nucleophile that can attack the carbonyl carbon of the substrate. The hydrogen bonds between the negatively charged oxygen of the tetrahedral intermediate and the NH group of Gly123 and Gly124 then stabilize the negatively charged oxygen. This configuration, in which the negatively charged car-

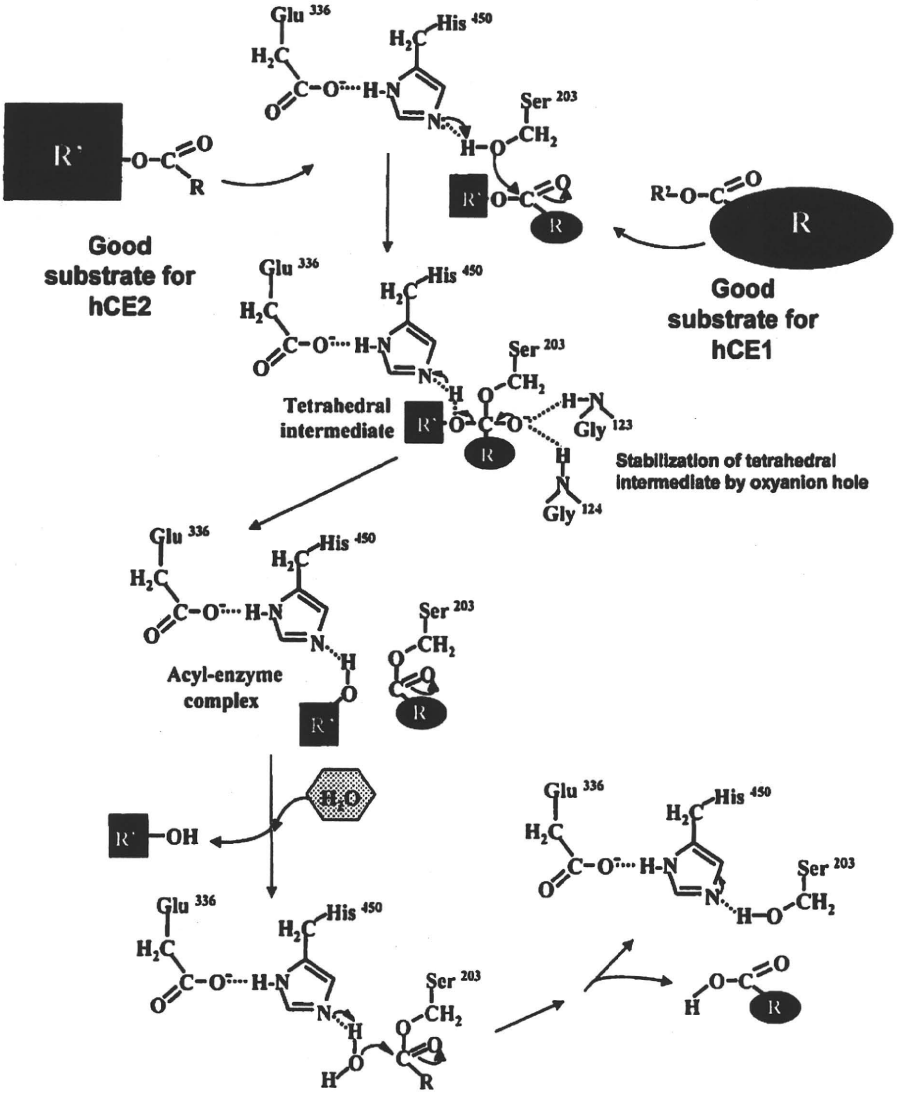

Fig. 4. Hydrolyzing mechanism of CES.

boxyloxygen is hydrogen bonded to two NH groups, is called an oxyanion hole. When the tetrahedral intermediate of an acyl group is formed, the alcohol product is released from the enzyme. The acyl-enzyme intermediate is then attacked in an identical fashion with water acting as the nucleophile, leading to release of the carboxylic acid and return of the catalytic amino acids to their original state.

Steric hindrance in the vicinity of the reaction site of hCE2 may occur with substrates containing a bulky acyl moiety in the process of the formation of the acyl-hCE2 intermediate in the first step of hydrolysis.[16] Interestingly, PL derivatives, generally good substrates for hCE2, are irregularly hydrolyzed by hCE2 depending on the structure of the acyl group. PL derivatives substituted by 3-methyl acyl group are scarcely hydrolyzed by recombinant hCE2, while propranolol derivatives with 2-methyl acyl groups are easily hydrolyzed at almost the same rate as the corresponding straight acyl derivatives.[16] In general, the chemical hydrolysis of ester bonds is sterically hindered by the substituent methyl group at the 2-position rather than the 3-position. Both findings, specific reduction of the hydrolysis rate by substitution of a methyl group at the 3-position and the low hydrolysis rate for a substrate with a large acyl group, suggest that the acyl-enzyme intermediate is difficult to form due to the presence of steric interference in the active site region of hCE2.

In contrast to hCE2, hCE1 preferentially recognizes a substrate with a large acyl moiety, and also catalyses the hydrolysis of prodrugs modified with a small acyl group even with limited activity. The substrate-binding site of hCE1 consists of a "small, rigid" pocket and a "large, flexible" pocket,[29,30] and these pockets allow hCE1 to act on structurally distinct compounds containing either large or small alcohol moieties. The distinct active site between hCE1 and hCE2 might be caused by different amino acid sequences, especially the lack of a loop structure consisting of 15 amino acids.

Interestingly, hCE1 enantioselectively catalyzes the hydrolysis of a substrate. For example, *S*-PL derivatives,[16] *S*-cocaine,[31] *d*-methylphenidate[32] and *cis*-cypermethrin analogues[33] are poor substrates of hCE1, in contrast to the opposing enantiomer. The differences in the hydrolysis rate between these enantiomers have been explained by steric clashes with the loop containing Gly123 and Gly124 in the rigid pocket,[30] where Gly residues form the oxyanion hole to stabilize the transition state of substrate via their amide nitrogen. Bencharit *et al.* also identified the Z-site surface as a ligand binding site for an inactive substrate.[34]

5. Species Difference of Activity and Expression Level of CES

The CES1 and CES2 families include a number of CES isozymes identified in several animals. CES isozymes within the same family show 60–95% homology in all animals including humans, and the CES 2 family shows 40–50% homology with human hCE1. Monkey CES1 (AB010633), rabbit 1 (AF036930) and dog CES D1 (AB023629) show 92.9, 81.1 and 79.7% homology with hCE1, respectively. The rat CES1 family includes four isozymes, Hydrolase A (ES10; X51974), Hydrolase B (X81825), Hydrolase C (RL1; U10698) and rat egasyn (X81395), and the mouse CES1 family includes at least three isozymes, Es-x (Y12887), mouse CES mMH1 (AB023631) and mouse egasyn (S80191). Thus, a number of CES1 isozymes have been identified as proteins. In contrast, few CES2 isozyme have been identified as proteins, such as rabbit CES2 (P14943), mouse mCES2 (ML3; BC031170) and two rat major CES2 isozyme, rCES2 (RL4; AB010635) and AY034877.

It is expected that orthologous isozymes will show similar substrate specificity, because of their 60–95% homology. For example, mouse mCES1, a mouse CES1 family isozyme, hydrolyzes temocapril, similar to human hCE1,[35] and rat CES1 isozyme (Hydrolase A and Hydrolase B) hydrolyzes deltamethrin and esfenvalerate, such as human hCE1.[36] Furthermore, rat rCES2, a rat CES2 family isozyme, hydrolyzes methylprednisolone hemisuccinate, as does human hCE2[37]; however, these examples are limited. In most cases, substrates are hydrolyzed with markedly different affinity among the same CES family isozymes. 1-*RS* *cis*-permethrin is hydrolyzed by CES1 isozyme of rats and rabbits, but not by human hCE1, although 1-*RS* *trans*-permethrin is hydrolyzed by rabbit, rat and human CES1 isozymes.[38] Rabbit CES1 is 100- to 1000-fold more efficient at converting CPT-11 to SN-38 than human hCE1.[39] Thus, the variation of hydrolase activity among the same CES family depends on the substrate, and it is difficult to predict the affinity of the isozyme to the substrate.[40]

Furthermore, the expression level is an important factor in tissue hydrolase activity. In order to clarify the expression of the CES family in several tissues, CES1 and CES2 levels were measured by Northern blots, RT-PCR and real-time PCR.[8] The human hCE1 is highly expressed in the liver, lung and other tissues, but human hCE2 is limitedly expressed in the small intestine and kidney at a high level. In most animals, CES1 isozyme is highly expressed in the liver and lung, and CES2 is present in the small intestine; however, in the kidney, only CES1 is expressed in the rat and hamster, and both CES1 and CES2 are present in the mouse, although CES2 is preferentially expressed in humans, monkeys and dogs. Interestingly, no CES isozyme is present in the dog small intestine;[40] therefore, dogs show different absorption kinetics of some prodrugs to other animals. In addition, high levels of CES activity can be detected in the blood of the majority of mammals, whereas no such activity is detected in the blood of humans, monkeys and dogs.[41]

6. Species Different Pharmacokinetics of Prodrug *via* CES Activity

From the substrate specificity and expression level of CES isozyme, it is expected that CES-mediated tissue hydrolase

activity will differ among several species. The different tissue hydrolase activity causes diverse pharmacokinetics of the prodrug among species. Oseltamivir exhibits good oral bioavailability compared to the parent acid form in most experimental animals and humans. In humans, oseltamivir is rapidly absorbed and almost completely hydrolyzed to the active form by hCE1 in the liver after oral administration;[42] however, oseltamivir is hardly hydrolyzed by rat CES1 isozymes in the liver, but easily hydrolyzed in the blood. Although oseltamivir is extensively absorbed in rats after oral dosing, hydrolase activity in blood is not sufficient to achieve complete conversion of oseltamivir to the active form;[43] therefore, another metabolites mediated by cytochrome P450 were observed in the blood and liver in rats.[44] The blood concentration profile in Fig. 2A is similar to that after administration of oseltamivir in rats.

We reported species difference in the pharmacokinetics of propranolol (PL) derivatives after oral and intravenous administration.[45] When isovaleryl-PL was orally administered to dogs, significantly higher plasma concentration of PL was observed than that following administration of PL (parent drug). Hydrophobic isovaleryl-PL is easily absorbed as an intact prodrug from dog intestine due to a lack of esterase and completely converted to PL in the liver, resulting in high plasma concentration of PL, such as in Fig. 2B. However, rats showed nearly the same plasma PL concentration as oral dosing of PL, because of the complete hydrolysis of isovaleryl-PL in the rat intestine,[21,45] such as in Fig. 2C. When isovaleryl-PL was intravenously administered to dogs, a negligible low concentration of intact prodrug and markedly high concentration of PL were detected in dog plasma due to first-pass hydrolysis by CES D1 in the lung. In rats, higher plasma concentration of intact prodrug than PL was observed after intravenous administration of isovaleryl-PL, because it was hydrolyzed in the liver and blood, but not in the lung.[46] Thus, first-pass hydrolysis in the lung and small intestine results in markedly different pharmacokinetics between rats and dogs. CES is distributed in almost all organs of the body; therefore, hydrolysis of the prodrug in the administered site and the first reached organ significantly affects the fate of prodrugs.

7. Gene Structure and Regulation of CES Isozymes

The *CES* genes comprise a multigene family and isozymes are classified into at least five groups (*CES1–CES5*) and several subgroups according to the homology of the amino acid sequence.[4,47] Genomic structures of the genes encoding these enzymes have been determined: *CES1* genes are located on chromosome 16 containing 14 exons and span about 30Kb[48,49] and *CES2* genes are also located on chromosome 16 containing 12 (15) exons and span about 11 kb.[50,51] Recently, two *CES1* genes, *CES1A1* (AB119997) and *CES1A2* (AB119998) have been identified in the human genome.[47] Both genes reside in chromosome 16q13–q22.1,[52,53] in a tail-to-tail manner, separated by about a 9 kb intergenic region. The exon–intron structure is totally conserved between the two genes and the homology of the exon and promoter regions is 98% and 91% at the nucleotide level, respectively.

Tables 1 and 2 shows the known or predicted *CES1* and

Table 1. Carboxylesterase (CES1) Genes and Enzymes Examined[a]

Mammal	CES gene	CES family	GenBank mRNA (or *N-scan ID)	No. of Amino acids	Chromosome location	Strand
Human	CES1	CES1	L07765	567	16	Negative
Chimp	CES1	CES1	*16.56.002	567	16	Negative
Orangutan	CES1	CES1	CR857194	566		
Baboon	CES1	CES1		567		
Rhesus	CES1	CES1	*20.55.002	566	20	Negative
Cow	CES1	CES1	NP031980	558	18	Negative
Pig	EST1	CES1	X63323	566		
Dog	CES1	CES1	AB023629	565	2	Positive
Cat	CES1	CES1	AB114676	566		Negative
Rabbit	EST1	CES1	AF036930	565		
Rat	CES3	CES1	X51974	565	19	Negative
Mouse	CES1	CES1	NP067431	565	8	Negative
Mouse	CES3	CES1	NM053200	565	8	Negative
Mouse	CES22	CES1	NP598421	562	8	Negative
Mouse	CESN	CES1	NP031980	551	8	Negative

[a] Ref. 54

Table 2. Carboxylesterase (CES2) Genes and Enzymes Examined[a]

Mammal	CES gene	CES family	GenBank mRNA (or *N-scan ID)	No. of Amino acids	Chromosome location	Strand
Human	CES2	CES2	BX538086	559	16	Positive
Chimp	CES2	CES2	*20.66.008	559	16	Positive
Baboon	CES2	CES2		561		
Rhesus	CES2	CES2	*20.66.008	561	20	Positive
Cow	CES2	CES2	BC102288	533	18	Positive
Rabbit	EST2	CES2	P14943	532		
Hamster	CES2	CES2	D28566	561		
Hamster	CES6	CES2	D50577	559		
Rat	CES2	CES2	AB010632	560	1	Positive
Rat	CES2.1	CES2	AB010635	561	1	Positive
Rat	CES6	CES2	AY034877	558	19	Negative
Mouse	CES2	CES2	NP663558	561	8	Positive
Mouse	CES5	CES2	BC055622	559	8	Positive
Mouse	CES6	CES2	NP598721	558	8	Positive

[a] Ref. 54

CES2 gene locations of three primate species and four non-primate eutherian mammals based upon published reports, and BLAT interrogation of human, chimp, rhesus, mouse, rat, cow, dog and cat genomes.[54] With the exception of rat *CES2* and *CES3* genes, which are located on chromosomes 1 and 19, respectively, *CES1* and *CES2* genes from the other examined five mammalian genomes were syntonic. In addition, 10 of 11 mammalian *CES1* genes were transcribed on the negative strand, and 9 of 10 *CES2* genes were transcribed on the positive strand.

Recently, we indentified and characterized dexamethasone-induced methylprednisolone hemisuccinate (MPHS) hydrolase in rat liver microsomes. Intraperitoneal injection of dexamethasone resulted in a significant increase in the level of MPHS hydrolase activity accompanied by the induction of a specific CES isozyme, AB0106353.[5] To confirm that AB010635 encodes the dexamethasone-induced CES isozyme, cDNA cloning was performed and the obtained cDNA was expressed in Sf9 cells using a baculovirus-mediated expression system. The recombinant CES protein could hydrolyze MPHS and exhibited biochemical characteristics similar to those of CES RL4. Collectively, the results indicated that dexamethasone-induced MPHS hydrolase in liver microsomes is a rat CES2 isozyme. Interestingly, the results also showed that this rat CES2 isozyme exists in plasma and that the amount of this protein is increased by dexamethasone.

The 5′-flanking regions of *CES1* and *CES2* genes were isolated from mouse, rat and human genomic DNA by PCR amplification. The mouse *CES* gene (*mCES1*), rat *CES* gene (*rCES1*) and two individual human *CES* genes (*CES1A1* and *1A2*) were found to belong to the CES1 family.[55] The mouse *mCES2*,[56] rat *rCES2*[57] and human *CES2A1* genes were found to belong to the CES2 family.[55] A TATA box does not precede the transcription start site of any of the *CES* promoters, as shown in Figs. 4 and 5. *CES* promoters share several common binding sites for transcription factors among the same CES families, suggesting that orthologous *CES* genes have evolutionally conserved transcriptional regulatory patterns. Potential binding sites of *CES* promoters for transcriptional factors include specificity protein (Sp) 1, Sp3, CCAAT box binding protein (C/EBP), upstream stimulatory factor (USF) 1, nuclear factor (NF) Y, nuclear factor kappa light chain enhancer of activates B cells (NFkB), peroxisome proliferator activated receptor (PPAR), glucocorticoid receptor (GR), and

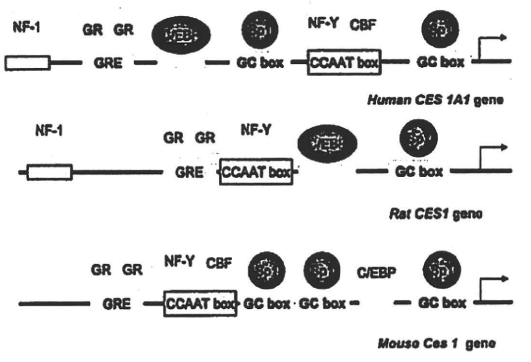

Fig. 5. Structure of the 5′ flanking region of mammal and human CES1 genes.

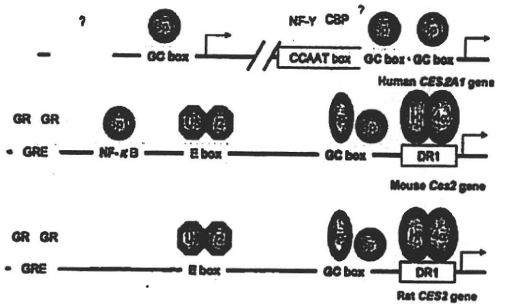

Fig. 6. Structure of the 5′ flanking region of mammal and human CES2 genes.

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),

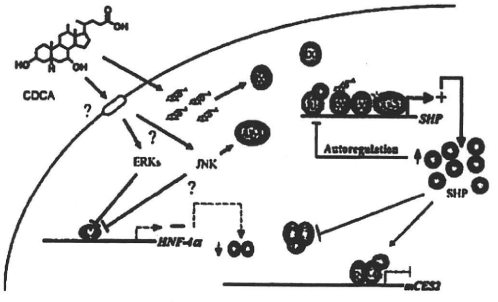

Fig. 7. Possible model for down-regulation of mCES2 gene transcription by CDCA treatment.

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

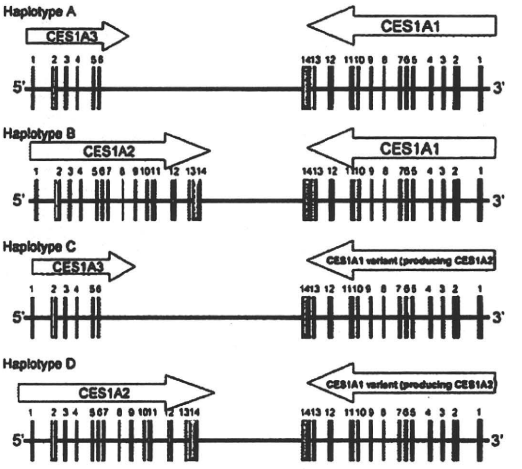

Fig. 8. CES1 gene structure and haplotype.

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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Masakiyo Hosokawa, Ph.D
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.

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

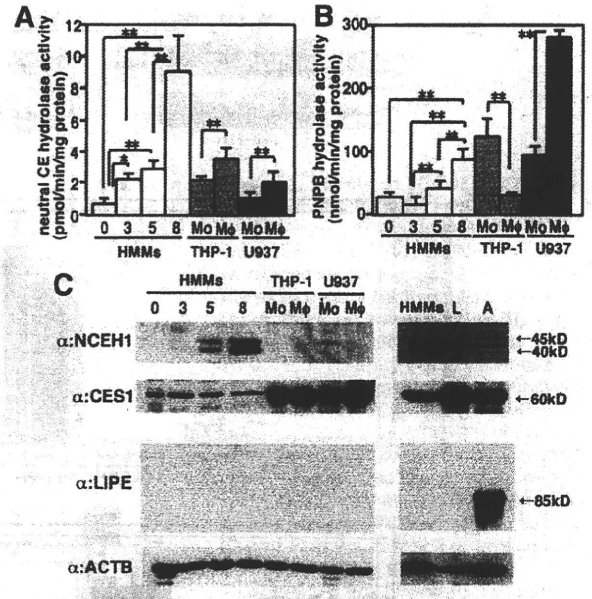

Figure 1. Endogenous neutral CE hydrolase in human macrophages. Neutral CE hydrolase activity **(A)**, PNPB hydrolase activity **(B)**, and protein expression of NCEH1, CES1, and LIPE **(C)** in peripheral blood monocytes, THP-1, and U937, before and after differentiation, was examined. Mo indicates 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).

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

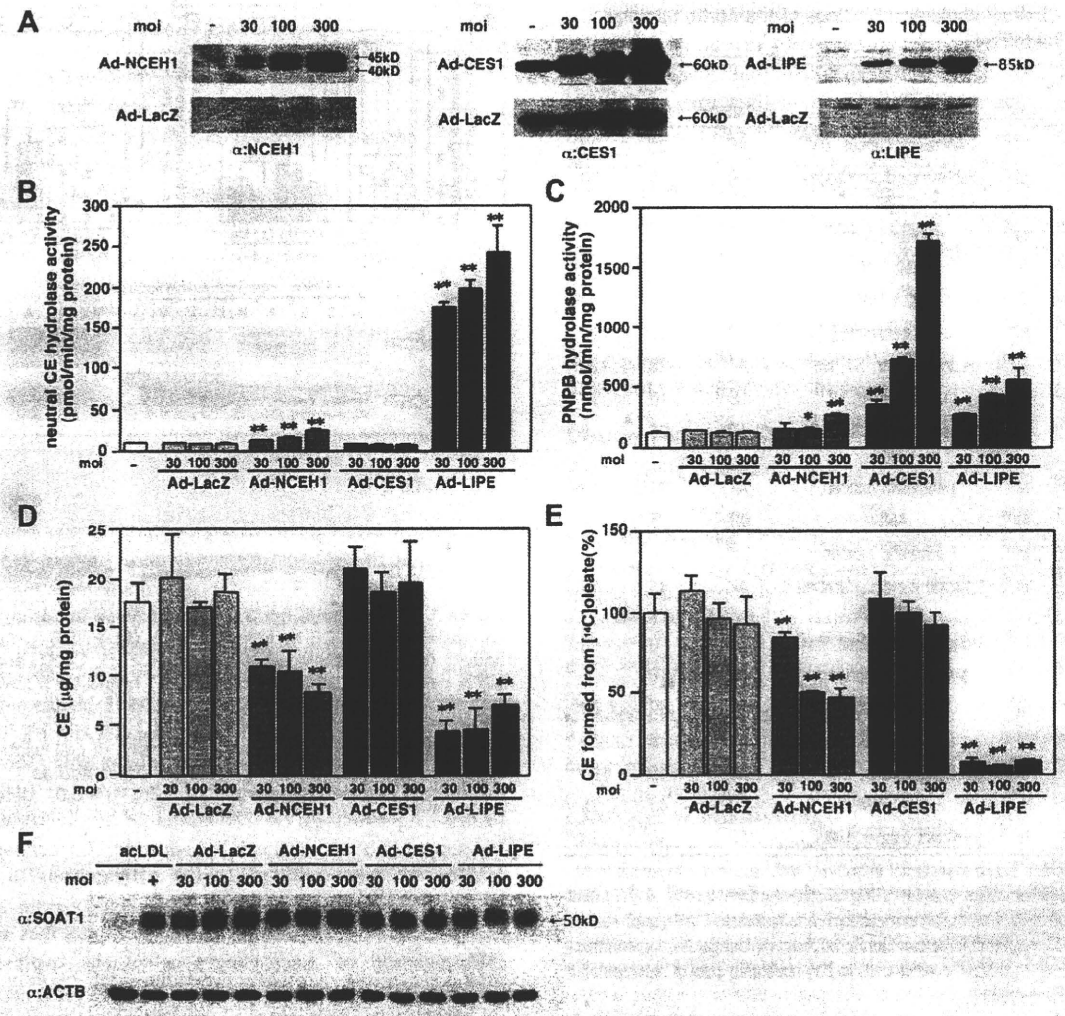

Figure 2. Enzymatic activity and cholesterol trafficking in cholesterol-loaded THP-1 macrophages overexpressing NCEH1, CES1, or LIPE. THP-1 macrophages were incubated with 100 μg/mL of acLDL for 24 hours. After infection with the recombinant adenovirus constructed to express LacZ (Ad-*LacZ*), NCEH1 (Ad-*NCEH1*), 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

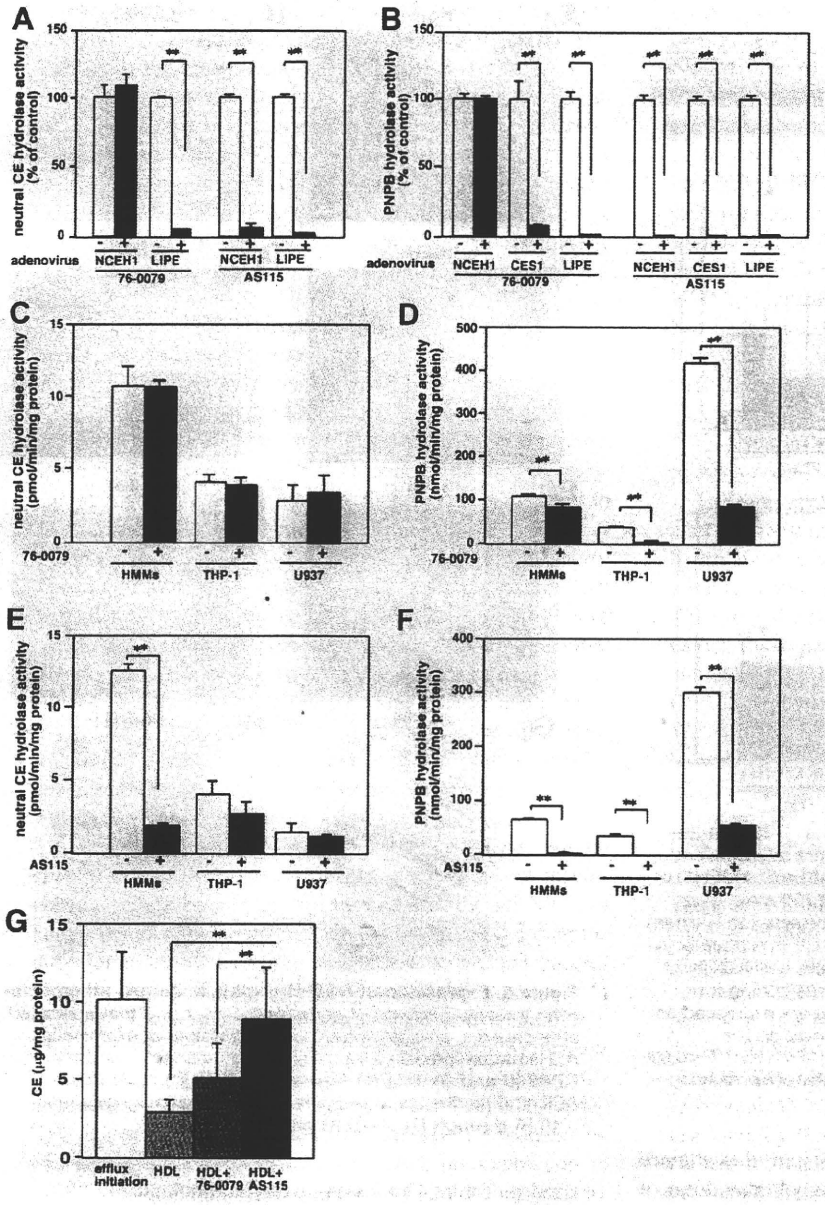

Figure 3. Effects of 76-0079 or AS115 on enzymatic activity. HEK293 cells were transfected with with Ad-*LacZ*, Ad-*NCEH1*, Ad-*CES1*, or Ad-*LIPE*, and whole-cell lysate was used for measurements of neutral CE hydrolase activity **(A)** or PNPB hydrolase activity **(B)** in the absence or presence of 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